The Accumulation, Distribution, and Toxicological Effects of Arsenic in Lake Whitefish (*Coregonus clupeaformis*) and Lake Trout (*Salvelinus namaycush*) Exposed by 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

Master Of Science

Department of Zoology
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Abstract

To address the need for dietary arsenic (As) research on freshwater fish, experiments were conducted to examine the uptake and toxicity of dietary As exposure in two species. First, a short-term preliminary experiment was conducted to compare the accumulation, distribution, and toxicological effects of dietary As exposure in lake whitefish (*Coregonus clupeaformis*) and lake trout (*Salvelinus namaycush*). Dietary As concentrations that fish would consume, and the influence of brine shrimp on consumption of As were also determined. Based on the results of this study, a longer-term experiment was conducted to investigate the uptake and toxicity of As in lake whitefish fed contaminated diets at nominal concentrations of 0, 1, 10, and 100 μg As/g food (d.w.) for 10, 30, and 64 days. The pattern of As accumulation in fish tissues was influenced by reduced feed consumption beginning on day 45 by fish fed the 100 μg As/g food. With the exception of the gallbladder, significant As accumulation occurred in all tissues examined from fish exposed to the 100 μg As/g food for 30 days. Significant accumulation of As occurred in livers and scales of fish fed the 10 μg As/g food for 30 and 64 days. At the molecular level of organization, metallothionein (MT) induction occurred in fish fed the 100 μg As/g food after 10 and 30 days, and in fish fed the 1 and 10 μg As/g food for 64 days. Plasma lipid peroxide concentrations were unaltered within the 64 days of exposure. At the tissue and organ level, liver somatic index was significantly decreased in fish fed the 100 μg As/g food for 64 days, however, blood parameters were not affected by As exposure. Liver and gallbladder histopathology was observed in fish fed all As contaminated diets after each duration of exposure. Both organs were sensitive to As toxicity, as damage occurred with exposure to concentrations

as low as 1 μg As/g. Whole organism parameters were unaltered by As exposure. As residues in pyloric caeca, intestine, liver, and scales, hepatic MT induction, and histopathological alterations in liver and gallbladder are recommended for use as indicators of As exposure and toxicity in environmental monitoring programs.

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List of Abbreviations

ANOVA	analysis of variance
AQUAMIN	Assessment of the Aquatic Effects of Mining
As	arsenic
cc	cubic centimeter
CD	cellular debris
CD	collecting duct
CF	condition factor
cm	centimetre
CSQG	Canadian sediment quality guidelines
CWQG	Canadian water quality guidelines
d	day
DDW	deionized distilled water
DL	detection limit
DO	dissolved oxygen
dolt-2	dogfish liver
dorm-2	dogfish muscle
dw	dry weight
E	eosinophilic hepatocytes
E	epithelium
EEM	environmental effects monitoring
F	folding
FBW	final wet body weight
FIB	fibrosis
g	gram
G	glycogen
G	glomerulus
G	goblet cell
GB	gallbladder
GI	gastrointestinal
h	hour
H	hematopoietic tissue
HD	hepatocyte degeneration
H&E	haematoxylin and eosin
I	inflammation
IBW	initial wet body weight
int.	intestine
kg	kilogram
kid.	kidney
L	litre
L	lumen
liv.	liver
LP	lamina propria

LPO	lipid peroxide
LSI	liver somatic index
LT	lake trout
LWF	lake whitefish
M	mucous cell
MAV	mean assessment value
MC	muscularis circularis
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean cell volume
mg	milligram
min	minute
mL	milliliter
ML	muscularis longitudinalis
mm	millimetre
mm^3	cubic millimetre
mM	millimole
MMLER	Metal Mining Liquid Effluent Regulations
MS-222	tricaine methane sulfonate
MSMA	monosodium methyl arsonate
MT	metallothionein
nmol	nanomole
NS	no shrimp
P1	first segment of the proximal tubule
PC	pyloric caeca
PEL	probable effect level
RBC	red blood cell
S	serosa
SC	stratum compactum
SE	standard error of the mean
SG	stratum granulosum
SM/E	submucosa/epithelium
SMR	standard metabolic rate
sto.	stomach
SS	subserosa
TD	tubular debris
TEL	threshold effect level
tort-2	lobster hepatopancreas
µg	microgram
µL	microlitre
µm	micron
µmol	micromole
VD	vascular dilation
WS	with shrimp
ww	wet weight
ww calc.	wet weight calculated

Introduction

Arsenic (As) is a metalloid belonging to the group of V(A) elements. It ranks 20th in elemental abundance in the earth's crust and is present in virtually all rocks, soils, and water (Eisler, 1988). As can exist in four valence states: the metalloid (0), the trivalent states (-3 or +3), and the pentavalent state (+5) (Cullen and Reimer, 1989). In nature, As typically occurs in combination with sulphur, either alone or combined with various other metals, such as copper, lead, iron, nickel, and cobalt (CEPA, 1993). In water, As can exist in both inorganic and organic forms, and in dissolved and gaseous states (Eisler, 1988).

Primarily, As is produced from flue dust generated during the smelting of copper, lead, and gold ores. In the past, As compounds were used as pesticides, herbicides, and insecticides in agriculture. Due to concerns about the risks to human health in the 1970s, agricultural applications of As have declined, however, As pesticides and herbicides are still in use in the cotton industry as well as for general weed and insect control. Presently, As is used mainly in the manufacturing of wood preservatives. It is also used in the production of glass and nonferrous alloys in the electronics industry (Eisler, 1988; CEPA, 1993; ATSDR, 1999).

Natural sources of As in the environment include the erosion of As-containing rocks and soils, volcanic activity, and the release of volatile methylarsines from soil. The principal anthropogenic sources of As to the Canadian environment are the mining, smelting, and refining of metals, including gold, uranium, and base metals. Mining operations release substantial amounts of As into the environment in a number of ways, including atmospheric emissions, liquid effluent discharges, unusable tailings, and solid

wastes on land (Eisler, 1988, CEPA, 1993). For example, the Mining Association of Canada (1999) reported that in 1998 mining operations released a total of 168 and 2.4 tonnes of As, to air and water, respectively. Other anthropogenic sources of As include the combustion of fossil fuels, the use of pesticides and herbicides containing As compounds, and disposal of domestic and industrial wastes (CEPA, 1993).

When As_2O_3 vapour is released into the atmosphere, it is removed by either dry deposition or rainfall, with rates of deposition being highest in areas closest to the source of emission. Once deposited into aerobic surface waters, As_2O_3 hydrolyses to form primarily arsenite (H_3AsO_3), which is thermodynamically unstable and tends to oxidize to form arsenates, mainly $H_2AsO_4^-$, and $HAsO_4^{-2}$ (Cullen and Reimer, 1989; CEPA, 1993). Dissolved As is removed from most surface waters, as a result of biotic uptake, adsorption onto iron and manganese hydroxides or clay particles, fixation by organic matter, or by precipitation or coprecipitation, and is deposited in sediment with settling organic and inorganic particles (Hindmarsh and McCurdy, 1986; Eisler, 1988; CEPA, 1993).

In Canada, the release of As into the aquatic environment by metal mining operations has been regulated by the Metal Mining Liquid Effluent Regulations (MMLER) since 1977, as part of the Fisheries Act. For the purpose of the regulations, effluent is defined as mine water effluent, mill process effluent, effluent from tailings, treatment pond effluent or treatment facility effluent, as well as seepage and surface drainage from the site. The MMLER regulate the maximum effluent concentrations of six substances, including As, considered to be deleterious to freshwater life. The

maximum acceptable concentration of As in treated liquid effluents is 500 μg/L (AQUAMIN 1996).

In 1993, the Assessment of the Aquatic Effects of Mining in Canada (AQUAMIN) was initiated to determine if the MMLER were providing adequate protection of the aquatic environment. One of the key recommendations of this review was to design and implement a national environmental effects monitoring (EEM) program for metal mining (AQUAMIN, 1996). To date, the EEM program has been developed and implementation is planned for the fall of 2000. The purpose of this program is to evaluate the effects of mine effluent on the aquatic environment, specifically fish, fish habitat, and the use of the fisheries resource, as defined in the Fisheries Act. To do this, the EEM program requires mines to monitor fish and benthic invertebrates, and to collect supporting information on water quality, sediment, effluent, and sublethal toxicity testing (Environment Canada, 1999).

Aquatic life in Canada is also protected from As by the Canadian water quality guidelines (CWQG) and the interim Canadian sediment quality guidelines (CSQG). The CWQG for As, based on the protection of freshwater aquatic life, is 50 μg/L (Environment Canada, 1979). The freshwater interim CSQG for As consist of a low and a high value. The threshold effect level (TEL) represents the concentration of As below which adverse effects are expected to occur rarely and is 5.90 μg/g (d.w.). The probable effect level (PEL) represents the concentration of As above which effects are expected to occur frequently and is 17.0 μg/g (d.w.) (Environment Canada, 1995).

The release of mining wastes into freshwater systems has resulted in concentrations of As exceeding the PEL by one to two orders of magnitude. For

example. Moira Lake, Ontario has been contaminated by mining and mineral processing wastes since the 1830's. Azcue and Nriaga (1993) found the mean concentration of As in surficial sediments of Moira Lake to be 545 μg/g (d.w.), and the maximal concentration of As (1000 μg/g, d.w.) occurred at depths of 23-27 cm. Great Slave Lake has received wastes from gold mining operations and concentrations of As over 2800 μg/g (d.w.) have been measured in sediments (Mudroch et al., 1989). Also, sediment As concentrations exceeding 5000 μg/g (d.w.) have been found in northern Saskatchewan lakes receiving effluents from uranium mining operations (Klaverkamp et al., 2000), and in lakes near Red Lake, Ontario, which have received gold-mining effluents for over 50 years (J.F. Klaverkamp, unpublished data).

As is biologically available to aquatic organisms, including freshwater fish, living in contaminated habitats. For example, the Clark Fork River, Montana, received large volumes of mining wastes from 1880 to 1972, resulting in concentrations of As as high as 404 μg/g (d.w.) in sediments (Brumbaugh et al., 1994) and 43.1 μg/g (d.w.) in benthic invertebrates (Woodward et al., 1994). Brown trout (*Salmo trutta*) collected from a contaminated site on the Clark Fork River had significantly higher concentrations of As in gill, liver, kidney, and pyloric caeca as compared to brown trout collected from a reference site (Farag et al., 1995). In addition, rock bass (*Ambloplites rupestris*) collected from Moira Lake were found to have elevated concentrations of As in intestine and bone plus scales (Azcue and Dixon, 1994).

Laboratory and field exposures of freshwater fish to various forms of As have resulted in a number of toxicological effects, including feed refusal, decreased growth, impaired reproduction, moderate anemia, and histopathological damage to organs,

including liver, kidney, and gallbladder (Cockell et al, 1990; Sorenson, 1991; CEPA, 1993).

The main route of As uptake by benthic-feeding fish is through ingestion of contaminated sediments and food (Sorenson, 1991; Handy, 1996; ATDSR, 1998). The majority of As research on freshwater fish has, however, examined the uptake and effects of exposure to waterborne As (Sorenson, 1976; 1979a; 1979b; McGeachy and Dixon, 1990; 1992; Rankin and Dixon, 1994), with the exception of a series of experiments that examined the effects of dietary As exposure in juvenile rainbow trout (*Oncorhynchus mykiss*) (Cockell, 1990). It is important, therefore, to conduct experiments that examine both the uptake and toxicological effects of dietary As exposure in freshwater fish found in Canadian aquatic habitats contaminated by As, in order to determine tissues and parameters that can be used as sensitive and reliable indicators of As bioavailability and toxicity in environmental monitoring programs (AETE, 1999; Environment Canada, 1999).

To address these research needs, experiments were conducted that examined the uptake and toxicity of dietary As exposure in two freshwater fish species. First, a short-term preliminary experiment, with the following objectives, was completed. The first objective was to compare the accumulation, distribution, and toxicological effects of dietary As exposure in lake whitefish (*Coregonus clupeaformis*) and lake trout (*Salvelinus namaycush*). The second objective was to determine concentrations of As in food that the fish would consume and the third objective was to evaluate whether the addition of brine shrimp to food would increase feed consumption of As contaminated

diets. The results of the preliminary experiment (Chapter 1) were used to determine the fish species, concentrations of As, and diet type used in the longer-term experiment. The longer-term experiment had two objectives. The first objective was to investigate the accumulation, and distribution of As in lake whitefish exposed by the dietary route of uptake, in order to determine tissues that can be used as indicators of As bioavailability in environmental monitoring programs (Chapter 2). The second objective was to examine the toxicological effects of dietary As exposure in lake whitefish at the molecular, tissue, organ, and whole organism levels of biological organization, in order to determine parameters that can be used as sensitive and reliable indicators of As toxicity in environmental monitoring programs (Chapter 3).

Chapter One

The accumulation, distribution, and toxicological effects of dietary arsenic exposure in lake whitefish (*Coregonus clupeaformis*) and lake trout (*Salvelinus namaycush*).

Abstract

A 20-day preliminary experiment was conducted to compare the accumulation, distribution, and toxicological effects of dietary As exposure in lake whitefish (*Coregonus clupeaformis*) and lake trout (*Salvelinus namaycush*). Toxicological effects of As were examined at the molecular, tissue, organ, and organism levels of biological organization. The influence of brine shrimp on the consumption of As contaminated diets was also examined. Each treatment group was exposed to a unique combination of one of three doses of As (0, 100, or 1000 μg As/g) and one of two types of diet (no shrimp (NS) or with shrimp (WS)), for a total of 6 treatment groups for each species. Modified feeding behavior occurred in lake whitefish and lake trout fed all of the As contaminated diets, with the exception of the 100 μg As/g NS food. Therefore, the addition of brine shrimp did not increase feed consumption of the As contaminated diets. Significant sites of As accumulation included stomach, pyloric caeca, intestine, liver, kidney, and gallbladder. Significant accumulation of As was not observed in bile or muscle. The pattern of As tissue distribution differed between lake trout and lake whitefish. At the molecular level of organization, dietary As exposure did not have a significant effect on metallothionein induction in either species. Concentrations of lipid peroxides were only significantly elevated in the plasma of lake trout fed the 1000 μg As/g WS food, however, this effect may have been partly the result of starvation. At the tissue and organ level, liver somatic indices decreased significantly in both species, whereas blood parameters were not affected in either lake trout or lake whitefish. There was a pronounced difference between the sensitivity of lake whitefish and lake trout tissues to histological alterations caused by As exposure, with damage occurring in

gallbladder, liver, kidney, pyloric caeca and intestine examined from lake whitefish. Histological alterations were not observed in similar tissues examined from lake trout. At the organism level, growth was considerably lower in both species fed As contaminated diets, however, condition factors were not affected. Based on the results of this study, lake whitefish were chosen to use in a longer-term experiment, which examined the accumulation, distribution, and toxicological effects of exposure to diets containing 0, 1, 10, or 100 μg As/g for 10, 30, and 64 days of exposure (Chapters 2 and 3).

1. Introduction

Arsenic (As) ranks twentieth in elemental abundance in the earth's crust and is present in virtually all rocks, soil, and water (Eisler, 1988). The erosion of As-containing rocks and soils, and volcanic activity are natural sources of As to the aquatic environment. Anthropogenic sources of As include the mining, smelting, and refining of metals, the use of pesticides and herbicides containing As compounds, and the generation of power from coal (CEPA, 1993).

The release of As into the aquatic environment as a result of anthropogenic activities has led to high concentrations of As in sediments and benthic invertebrates. For example, the Coeur d'Alene River in Idaho has received contamination from mining and smelting operations since 1885, resulting in concentrations of As as high as 179 and 47.8 μg/g (d.w.) in sediments and benthic macroinvertebrates, respectively (Farag et al., 1998). Surficial sediment As concentrations in a coal-ash settling basin and downstream drainage basin in South Carolina were reported to be as high as 70.8 and 116.6 μg/g (d.w.), respectively (Rowe et al., 1996). Also, lakes near Red Lake Ontario, which have received gold-mining effluents for over 50 years, contain concentrations of As exceeding 5000 μg/g (d.w.) in surficial sediment (J.F. Klaverkamp, unpublished data).

As is biologically available to aquatic organisms, including freshwater fish, living in contaminated habitats. For example, a number of organisms, including bullfrog (*Rana catesbeiana*) tadpoles, bullfrog metamorphs, juvenile bluegill sunfish (*Lepomis macrochirus*), adult mosquito fish (*Gambusia affinis*), and juvenile largemouth bass (*Micropterus salmoides*), collected from the coal-ash settling basin were found to contain mean whole-body As concentrations of 31.87, 15.55, 2.61, 2.89, and 1.92 μg/g (d.w.),

respectively. Concentrations of As were elevated compared to organisms collected from a reference site (Hopkins et al., 1999). Also, gizzard shad (*Dorosoma cepedianum*) collected from Lake Texoma, which has been contaminated by agricultural run-off and contains up to 209 μg As/g (d.w.) in sediment, were found to have whole-body As concentrations as high as 34.0 μg/g (w.w.) (Hunter et al., 1982).

Exposure of aquatic organisms to various forms of As has resulted in toxicological effects, including feed refusal, decreased growth, impaired reproduction, moderate anemia, and histopathological damage to organs, including liver, kidney, and gallbladder (Cockell, 1990; Sorenson, 1991; CEPA, 1993). As exposure has also been reported to cause metallothionein (MT) induction in freshwater fish (Schlenk, et al., 1997).

As a result of the high concentrations of As in sediments and invertebrates, the main route of As uptake by fish is dietary (Sorenson, 1991; Handy, 1996; ATSDR, 1998). However, few studies have examined the uptake and effects of dietary As exposure in freshwater fish, with the exception of work by Cockell (1990). Therefore, it is important to conduct studies that link exposure and effects of As in order to determine reliable indicators of As toxicity to be used in environmental monitoring programs (AETE, 1999, Environment Canada, 1999).

The lack of dietary As research indicated a study, which investigated the uptake and effects of dietary As exposure in a freshwater fish, was needed (Chapters 2 and 3). Prior to conducting a long-term exposure, a preliminary experiment, which had the following objectives, was first completed. The first objective was to compare the accumulation, distribution, and toxicological effects of dietary As exposure in lake

whitefish (*Coregonus clupeaformis*) and lake trout (*Salvelinus namaycush*). The effects of As were assessed at several levels of biological organization. At the molecular level, MT concentrations in liver and kidney and lipid peroxides (LPO) in plasma were measured. At the tissue and organ levels, hematological parameters, liver somatic indices (LSI), and histopathology of liver, anterior and posterior kidney, stomach, pyloric caeca, intestine, gallbladder, and spleen were evaluated. At the whole organism level, growth and condition factor were assessed. The second objective was to determine concentrations of As in food that the fish would consume and the third objective was to evaluate whether the addition of brine shrimp to food would improve the consumption of As contaminated diets. The form of As incorporated into the diet was arsenate, as this is the most common form of As in oxic littoral zones of freshwater habitats (Cullen and Reimer, 1989; AQUAMIN, 1996).

2. Materials and Methods

2.1 Fish

Lake whitefish were reared at the Freshwater Institute and were 3.5 years of age at the beginning of the experiment. Initial body weights and fork lengths were 240 ± 14.0 g and 25 ± 0.42 cm (mean ± SE), respectively. Lake trout, reared at the Freshwater Institute, were 2 years of age at the beginning of the experiment. Initial body weights and fork lengths were 289 ± 5.77 g and 30 ± 0.20 cm (mean ± SE), respectively. Fish were fed an amount of No. 3 trout pellets (Martin Feed Mills, Elmira, Ont.) equal to 0.5% of the total body weight per tank every Monday, Wednesday, and Friday of each week.

Lake trout and lake whitefish were acclimated to all experimental conditions for 2 and 4 weeks, respectively.

2.2 Tanks

Twenty-four lake whitefish were randomly distributed, 4 fish/tank, among six, 200 L fiberglass tanks. Twenty-four lake trout were also randomly distributed, 4 fish/tank, among 6 additional, 200 L fiberglass tanks. Dechlorinated City of Winnipeg tap water, maintained at or below 8 μg Cl_2/L by activated charcoal filtration and ozonation (Wagemann et al., 1987), was supplied to the tanks. The tanks were individually aerated, and maintained on a one-pass water flow at 1.2 L/min, yielding 95% replacement of tank water in 9 hours (Sprague, 1973). Tank temperature, pH, and dissolved oxygen (DO) concentrations were measured each day, and are expressed as mean ± SE. Temperature and pH were 11 ± 0.02 °C and 7.6 ± 0.01, respectively. The percent saturation of dissolved oxygen was 89 ± 0.23 %. Concentrations of major ions, total anions and cations, organic acids, total dissolved inorganic and organic carbon, total suspended solids, conductivity, and alkalinity of water supplied to the tanks are presented in Cooley and Klaverkamp (2000). Photoperiod was held constant at 11.5 hours of light and 11.5 hours of darkness, with 30-minute periods of 'dusk' and 'dawn'.

2.3 Processing of Diets

As was obtained as disodium arsenate heptahydrate from Sigma Chemical Co. (St. Louis, MO). Six diets were formulated using trout chow flour, which contained 42% crude protein, 16% crude fat, 2% crude fiber, 5% ash, and 0.9% calcium (Martin Feed

Mills, Elmira, Ont.) and deionized distilled water (DDW). Brine shrimp were added to 3 of the 6 diets. Diets 1, 2, and 3 were constructed as follows: 2 parts of trout chow flour and 1 part of DDW were combined to produce a 0 μg As/g control NS (no shrimp) diet, and disodium arsenate heptahydrate was combined with 2 parts of trout chow flour and 1 part DDW to produce nominal As concentrations of 100 μg As/g NS food, and 1000 μg As/g NS food. Brine shrimp were incorporated into diets 4, 5, and 6 as follows: 1 part of brine shrimp was combined with 3 parts of trout chow flour and 1 part of DDW to produce a 0 μg As/g control WS (with shrimp) diet, and disodium arsenate heptahydrate was combined with 1 part of brine shrimp, 3 parts of trout chow trout flour and 1 part DDW to produce nominal As concentrations of 100 μg As/g WS food, and 1000 μg As/g WS food. The diets were combined using a commercial electric mixture, pelleted using a laboratory pellet mill, dried in a fan-ventilated chamber, and stored at -20°C until required for feeding. The measured concentrations of As in the NS diets were 0.69 ± 0.06, 120 ± 21, and 1100 ± 61 μg As/g, in the control, low, and high dose food, respectively. The measured concentrations of As in the WS diets were 0.94 ± 0.02, 160 ± 17, and 1300 ± 50 μg As/g, in the control, low, and high dose food, respectively. The WS diets contained higher As concentrations after they were dried because they initially contained more water than the NS diets, due to the addition of brine shrimp when the diets were prepared.

2.4 Experimental Design

The treatments consisted of one of the three dose groups (0, 100, or 1000 μg As/g) and one of the two types of diets (no shrimp or with shrimp), for a total of 6

treatment groups. Each of the 6 tanks of lake whitefish and each of the 6 tanks of lake trout were randomly assigned to one of the treatments. The sample size was 4 fish per treatment group. Lake whitefish and lake trout were fed the As-contaminated diets at 0.5% of the total body weight per tank every Monday, Wednesday, and Friday for 20 days. Fish were administered the diets 8 times over the course of the experiment.

2.5 Blood Sampling and Analyses

On sampling day, fish were anesthetized in a pH-neutralized tricaine methane sulfonate (MS-222) (Sigma Chemical Co., St. Louis, MO) solution (338 mg/L). After anaesthetization, fish lengths and weights were measured, and a sample of blood was drawn from the caudal artery and vein using a 20-gauge needle and a 5 cc syringe rinsed with ammonium heparin. The blood sample was transferred to vacutainers containing ammonia heparin. For each fish, a portion of blood was diluted 1:200 in a standard red blood cell (RBC) diluting pipette with Hendrick's diluting solution. The RBC diluting pipette was shaken and placed on ice. After 12 fish were sampled, RBC counts were performed following the method described in Schreck and Moyle (1990). A portion of blood was also transferred to hematocrit capillary tubes, sealed, and centrifuged at 8000 g for 5 min using a Damon/IEC Division Clinical Centrifuge (Needham, MA). Hematocrit was measured using a Damon/IEC Division microcapillary hematocrit reader (Needham, MA). Next, 20 μL of whole blood was pipetted into test tubes containing Drabkin's solution for measurement of hemoglobin concentrations. After all fish were sampled, hemoglobin measurements were made using the Sigma Diagnostics Total Hemoglobin Procedure No. 525 (St. Louis, MO). The vacutainers containing the remainder of the

whole blood for each fish were centrifuged for 3 min at 10 000 g. The plasma was pipetted into 1.5 mL microcentrifuge tubes and frozen at –90°C until lipid peroxide analysis was conducted using the K-Assay LPO-CC kit (Kamiya Biomedical Company, Seattle, WA). The detection limit of this kit was 2.0 nmol/mL of plasma. Mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were determined from RBC counts, hemoglobin concentrations and hemoglobin values by standard equations (Schreck and Moyle 1990).

2.6 Sampling, Removal, and Preparation of Tissues

Following blood withdrawal, tissues were removed for As, MT, and histological analyses according to the following procedures. The gallbladder was separated from the liver. The bile was removed with a 23-gauge needle and 3 cc syringe, placed in a 1.5 mL microcentrifuge tube, and frozen. A sub sample of the gallbladder was cut out and placed in Bouin's fixative and the remainder of the gallbladder was rinsed thoroughly with physiological saline and frozen for As analysis. The liver was weighed, a sub sample was taken and placed in Bouin's fixative, and the remainder of the liver was frozen. The entire gastrointestinal (GI) tract was removed from the abdominal cavity. The spleen was removed and subsamples of cardiac stomach, pyloric caeca, and intestine (sampled just after the pyloric caeca) were taken and placed in Bouin's fixative. The remaining stomach, pyloric caeca, and intestine were frozen. Following removal of the swim bladder, the anterior kidney was removed and a subsample of the posterior kidney from the mid region was excised and placed in Bouin's fixative. The remainder of the posterior kidney was removed and frozen. A sample of the skeletal muscle posterior to

the opercular bone and dorsal to the abdominal cavity was taken and frozen. Prior to As analyses, the stomach and intestine were thawed and cleaned by squeezing out the contents, making a longitudinal incision, and scraping and blotting to remove As-containing food particles and feces. Liver and kidney were split into two samples and one sample was stored at -90°C until analyses of MT concentrations, while the other sample was stored at -20°C until As analysis. All remaining tissues were stored at -20°C immediately after sampling until As analysis.

2.7 Analysis of Arsenic

Eight tissues (stomach, pyloric caeca, intestine, gallbladder, bile, liver, kidney, and muscle) from lake whitefish and lake trout and food samples were analyzed for As using a borohydride reduction method (Vijan and Wood, 1974) according to the procedures outlined in Chapter 2.

The detection limit (DL) of the Varian SpectrAA-20 atomic absorption spectrophotometer is 2.0 μg/L or 0.05 μg/g based on 1.0 g tissue weight. Due to the fact that the weight of tissues used for the As analyses varied, the DL were tissue weight-dependent. The DL calculated for the 8 tissues examined from lake trout (LT) and lake whitefish (LWF) are reported as mean wet weights (μg As/g) ± SE and were as follows: LT gallbladder 1.3 ± 0.21, LWF gallbladder 1.3 ± 0.15, LT liver 0.07 ± 0.01, LWF liver 0.09 ± 0.01, LT and LWF pyloric caeca 0.09 ± 0.01, LT stomach 0.08 ± 0.00, LWF stomach 0.10 ± 0.00, LT intestine 0.09 ± 0.00, LWF intestine 0.18 ± 0.01, LT kidney 0.09 ± 0.01, LWF kidney 0.14 ± 0.01, and LT and LWF muscle 0.08 ± 0.00. The DL for fish food was 0.27 ± 0.04.

2.8 Comparison of As Concentrations to Literature Values

In order to compare concentrations of As measured in the current study as wet weight to concentrations of As measured in previous studies as dry weight, dry weights were converted to wet weights based on the following calculation: dry weight x 0.2 = wet weight calculated (Jarvinen and Ankley, 1999). The converted values are indicated by (w.w. calc.) in the discussion.

2.9 Histopathological Analysis of Tissues

Samples of gallbladder, liver, spleen, stomach, pyloric caeca, intestine, anterior kidney, and posterior kidney were fixed in Bouin's fixative for 48 h, rinsed in 3 changes of 70% ethanol over the next 72 h, and stored in 70% ethanol until processing. Tissues were processed in an automated tissue processor (IL MVP Tissue Processor) using an ethanol/butanol series. Following processing, tissues were embedded in paraffin (Tissue Prep II), sectioned at 7 μm, and mounted on glass slides. Tissue sections were stained with Harris' haematoxylin and eosin, following the method described in Edwards (1967). All chemicals were obtained from the Fisher Scientific Company (Fair Lawn, NJ).

Qualitative histopathological examinations of lake whitefish and lake trout tissues were completed using a Zeiss Photomicroscope III. The following tissues were examined from lake whitefish: liver (all fish), kidney (all fish), gallbladder (all fish fed NS diets), pyloric caeca (all fish fed NS diets, 2 of 4 fish fed the 100 and 1000 μg As/g WS diets), intestine (all fish fed NS diets), anterior kidney (all fish fed NS diets), spleen (all fish fed NS diets), and stomach (all fish fed NS diets). The following tissues were examined for

lake trout: liver (all fish), kidney (all fish), pyloric caeca (2 of 4 fish fed the control NS diet, and all fish fed the 100 μg As/g NS diet), anterior kidney (all fish fed NS diets), and spleen (all fish fed NS diets, 1 of 4 fish fed the 100 and 100 μg As/g WS diets). Photomicrographs were taken with a Kodak DC120 Zoom digital camera using the Kodak Digital Science Microscopy Documentation 120 System (MDS120) and Adobe PhotoDeluxe (version 2.0) software (Adobe Systems, Inc.).

2.10 Metallothionein Analysis

Liver and kidney of lake whitefish and lake trout were prepared and analyzed for MT by the mercury saturation assay of Dutton et al. (1993) as modified by Klaverkamp et al. (2000b).

2.11 Statistical Analyses

Statistical analyses were performed using SPSS version 9.0 and were based on the following references, Neter et al. (1996), Stevens (1992) and SPSS (1999). For each data set, the assumptions of an analysis of variance (ANOVA), normality and equal variance, were checked. Because the assumptions were not met, the data sets were analyzed using a Kruskal-Wallis one-way ANOVA on ranks. Where differences were significant, treatment groups were further compared to controls using the Dunnett's multiple comparison method. All statistical analyses were conducted at an alpha level of 0.05.

3. Results

3.1 Feed Consumption and Morphological Indices

A modification of feeding behavior, characterized by a fish taking a food pellet into its mouth and then spitting it out, was exhibited by lake trout and lake whitefish fed all of the As contaminated diets, with the exception of the 100 μg As/g NS (no shrimp) food. Complete feed refusal occurred in lake trout fed the 1000 μg As/g NS food after 10 days of exposure, and in both lake trout and lake whitefish fed the 1000 μg As/g WS (with shrimp) food after 8 days of exposure. A summary of the modified feeding behavior exhibited by lake trout and lake whitefish over the course of the experiment is shown in Table 1.

No mortalities occurred during the experiment, however, on day 2, one lake whitefish fed the 1000 μg As/g WS food exhibited overt toxicity, including impaired buoyancy control, and lethargy. The fish was sampled on day 2, decreasing the sample size of this treatment group to three fish.

Growth of fish was calculated as the percentage change in wet weight from the initial body weight after 20 days of exposure. Growth of lake whitefish fed both NS and WS As contaminated diets was not significantly different from the controls. However, growth of lake whitefish fed the high dose diets, both NS and WS, was considerably less than in the other groups (Figure 1a). No significant differences in growth were observed in lake trout fed the NS As contaminated diets. Growth of lake trout fed the 1000 μg As/g WS food was significantly less than the control group (Figure 1b). Consumption of NS and WS As contaminated diets for a 20-day period did not have a significant effect on the condition factors of lake whitefish or lake trout. The mean condition factors of lake

whitefish fed the control, 100 μg As/g, and 1000 μg As/g NS diets and the control, 100 μg As/g, and 1000 μg As/g WS diets were 1.3 ± 0.06, 1.3 ± 0.02, 1.2 ± 0.04, 1.3 ± 0.05, 1.3 ± 0.06, and 1.4 ± 0.07, respectively. The mean condition factors of lake trout fed the control, 100 μg As/g, and 1000 μg As/g NS diets were all 1.0 ± 0.03. The mean condition factors of lake trout fed the control, 100 μg As/g, and 1000 μg As/g WS diets were 1.0 ± 0.01, 0.92 ± 0.04, and 1.0 ± 0.03, respectively. Liver somatic indices (LSI) of both species were, however, significantly effected by consumption of As contaminated food. Lake whitefish fed both 100 and 1000 μg As/g WS food had significantly lower LSI than the control group (Figure 2a). Lake trout fed the 1000 μg As/g NS food were also found to have a significantly lower mean LSI than the control group. Although mean LSI of the lake trout fed the two As WS diets were less than the corresponding control group, no significant differences were observed (Figure 2b).

3.2 Accumulation of Arsenic

Exposure of lake whitefish and lake trout to As contaminated food resulted in significant accumulation of As in the GI tracts of both species. Although concentrations of As increased with exposure to higher doses of As in the stomachs of lake whitefish fed the NS diets, no significant differences were observed. Lake whitefish fed the 1000 μg As/g WS food accumulated significant concentrations of As in stomach after 20 days of exposure (Figure 3a). Stomachs of lake trout fed both 100 and 1000 μg As/g NS food accumulated significant concentrations of As, whereas concentrations of As in stomachs of lake trout fed the WS diets were not significantly different (Figure 3b).

Significant accumulation of As occurred in pyloric caeca of lake whitefish fed the 1000 μg As/g NS food, as well as the 100 and 1000 μg As/g WS food (Figure 3c). Accumulation of As was significant in pyloric caeca of lake trout fed all of the As contaminated diets (Figure 3d).

Lake whitefish fed the 100 and 1000 μg As/g NS food, and the 1000 μg As/g WS food had significantly higher concentrations of As in intestine (Figure 3e). Significant accumulation of As only occurred in intestines of lake trout fed the 100 and 1000 μg As/g NS food, as concentrations of As in intestines of lake trout fed the WS diets were not significantly different (Figure 3f).

As was absorbed internally by lake whitefish and lake trout, as significant As accumulation occurred in the livers and kidneys of both species. Accumulation of As was significant in livers of lake trout and lake whitefish fed all As contaminated diets, with the exception of lake trout fed the 1000 μg As/g WS food (Figure 4a, and b). Significant accumulation of As occurred in kidneys of both species fed all As contaminated diets, with the exception of lake whitefish fed the 100 μg As/g NS food (Figure 4c, and d).

Accumulation of As occurred in gallbladders and bile of lake whitefish and lake trout fed As contaminated diets, however, the only concentration of As that was significant occurred in gallbladders of lake trout fed 100 μg As/g NS food (Figure 5). Although mean As concentrations were higher in several other groups, the high degree of variability between fish, low numbers of fish in each group, and the absence of bile samples from many fish contributed to a lack of statistical significance. The concentrations of As in muscle of lake whitefish fed the NS and WS control diets and

lake trout fed the NS and WS control diets were 0.35 ± 0.06, 0.38 ± 0.02, 0.46 ± 0.07, and 0.47 ± 0.02 μg As/g, respectively. Feeding lake trout and lake whitefish As contaminated diets had no significant effect on As concentrations in muscle.

3.3 Hematology

No significant changes were observed in the hematological variables of hematocrit, hemoglobin concentration, red blood cell count, mean cell volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration for lake whitefish or lake trout exposed to As contaminated diets for 20 days (Table 2).

3.4 Biochemistry

MT concentrations in livers and kidneys of lake trout and lake whitefish were not significantly different from the control groups after 20 days of exposure to As contaminated diets (Table 3).

No significant differences in lipid peroxide (LPO) concentrations were observed in the plasma of lake whitefish fed either NS or WS As contaminated diets. Significantly higher concentrations of LPO were observed in the plasma of lake trout fed the 1000 μg As/g WS food. No other differences were observed between exposed and control concentrations of LPO in lake trout plasma (Table 3).

3.5 Histopathology

Macroscopically visible lesions of the gallbladder wall were observed in lake whitefish and lake trout fed 100 and 1000 μg As/g NS and WS food. These overt lesions

were observed more frequently in both lake trout and lake whitefish fed the higher doses (NS and WS) of As. Lesions were characterized by hemorrhaging, diffuse whitening of the gallbladder wall, and a thickened appearance. The gallbladders contained very little or no bile.

Histological alterations were not observed in any of the other tissues (pyloric caeca, liver, anterior and posterior kidney, and spleen) examined from lake trout exposed to As contaminated diets. Alterations were, however, observed in liver, kidney, pyloric caeca, intestine, and gallbladder examined from lake whitefish exposed to As contaminated diets. Alterations were not observed in anterior kidney, spleen, or stomach of lake whitefish. Histological damage was observed in the pyloric caeca of lake whitefish fed all As contaminated diets. This damage was characterized by an increase in the number of goblet (mucosal) cells, as well as an increase in the width of the lamina propria in the exposed fish, as compared to control lake whitefish (Figure 6). Similar alterations were observed in the intestines of lake whitefish fed the 1000 μg As/g NS food (Figure 7). Histological alterations, including sloughing of the epithelium, vascular dilation, and edema of the submucosal tissues, were observed in gallbladder walls of lake whitefish fed all As contaminated diets (Figure 8a, and b). Fibrosis of the gallbladder wall was also observed in lake whitefish fed the 1000 μg As/g NS food (Figure 8c). Lesions were observed in both liver and kidney of lake whitefish fed the 100 μg As/g NS food. Areas of focal necrosis, characterized by pyknotic nuclei, degenerating hepatocytes, and cellular debris, were observed in the liver (Figure 9). Small areas of cellular degeneration, characterized by the presence of pyknotic nuclei and tubular debris, were observed in the collecting ducts of kidneys (Figure 10).

4. Discussion

A reduction in feed consumption in response to a diet contaminated with As has been previously observed in freshwater fish, birds, and mammals (Neiger and Osweiler, 1989; Camardese et al., 1990; Cockell, 1990). The characteristics of the modified feeding behavior of lake whitefish and lake trout seen in the current study are consistent with the modified feeding behavior described for juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to dietary disodium arsenate (Cockell and Hilton, 1988; Cockell et al., 1991; Cockell et al., 1992, Cockell and Bettger, 1993). Both the type of feed refusal, where a food pellet is taken into the mouth and subsequently rejected, and the early onset of modified feeding behavior observed in the current study suggest that chemoreceptory detection of As in the food was occurring. The addition of brine shrimp to the diets did not increase feed consumption of the As contaminated food by either lake whitefish or lake trout, as feed refusal occurred in both species fed diets with and without shrimp.

Due to the reduction in feed intake that occurred to varying degrees in different treatment groups, the accumulation of As in lake whitefish and lake trout tissues is difficult to interpret according to dose-dependencies. However, some statements regarding which tissues accumulated As can still be made. As was absorbed along the GI tract of both species, as significant accumulation of As occurred in the stomach, pyloric caeca, and intestine of both lake whitefish and lake trout. The greatest accumulation of As in these three tissues occurred in the pyloric caeca of both species. The concentrations were 1.9 and 1.8 μg/g (w.w.) in lake whitefish and lake trout, respectively. In a previous study, brown trout (*Salmo trutta*) collected from the Clark Fork River,

which contained 102 μg As/g (d.w.) in sediments (Brumbaugh et al., 1994), were found to have significantly higher concentrations of As in pyloric caeca (0.29 μg/g, w.w. calc.) as compared to fish collected from a reference site (Farag et al., 1995). This concentration is an order of magnitude lower than the concentrations observed in the current study. In another study, rock bass (*Ambloplites rupestris*) collected from Moira Lake, Ontario, which contained 1000 μg As/g (d.w.) in sediments, were found to have increased As concentrations in intestine with contents (0.274 μg/g, w.w.) (Azcue and Dixon, 1994). Similar concentrations of As in intestine were observed in the current study, even though concentrations of As were measured in the intestine without contents. The highest concentrations of As in the intestine of lake whitefish and lake trout were 0.45 and 0.87 μg/g (w.w.), respectively.

After absorption from the GI tract, As is distributed to the internal organs of lake trout and lake whitefish, as significant accumulation occurred in liver and kidney of both species. Previous laboratory and field studies have found liver and kidney to be important sites of As accumulation in freshwater fish. For example, juvenile rainbow trout (*O. mykiss*) exposed to dietary sodium arsenate at a dose of 104 μg As/g food for 16 weeks accumulated significantly higher concentrations of As in liver (3.25 μg/g, w.w. calc.) and kidney (5.71 μg/g, w.w. calc.) as compared to fish fed a control diet (Cockell et al., 1991). Brown trout collected from the Clark Fork River also accumulated significantly higher concentrations of As in liver (0.32 μg/g, w.w. calc.) and kidney (2.26 μg/g, w.w. calc.) as compared to fish collected from a reference site (Farag et al., 1995). The highest concentrations of As observed in the liver (1.14 and 1.81 μg/g, w.w. in LWF and LT, respectively) and kidney (0.82 and 1.59 μg/g, w.w. in LWF and LT,

respectively) of lake trout and lake whitefish were similar to those observed in the previous studies, however, higher concentrations were observed in the liver as opposed to the kidney in the current study.

Comparisons between the accumulation and distribution of As in lake whitefish and lake trout tissues are difficult to make due to the reduction in feed intake. However, modified feeding behavior was not observed in either species fed the 100 μg As/g NS food. Therefore, modified feeding behavior was not a confounding factor on the results for these two treatment groups and valid comparisons between them can be made. The distribution of As in tissues of lake whitefish fed the 100 μg As/g NS food was as follows: pyloric caeca (1.6 μg/g, w.w.) > liver (1.1 μg/g, w.w.) > bile (0.60 μg/g, w.w.) > muscle (0.41 μg/g, w.w.) > kidney (0.29 μg/g, w.w.) > intestine (0.13 μg/g, w.w.) > stomach (0.11 μg/g, w.w.) > gallbladder (0.07 μg/g, w.w.). Concentrations of As were only significant in liver and intestine when compared to lake whitefish fed the control NS food.

In comparison, the pattern of As distribution in tissues of lake trout fed the 100 μg As/g NS food was different from that of lake whitefish and was as follows: bile (2.3 μg/g, w.w.) > pyloric caeca (1.8 μg/g, w.w.) = liver (1.8 μg/g, w.w.) > kidney (1.1 μg/g, w.w.) > intestine (0.87 μg/g, w.w.) > gallbladder (0.79 μg/g, w.w.) > muscle (0.59 μg/g, w.w.) > stomach (0.56 μg/g, w.w.). In this case, concentrations of As in pyloric caeca, liver, kidney, intestine, and stomach were significantly higher than in the same tissues of lake trout fed the control NS diet. The differences in As accumulation and distribution between lake whitefish and lake trout may be due to variation in uptake, storage, and elimination of As in the two species (Lowe et al., 1985). For example, pronounced

mucosal sloughing of the GI tract was observed in all lake whitefish fed As contaminated diets beginning on day 3 of exposure. The sloughing was not observed to occur in lake trout. Lake whitefish may be able to decrease the entry of As into the GI tract by increasing mucus production in response to As exposure. As is known to accumulate in the mucosal tissue of chick intestines (Fullmer and Wasserman, 1985). Continuous production and shedding of mucus layers in the GI tract can, therefore, substantially decrease absorption of As (Langston and Spence, 1995).

Reduced growth as a result of both aqueous and dietary As exposure has been previously observed in a number of organisms, including freshwater fish, and mallards (Gilderhaus, 1966; Pandey and Shukla, 1982; Oladimeji et al., 1984b; Camardese et al., 1990; McGeachy and Dixon, 1990; Cockell et al., 1991; Hoffman et al., 1992; Rankin and Dixon, 1994; Stanley et al., 1994). In the current study, decreases in growth were evident in the majority of lake whitefish and lake trout fed As contaminated diets. However, a significant reduction in growth only occurred in lake trout fed the 1000 μg As/g WS food. The lack of significant differences in other groups may be due to inherent species differences and/or the high degree of variability within treatment groups.

The reduction in growth observed in the current study may be due primarily to reduced feed consumption, however, there is increasing evidence that As has a direct effect on growth, independent of an effect mediated by feed refusal (Cockell et al., 1991; Rankin and Dixon, 1994; Rowe et al., 1998; Hopkins, et al., 1999). For example, Cockell et al. (1991) conducted a pair-feeding study in which juvenile rainbow trout (*O. mykiss*) were fed diets containing 0, 49, or 182 μg As/g as sodium arsenate. The feed intake of the 49 and 182 μg As/g treatment groups was determined and the same weight of the 0

μg As/g diet was fed to additional groups of fish, which were the "pair-fed control" groups. Cockell et al. (1991) observed a reduction in growth in the "pair-fed control" groups, and a further reduction in growth in the fish fed comparable amounts of As contaminated diets, indicating As had a direct effect on growth independent of feed refusal.

Studies that have compared the standard metabolic rates (SMR) of bullfrog tadpoles (*Rana catesbeiana*) (Rowe et al., 1998) and banded water snakes (*Nerodia fasciata*) (Hopkins et al., 1999) collected from a coal ash deposition basin and a downstream drainage swamp to the SMR of tadpoles and snakes collected from a reference pond, have shed light on the cause of reduced growth in As exposed organisms. Sediments at the polluted site contained concentrations of As as high as 116.6 μg/g (d.w.) (Rowe et al., 1996). Tadpoles and snakes collected from the polluted sites had 40%-97%, and 32% higher SMR, respectively, than animals collected from the reference site, indicating that maintenance costs were substantially increased in individuals exposed to coal combustion wastes (Rowe et al., 1998; Hopkins et al., 1999). In ectotherms, maintenance can account for greater than 80% of the total energy budget (Congdon et al., 1982), therefore, an increase in allocation of energy to maintenance will reduce the total amount of energy available to production processes, including growth. Individuals could compensate for the increased energy required for maintenance by increasing the total amount of energy assimilated, however, reduced feed consumption as a result of modified feeding behavior makes this possibility unlikely. Therefore, one of the end effects in As exposed organisms is decreased growth, as observed in lake whitefish and lake trout in the current study. Rowe et al. (1998) and Hopkins et al. (1999) have speculated that an

increase in energetically costly processes including, transport and excretion of pollutants, stress-protein formations, and/or cellular repair mechanisms, resulted in the elevated metabolic rates observed in organisms exposed to coal combustion wastes, including As. Similar energetically costly processes likely occurred in lake whitefish and lake trout exposed to dietary As.

Typically, LSI have not been measured in experiments that have examined the effects of dietary As exposure on freshwater fish (Oladimeji et al., 1984b; Cockell and Hilton, 1988; Cockell et al., 1991; Cockell et al., 1992; Cockell and Bettger, 1993). However, McGeachy and Dixon (1990) measured LSI of rainbow trout (*O. mykiss*) exposed to aqueous sodium arsenate at concentrations of 0, 1.5, 18, and 36 mg/L at 5°C and 0, 1.5, 9, and 18 mg/L at 15°C for 11 weeks and found no significant differences in LSI between control and exposed fish at either temperature. Although a decrease in LSI was observed in mallard (*Anas platyrhynchos*) ducklings fed a diet containing 200 μg As/g as sodium arsenate for 4 weeks without the occurrence of feed refusal (Hoffman et al., 1992), the significant reduction in LSI of lake whitefish fed the 100 and 1000 μg As/g WS food and lake trout fed 1000 μg As/g NS food is likely due to the reduction in feed intake observed in the current study. Steffens (1989) reported that the hepatic effects of reduced feed intake and starvation become apparent quickly in fish. For example, Albrecht (1967 in Steffens, 1989) found that the liver weight of 1-2 year old carp (*Cyprinus carpio*) decreased by 30% after 7 days of fasting, and by over 40% after 15 days. Lake whitefish fed the 1000 μg As/g WS food and lake trout fed the 1000 μg As/g NS food were starved for 12 and 10 days, respectively, as a result of complete feed

refusal. This is likely the main factor contributing to the decreased LSI observed in the current study.

Exposure of freshwater fish to As has previously resulted in moderate anemia (Oladimeji et al., 1984b; Goel and Sharma, 1987; Cockell et al., 1991; 1992), however, no significant alterations in blood parameters of lake whitefish or lake trout were observed. This is likely due to the short duration of exposure to As in the current study. For example, rainbow trout (*Salmo gairdneri*) exposed to 10, 20, and 30 μg As/g diets did not show significant decreases in hemoglobin concentration until after 8 weeks of exposure (Oladimeji et al., 1984b). Cockell et al. (1992) observed reduced blood hemoglobin concentrations, red blood cell counts, hematocrit, MCH, and MCHC in juvenile rainbow trout (*O. mykiss*) fed a 55 μg As/g diet, however, these parameters were only measured after 8 weeks of exposure, therefore, it is not known when these alterations began. If a particular level of As must be maintained in blood for As to cause an effect on blood parameters, the absence of an effect in the current study may also be due to the feed refusal observed. Fasting of fish, however, is reported to cause an initial rise in RBC count, hemoglobin content, and hematocrit due to water loss. Afterwards, these parameters show a continual decline (Steffens, 1989). Neither effect was observed in the current study, even though complete feed refusal occurred for as long as 12 days in two treatment groups.

The most pronounced difference between lake whitefish and lake trout exposed to dietary As is the presence or absence of histological alterations in the tissues examined. Histopathology was observed in five of the eight tissues examined from lake whitefish, including gallbladder, liver, kidney, pyloric caeca, and intestine, whereas no alterations

occurred in the tissues examined from lake trout. It must be noted, however, that gallbladders of lake trout were not assessed. Previous studies have found gallbladder lesions to be the most sensitive and reliable indicator of chronic dietary As toxicity in rainbow trout (*O. mykiss*) (Cockell, 1990). The absence of histological damage in tissues of lake trout was, however, not surprising, as previous studies examining the effects of both aqueous and dietary As exposures in rainbow trout have reported no histological changes in kidney, liver, spleen, stomach, pyloric caeca, or intestine (Cockell, 1990; McGeachy and Dixon, 1990; Rankin and Dixon, 1994). Interestingly, lake whitefish appear to be more sensitive to dietary As exposure, with respect to histological effects, than either lake trout or rainbow trout.

The macroscopic lesions observed in gallbladders of lake whitefish and lake trout exposed to all of the As contaminated diets had the same characteristics as lesions previously described for rainbow trout (*O. mykiss*) exposed to aqueous sodium arsenite (Rankin and Dixon, 1994) and sodium arsenate (McGeachy and Dixon, 1990), as well as dietary sodium arsenate (Cockell et al., 1991). Histological alterations, including sloughing of the epithelium, edema and fibrosis of the submucosal tissues, observed in gallbladders of lake whitefish have also been previously described for juvenile rainbow trout (*O. mykiss*) exposed to concentrations of dietary arsenate ranging from 33 to 182 μg As/g (Cockell et al., 1991).

Both laboratory and field exposures of fish to As have resulted in similar alterations in liver and kidney as described in the current study for lake whitefish (Gilderhaus, 1966; Sorenson et al., 1979a; 1979b; Sorenson et al., 1980; Chang et al., 1998; Kotsanis and Iliopoulou-Georgudaki, 1999). Other liver alterations, including fatty

infiltration, central or focal necrosis, cirrhosis, cytoplasmic vacuolation, nonspecific autolytic changes, hemosiderin granules, necrotic and fibrous bodies and nuclear and cytoplasmic As inclusions, have also been reported for green sunfish (*Lepomis cyanellus*) exposed to aqueous concentrations of sodium arsenate for various durations (Sorenson, 1991).

Histological alterations in pyloric caeca and intestine of lake whitefish were also observed in the current study. Similar changes have not been previously reported for freshwater fish exposed to As, however, gastrointestinal hemorrhage and necrosis were observed in monkeys given arsenate for 2 weeks, and inflammation of the small intestine was observed after 4 weeks of exposure (ATSDR, 1999).

MT is a low molecular weight, cysteine-rich, metal-binding protein that has been identified in vertebrates, invertebrates, and microorganisms (Mason and Jenkins, 1995). It functions in the regulation of the essential metals Zn and Cu and the detoxification of these essential metals and certain non-essential metals, including Cd and Hg (Roesijadi, 1992). MT induction has been promoted for use in environmental monitoring programs as a biomarker of metal exposure (Benson et al., 1990; Couillard, 1997; AETE, 1999). Previous studies have shown As induces hepatic MT in freshwater fish (Schlenk et al., 1997; Das et al., 1998; Eller-Jessen and Crivello, 1998a; 1998b). For example, channel catfish (*Ictalurus punctatus*) were treated with monosodium methyl arsonate (MSMA), sodium arsenite, and sodium arsenate. Fish were exposed to 0.01, 0.1, and 1.0 mg/L of each compound for 1 week. Exposure to all compounds resulted in dose-dependent induction of hepatic MT, with significant induction occurring in fish exposed to 1.0 mg/L of MSMA and sodium arsenite (Schlenk et al., 1997). In the current study, however,

significant induction of MT in liver or kidney of lake whitefish or lake trout did not occur.

The lack of MT induction in lake whitefish and lake trout liver and kidney may be due to one or more of the following reasons: (1) a progressive decline in As dose, (2) the length of exposure, (3) the induction of MT in other organs, and/or (4) the small sample size and high variability within treatment groups. First, due to reduced feed consumption of fish fed the As contaminated diets, the dosage of As was continually decreasing and eventually ceased in some treatment groups. If a critical minimum concentration of As is necessary to cause induction of MT, it may not have been reached during this exposure. Also, the length of exposure to As contaminated diets may not have been long enough to evoke MT induction, as concentrations of As necessary for induction may not have been reached after 20 days. The third explanation for the lack of MT induction is the possible induction of MT in tissues of lake whitefish and lake trout, which were not examined. MT induction is greatest in tissues that are involved in the uptake, detoxification, and excretion of metals, including liver, kidney, and intestine of aquatic animals (Roesijadi, 1992; Roesijadi and Robinson, 1994). MT may have been induced in the pyloric caeca, or intestine of lake whitefish and lake trout, as significant accumulation occurred in these tissues, with particularly high accumulation present in the pyloric caeca of both species. Finally, the absence of significant MT induction in lake whitefish and lake trout tissues may be explained by the small sample size used in the experiment, as well as the high variability observed within treatment groups, particularly in the liver of both species. Both factors make it difficult to detect significant differences between treatment groups.

The peroxidation of cellular lipids by oxyradicals (free radicals) is known as lipid peroxidation (LPO). This process can alter the balance of fluidity and structure of subcellular and cellular membranes by damaging polyunsaturated fatty acids located in the membrane. The end result of LPO is often tissue damage and cell death (Winston and Di Giulio, 1991; Kehrer, 1993). Metals that exist in more than one valence state and undergo intracellular oxidative-reduction reactions can initiate lipid peroxidation (Wills, 1985). These characteristics apply to As, however, results from previous studies that have examined the effect of As on LPO in fish, birds and mammals have not been consistent. For example, Yanez et al., (1991) found 10 μg/g of As to significantly induce LPO in heart tissue of rats after 24 hours, whereas Schinella et al. (1996) exposed rats to the same concentration of As for 2 days and induction of LPO in liver was not observed. In addition, Camardese et al. (1990) found LPO decreased in liver of mallard (*A. platyrhynchos*) ducklings exposed to diets containing 30, 100, or 300 μg As/g for 10 weeks. Hepatic LPO of channel catfish (*Ictalurus punctatus*) exposed to 0.01, 0.1, and 1.0 mg/L each of MSMA, sodium arsenite, and sodium arsenate was unaltered by any of the As treatments (Schlenk et al., 1997). Finally, hepatic LPO was induced in climbing perch (*Anadas testudineus*) exposed to 1.5 mg/L of As for 48 h, however, significant induction was not present after 30 days of exposure to 0.75 mg/L.

In the current study, a significant increase in LPO was only observed in the plasma of lake trout fed the 1000 μg As/g WS food. The induction of LPO in this treatment group may be due to feed refusal as opposed to As exposure, as starvation stimulates LPO in mammals (Shaheen et al., 1996). Feed refusal also occurred in lake whitefish, however, inherent differences between species may account for the lack of

LPO induction in lake whitefish. However, the lack of correlation between increased LPO concentrations and histopathological damage in lake whitefish is not consistent with the observations of Cooley et al. (2000), in experiments that examined the effects of dietary uranium exposure on lake whitefish.

The absence of LPO induction observed in the majority of treatment groups may be due to one or more of the following reasons. (1) LPO may have been induced in other tissues, such as liver, and the concentrations present in plasma were not high enough to detect significant differences. (2) As a result of feed refusal and/or length of exposure, the concentrations of As in tissues of lake whitefish and lake trout may not have been high enough to induce LPO. (3) LPO may have been initially increased and then declined, so that it was not present in plasma at the time of sampling. (4) Significant differences between treatment groups may not have been observed due to the small sample size used in the experiment, and the high variability observed within treatment groups.

5. Conclusions

In conclusion, modified feeding behavior occurred in lake whitefish and lake trout fed all of the As contaminated diets, with the exception of the 100 μg As/g NS food. Therefore, the addition of brine shrimp to the diets did not increase feed consumption of As. There were a number of differences between the pattern of As distribution in tissues of lake trout and lake whitefish fed the 100 μg As/g NS food for 20 days. At the molecular level of organization, the parameters examined were not altered by dietary As exposure, with one exception. LPO concentrations significantly increased in the plasma

of lake trout fed the 1000 μg As/g WS food for 20 days. At the tissue and organ level, LSI were significantly decreased in both species due primarily to feed refusal. Blood parameters were not affected by As exposure in either species. There was a dramatic difference in the histological alterations observed in the tissues of lake whitefish and lake trout, with lake whitefish being the more sensitive species. At the whole organism level of organization, decreased growth was only significant in lake trout fed the 1000 μg As/g WS food, however, growth also appeared to be considerably lower in lake whitefish fed As contaminated diets. Condition factors were not affected by As exposure in either species.

Based on these results, particularly the greater sensitivity to histological damage, lake whitefish were selected for use in a longer-term experiment that examined the accumulation, distribution, and toxicological effects of dietary As at 10, 30, and 64 days of exposure. Concentrations of As exposure chosen for the study were 0, 1, 10, and 100 μg As/g to reduce the risk that feed refusal would not occur. Further discussion of the accumulation and distribution of As in tissues of lake whitefish, as well as mechanisms of As uptake, can be found in Chapter 2. The toxicological effects of dietary As exposure in lake whitefish, including blood parameters, MT induction, LPO, and histopathology of liver and gallbladder are discussed in more detail in Chapter 3.

Table 1. A summary of when modified feeding behavior and/or complete feed refusal were observed over the course of a 20-day exposure of lake whitefish (LWF) and lake trout (LT) to 0, 100 and 1000 μg As/g NS (no shrimp) food and 0, 100 and 1000 μg As/g WS (with shrimp) food.

Species	Treatment Group		Modified Feeding Behavior Began	Complete Feed Refusal Began
	Dose (μg As/g)	NS or WS		
LWF	0	NS	-	-
LWF	100	NS	-	-
LWF	1000	NS	day 3	-
LWF	0	WS	-	-
LWF	100	WS	day 8	-
LWF	1000	WS	day 3	day 8
LT	0	NS	-	-
LT	100	NS	-	-
LT	1000	NS	day 6	day 10
LT	0	WS	-	-
LT	100	WS	day 8	-
LT	1000	WS	day 3	day 8

Table 2. The hematological parameters, hematocrit, hemoglobin concentration, red blood cell count (RBC #), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), of lake whitefish and lake trout fed two control diets (0 μg As/g NS, no shrimp and 0 μg As/g WS, with shrimp) and four As contaminated diets (100 and 1000 μg As/g NS; 100 and 1000 μg As/g WS) for a 20-day period. Data are expressed as mean (± SE).

Blood Parameter	Lake Whitefish						Lake Trout					
	NS Diet (μg As/g)			WS Diet (μg As/g)			NS Diet (μg As/g)			WS Diet (μg As/g)		
	0	100	1000	0	100	1000	0	100	1000	0	100	1000
RBC (10^6/mL)	0.94 (0.07)	0.91 (0.04)	0.95 (0.19)	0.97 (0.08)	1.1 (0.18)	0.9 (0.05)	0.60 (0.04)	0.63 (0.04)	0.69 (0.07)	0.67 (0.12)	0.67 (0.12)	0.74 (0.08)
Hematocrit (%)	42.1 (2.53)	41.6 (3.69)	44.8 (2.50)	40.5 (2.22)	41.1 (2.06)	39.2 (0.441)	32.8 (1.13)	32.6 (1.34)	32.6 (0.966)	30.4 (1.25)	36.8 (0.520)	32.0 (2.52)
Hemoglobin (g/dL)	9.5 (0.50)	10 (0.51)	9.8 (0.99)	9.6 (0.26)	9.9 (0.53)	11 (0.97)	9.9 (0.36)	8.9 (0.34)	9.4 (0.39)	8.6 (0.38)	10 (0.40)	8.4 (0.46)
MCH (pg)	100 (5.9)	110 (8.5)	110 (16)	100 (9.1)	110 (22)	120 (6.1)	170 (8.3)	140 (7.6)	140 (16)	140 (22)	170 (46)	120 (12)
MCHC (%)	23 (1.2)	25 (2.3)	22 (1.7)	24 (1.0)	24 (1.5)	27 (2.3)	30 (0.15)	27 (0.47)	29 (1.0)	28 (0.26)	27 (1.4)	26 (0.86)
MCV (fl)	460 (51)	460 (31)	520 (87)	420 (37)	440 (93)	440 (23)	550 (29)	520 (20)	490 (50)	490 (75)	630 (160)	440 (40)

Table 3. Metallothioncin (MT) concentrations in the livers and kidneys of lake whitefish and lake trout and lipid peroxide (LPO) concentrations in the plasma of lake whitefish and lake trout fed two control diets (0 μg As/g NS, no shrimp and 0 μg As/g WS, with shrimp) and four As contaminated diets (100 and 1000 μg As/g NS; 100 and 1000 μg As/g WS) for a 20-day period. Data are expressed as mean (± SE). Asterisks denote means are significantly different from the control NS group or the control WS group ($P < 0.05$).

Parameter	Lake Whitefish						Lake Trout					
	NS Diet (μg As/g)			WS Diet (μg As/g)			NS Diet (μg As/g)			WS Diet (μg As/g)		
	0	100	1000	0	100	1000	0	100	1000	0	100	1000
Liver MT (μg/g w.w.)	343 (102)	362 (65.0)	348 (114)	381 (88.0)	429 (119)	491 (102)	868 (218)	1090 (261)	1030 (118)	936 (302)	1270 (303)	957 (159)
Kidney MT (μg/g w.w.)	18.7 (2.89)	23.7 (5.07)	30.3 (8.23)	26.6 (4.46)	22.1 (5.52)	16.9 (2.47)	21.3 (1.43)	34.8 (2.55)	37.7 (7.99)	21.1 (3.69)	30.7 (7.20)	29.5 (1.34)
LPO (nmol/mL)	2.6 (0.28)	4.0 (1.0)	2.5 (0.50)	4.9 (1.6)	2.1 (0.49)	4.0 (0.82)	1.0 (0.00)	2.2 (0.71)	1.0 (0.00)	1.7 (0.39)	3.3 (0.22)	4.3* (0.78)

Figure 1. The growth of a) lake whitefish and b) lake trout fed two control diets (0 μg As/g NS, no shrimp and 0 μg As/g WS, with shrimp) and four As contaminated diets (100 and 1000 μg As/g NS; 100 and 1000 μg As/g WS) for a 20-day period. Growth was calculated as the percentage change in wet weight from the initial body weight. Data are expressed as mean (± SE). Asterisks denote means are significantly different from the control NS group or the control WS group ($P < 0.05$).

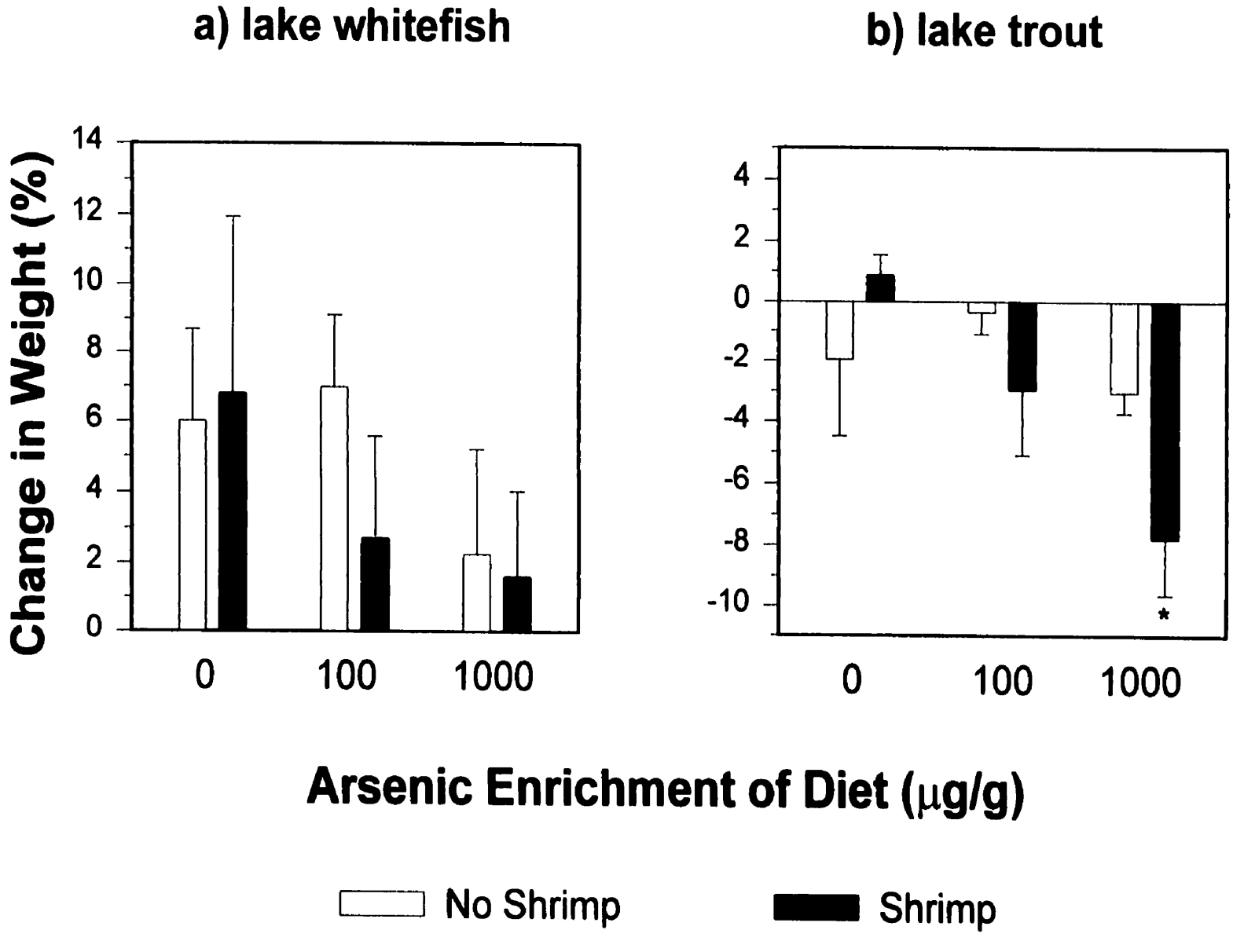

a) lake whitefish
b) lake trout
Change in Weight (%)
14
12
10
8
6
4
2
0
4
2
0
-2
-4
-6
-8
-10
*
0
100
1000
0
100
1000
Arsenic Enrichment of Diet (µg/g)
No Shrimp
Shrimp

Figure 2. The liver somatic indices of a) lake whitefish and b) lake trout fed two control diets (0 μg As/g NS, no shrimp and 0 μg As/g WS, with shrimp) and four As contaminated diets (100 and 1000 μg As/g NS; 100 and 1000 μg As/g WS) for a 20-day period. Data are expressed as mean (± SE). Asterisks denote means are significantly different from the control NS group or the control WS group ($P < 0.05$).

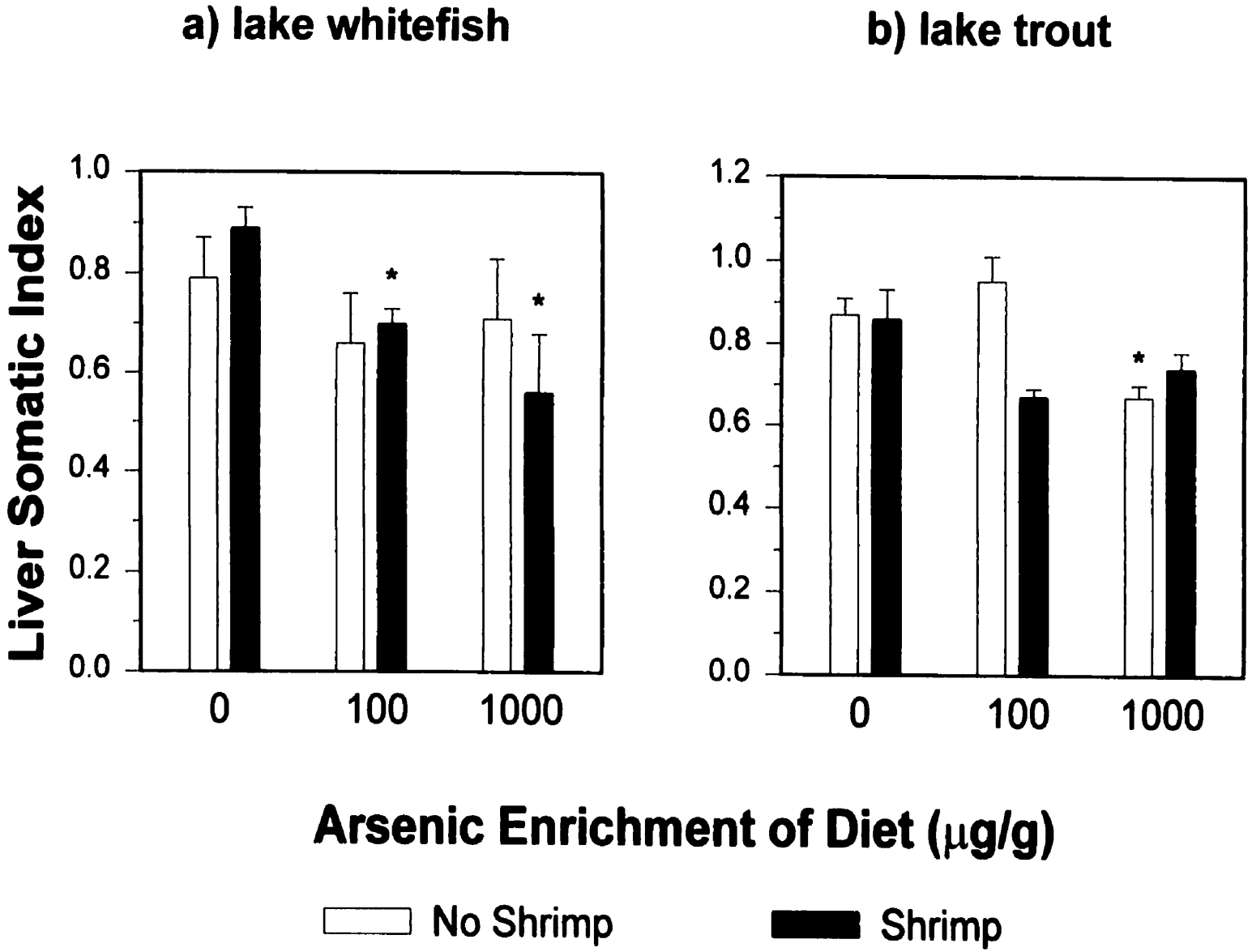

a) lake whitefish
b) lake trout
Liver Somatic Index
1.0
0.8
0.6
0.4
0.2
0.0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
0
100
1000
0
100
1000
Arsenic Enrichment of Diet (µg/g)
No Shrimp
Shrimp

Figure 3. The accumulation of As in the stomachs of a) lake whitefish and b) lake trout, the pyloric caeca of c) lake whitefish and d) lake trout, and the intestines of e) lake whitefish and f) lake trout fed two control diets (0 μg As/g NS, no shrimp and 0 μg As/g WS, with shrimp) and four As contaminated diets (100 and 1000 μg As/g NS; 100 and 1000 μg As/g WS) for a 20-day period. Data are expressed as mean (± SE). Asterisks denote means are significantly different from the control NS group or the control WS group ($P < 0.05$).

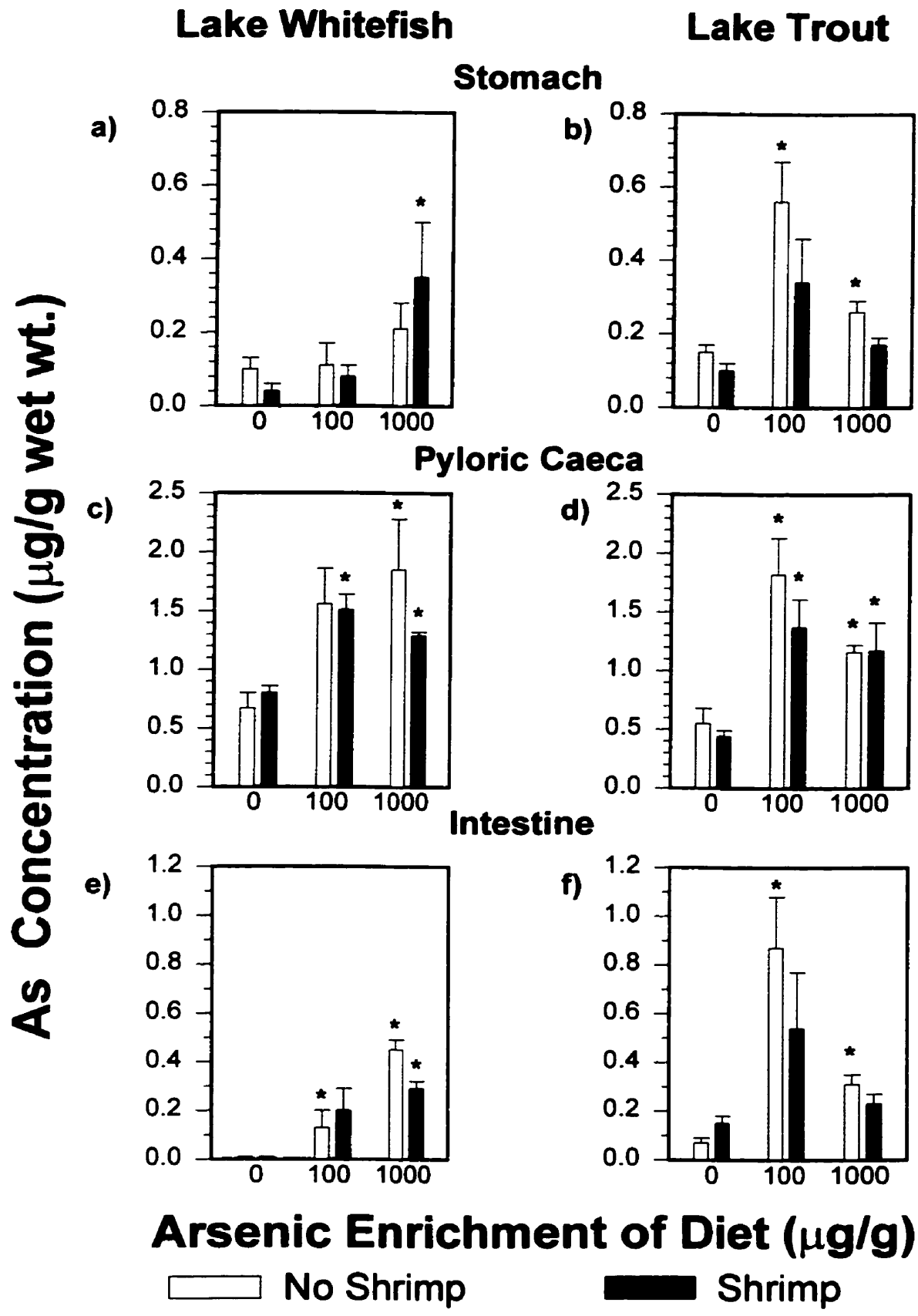

Lake Whitefish
Lake Trout
Stomach
Pyloric Caeca
Intestine
a)
b)
c)
d)
e)
f)
As Concentration (µg/g wet wt.)
Arsenic Enrichment of Diet (µg/g)
No Shrimp
Shrimp
0
100
1000

Figure 4. The accumulation of As in the livers of a) lake whitefish and b) lake trout and the kidneys of c) lake whitefish and d) lake trout fed two control diets (0 μg As/g NS, no shrimp and 0 μg As/g WS, with shrimp) and four As contaminated diets (100 and 1000 μg As/g NS; 100 and 1000 μg As/g WS) for a 20-day period. Data are expressed as mean (± SE). Asterisks denote means are significantly different from the control NS group or the control WS group ($P < 0.05$).

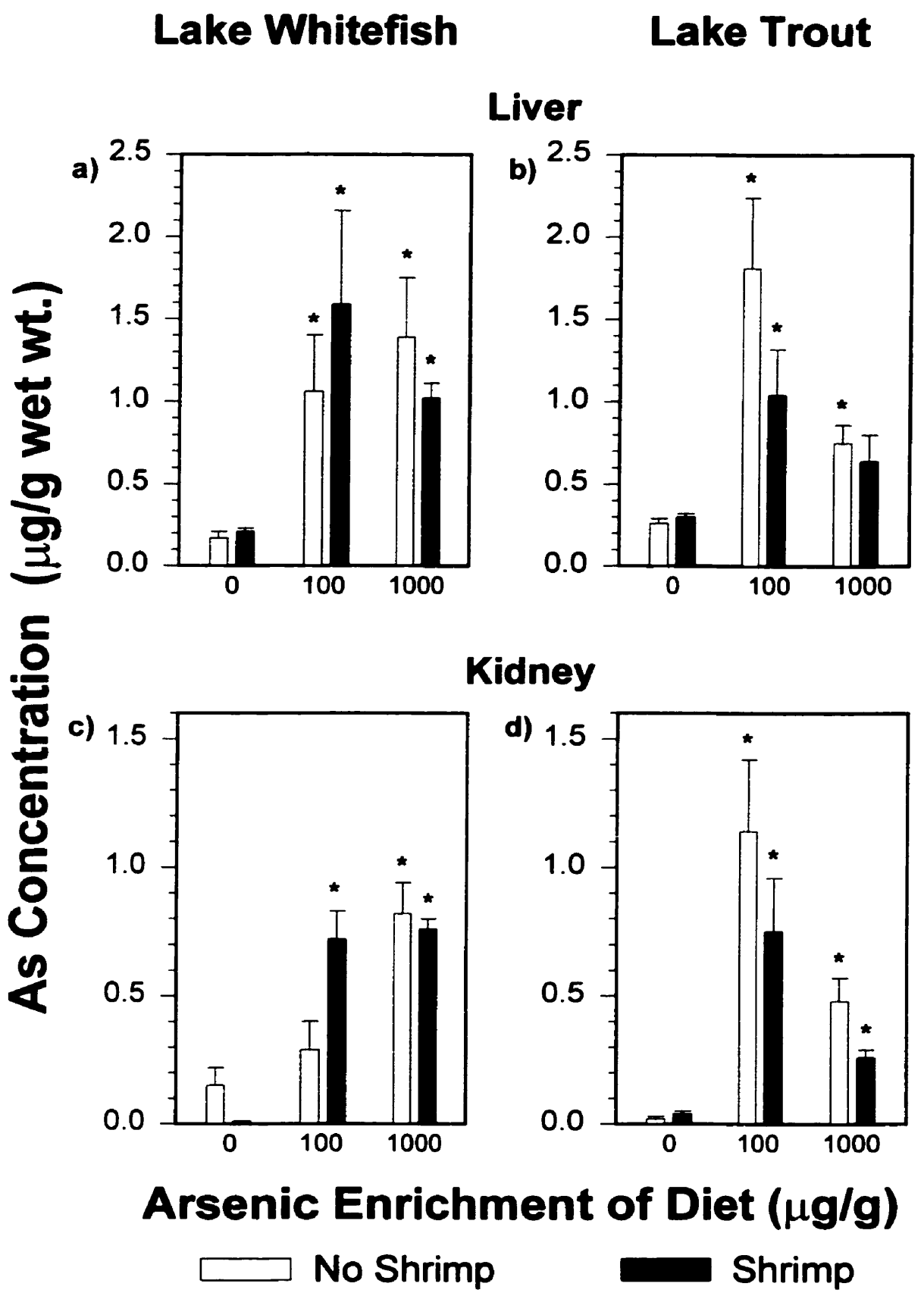

Lake Whitefish
Lake Trout
Liver
Kidney
a)
b)
c)
d)
As Concentration (µg/g wet wt.)
2.5
2.0
1.5
1.0
0.5
0.0
0
100
1000
Arsenic Enrichment of Diet (µg/g)
No Shrimp
Shrimp

Figure 5. The accumulation of As in the gallbladders of a) lake whitefish and b) lake trout and the bile of c) lake whitefish and d) lake trout fed two control diets (0 μg As/g NS, no shrimp and 0 μg As/g WS, with shrimp) and four As contaminated diets (100 and 1000 μg As/g NS; 100 and 1000 μg As/g WS) for a 20-day period. Data are expressed as mean (± SE). Asterisks denote means are significantly different from the control NS group or the control WS group ($P < 0.05$).

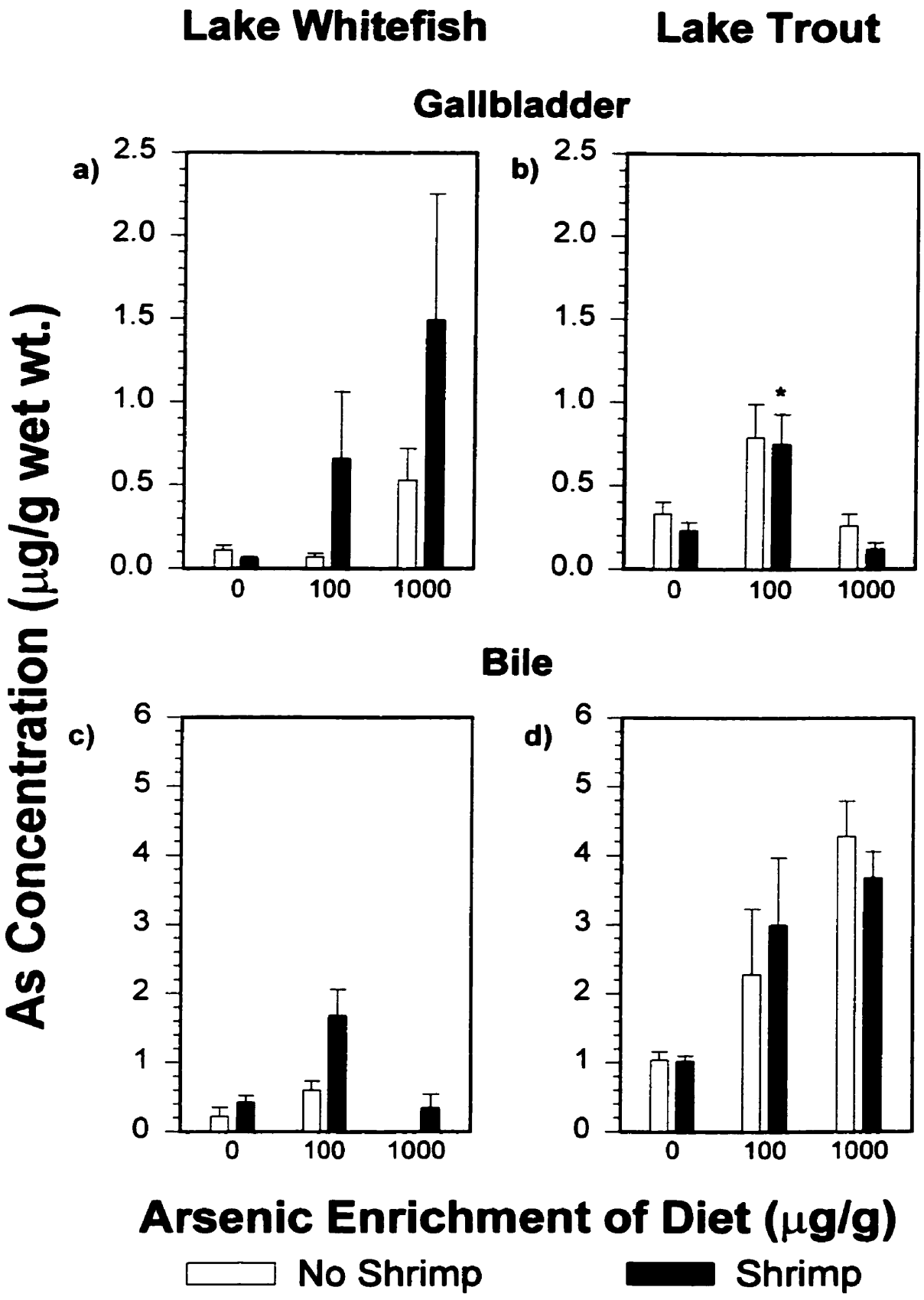
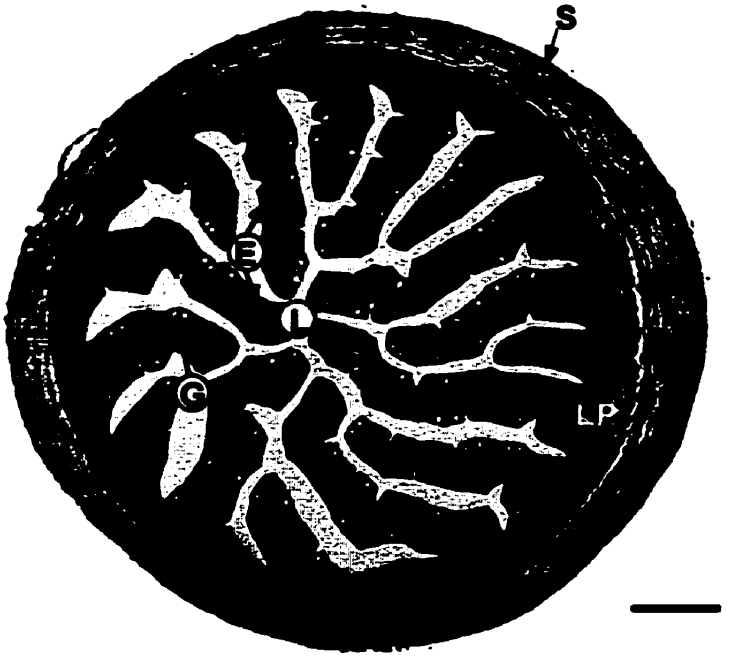

Lake Whitefish
Lake Trout
Gallbladder
a)
b)
Bile
c)
d)
As Concentration (µg/g wet wt.)
2.5
2.0
1.5
1.0
0.5
0.0
6
5
4
3
2
1
0
0
100
1000
*
Arsenic Enrichment of Diet (µg/g)
No Shrimp
Shrimp

Figure 6. Low magnification micrographs of pyloric caeca from lake whitefish fed a) an uncontaminated NS (no shrimp) diet and b) a 1000 μg As/g NS diet for a 20-day period. L = lumen, E = epithelium, LP = lamina propria, MC = muscularis circularis, S = serosa, and G = goblet (mucous) cells. Note the increase in number of goblet cells in the epithelium and increased width of the lamina propria in the pyloric caeca from the exposed lake whitefish. Bar = 160 μm. H&E stain.

a)



b)

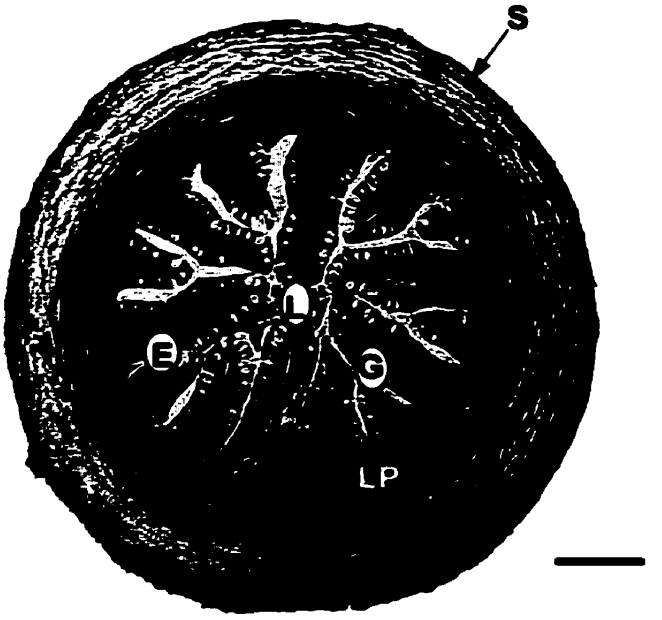
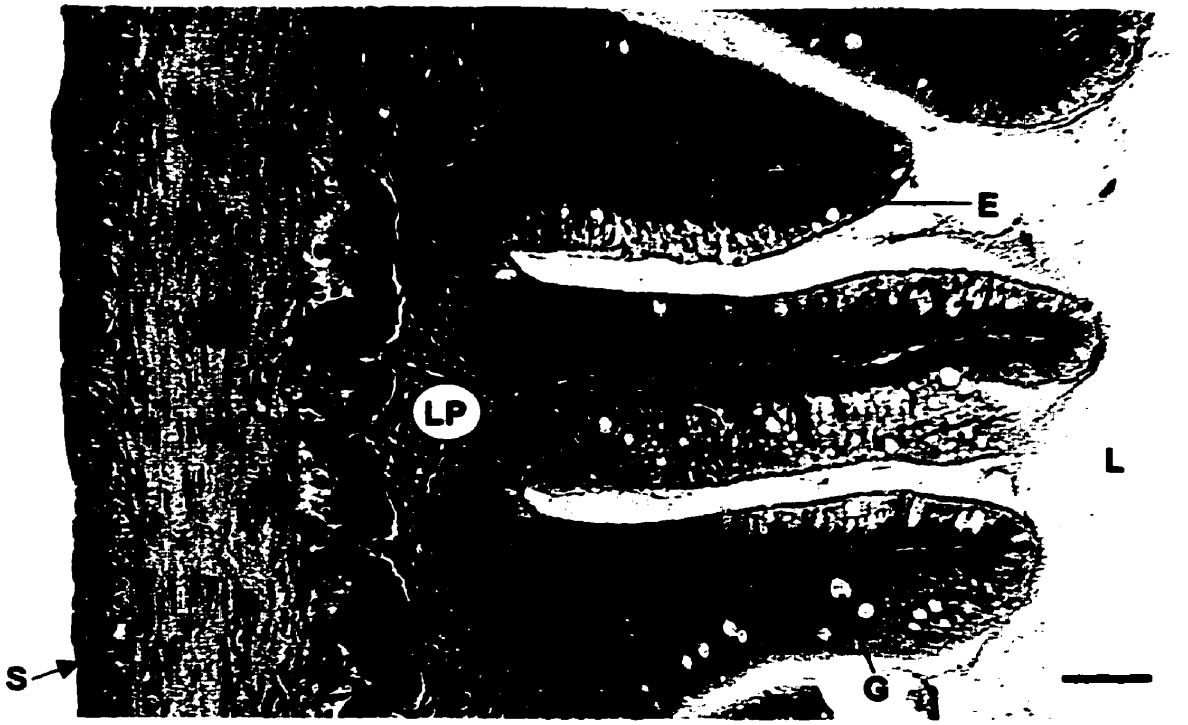


Figure 7. High magnification micrographs of intestine from lake whitefish fed a) an uncontaminated NS (no shrimp) diet and b) a 1000 μg As/g NS diet for a 20-day period. L = lumen, E = epithelium, LP = lamina propria, SC = stratum compactum, SG = stratum granulosum, MC = muscularis circularis, ML = muscularis longitudinalis, S = serosa, and G = goblet (mucous) cells. Note the increase in number of goblet cells in the epithelium and increased width of the lamina propria in the intestine from the exposed lake whitefish. Bar = 60 μm. H&E stain.

a)



b)

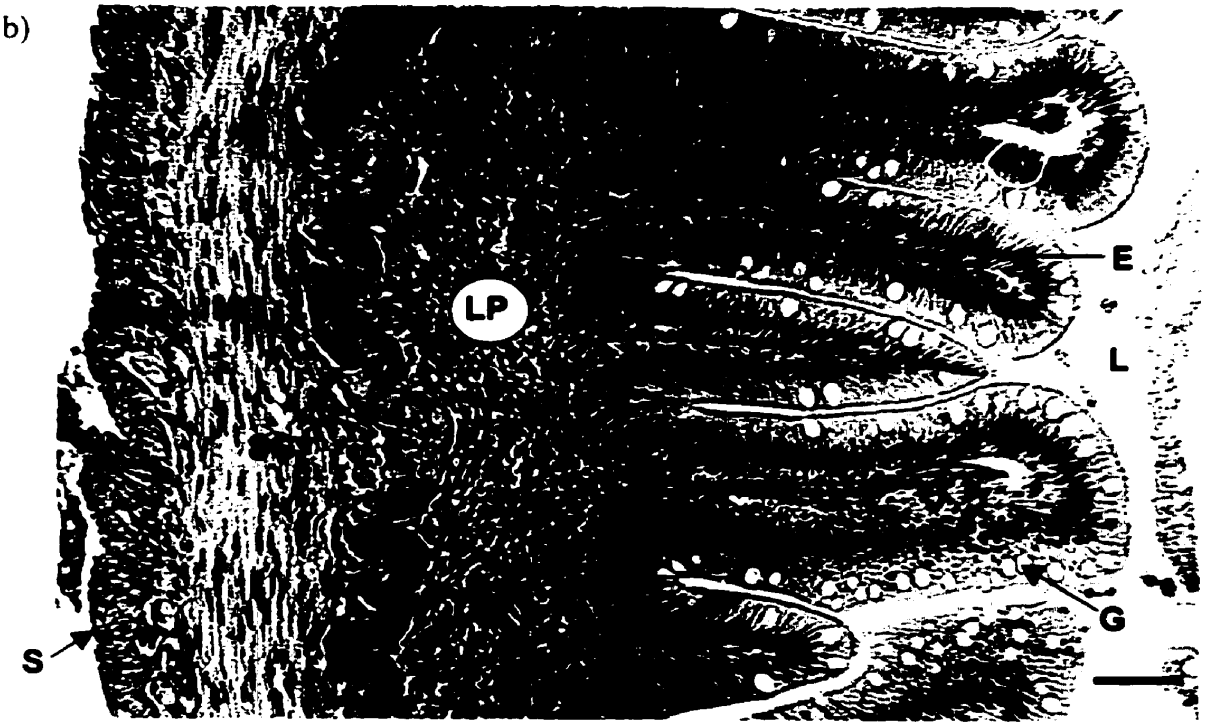
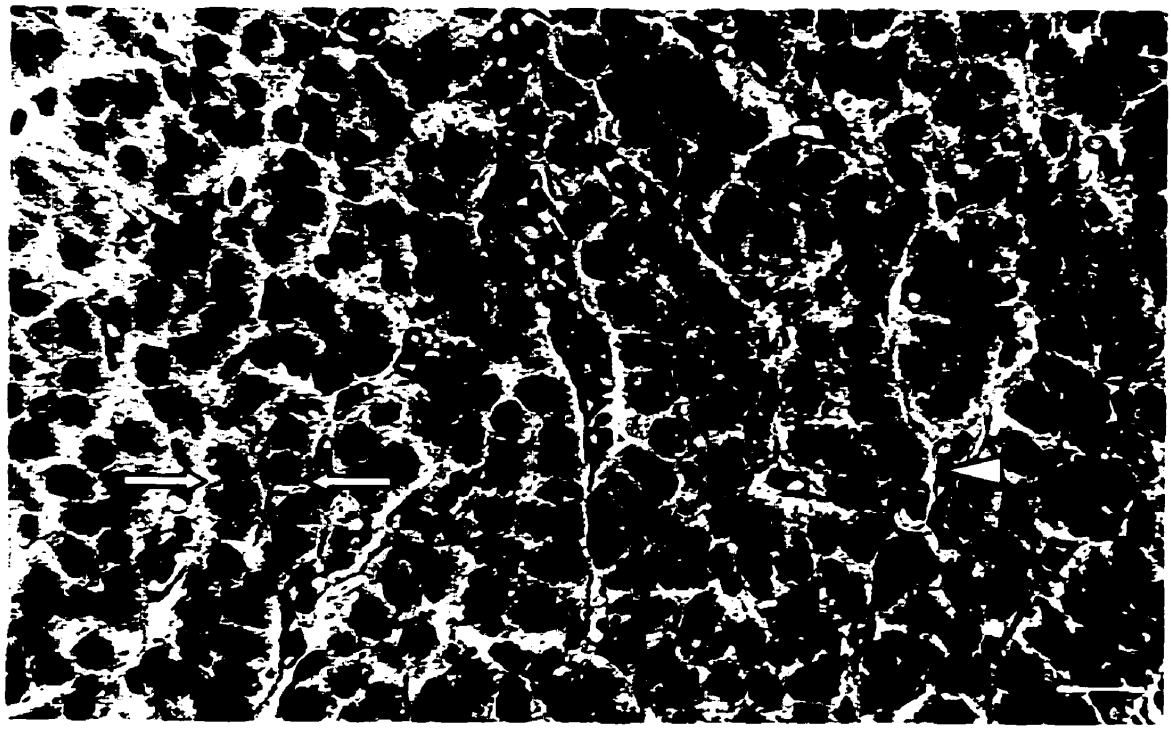


Figure 8. a) High magnification micrograph of a gallbladder from lake whitefish fed an uncontaminated NS (no shrimp) diet for a 20-day period demonstrating the lumen (L), epithelium (E), lamina propria (LP), lamina muscularis (LM), subserosa (SS), and serosa (S). Bar = 60 μm. b) High magnification micrograph of a gallbladder from lake whitefish fed a 100 μg As/g NS diet demonstrating the lumen (L), epithelium (E), lamina propria (LP), lamina muscularis (LM), subserosa (SS), and serosa (S). Note the increased width of the submucosal tissues (LP, LM, and SS) due to edema, and vascular dilation (VD). Bar = 60 μm. c) High magnification micrograph of a gallbladder from lake whitefish fed a 1000 μg As/g NS diet demonstrating the lumen (L), epithelium (E), lamina propria (LP), and lamina muscularis (LM). Note the inflammation (I), characterized by vascular dilation (VD) and presence of lymphocytes, as well as the fibrosis in the submucosal tissues. Bar = 60 μm. H&E stain.


a)
S
E
b)
S
E
L
c)
L
E
D
O

Figure 9. a) High magnification micrograph of a liver from lake whitefish fed an uncontaminated NS (no shrimp) diet for a 20-day period demonstrating the arrangement of hepatocytes in two-cell thick cords (between the block arrows), separated by liver sinusoids containing erythrocytes (arrowhead). Bar = 18 μm. b) High magnification micrograph of a liver from lake whitefish fed a 100 μg As/g NS diet demonstrating an area of focal necrosis, characterized by degenerating hepatocytes containing pyknotic nuclei (arrows), eosinophilic hepatocytes, and cellular debris (CD). Bar = 18 μm. H&E stain.

a)



b)

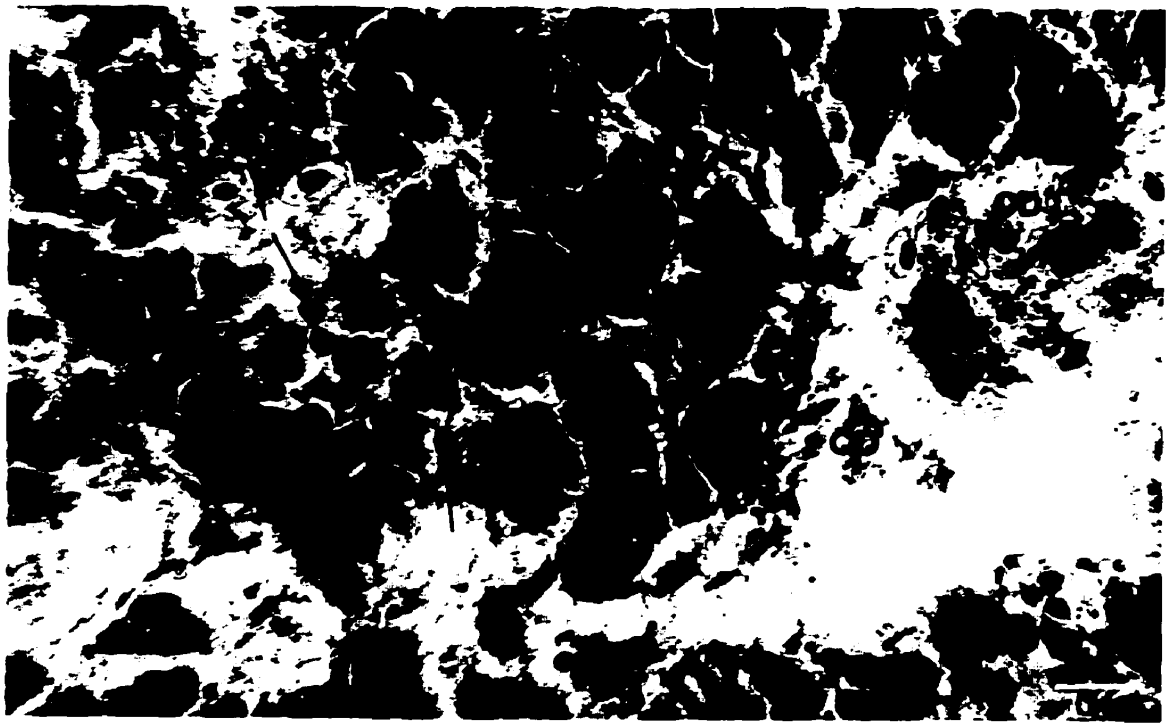
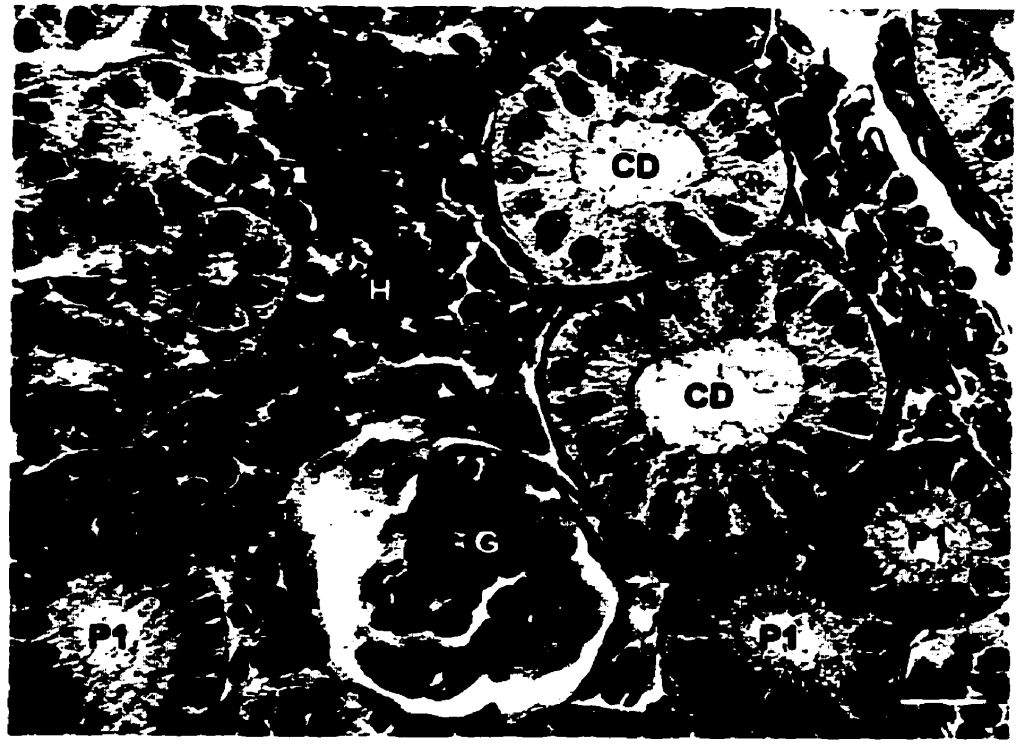
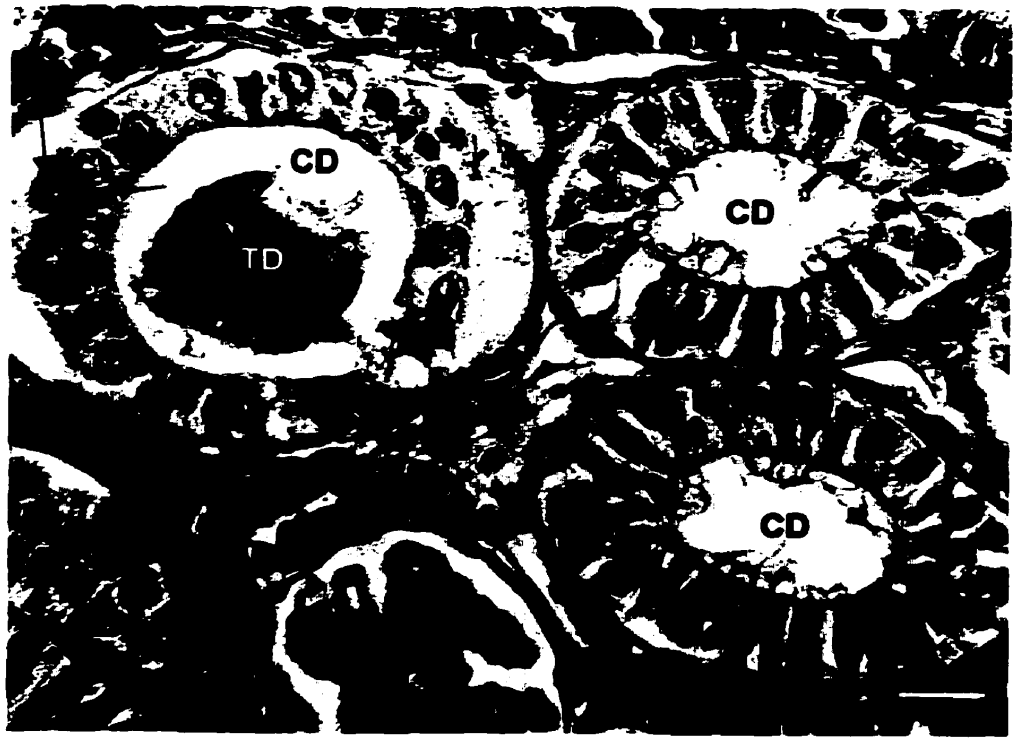


Figure 10. a) High magnification micrograph of a kidney from lake whitefish fed an uncontaminated NS (no shrimp) diet for a 20-day period demonstrating the first segment of the proximal tubule (P1), glomerulus (G), hematopoietic tissue (H), and collecting ducts (CD). Bar = 18 μm. b) High magnification micrograph of a kidney from lake whitefish fed a 100 μg As/g NS diet demonstrating small areas of cellular degeneration, characterized by pyknotic nuclei (arrows) and tubular debris (TD), in the collecting ducts (CD). Bar = 18 μm. H&E stain.

a)



b)



Chapter Two

Accumulation and distribution of arsenic in lake whitefish exposed by the dietary route of uptake (*Coregonus clupeaformis*).

Abstract

In contaminated habitats, freshwater fish are exposed to As through consumption of contaminated food organisms and sediments. Due to the need for dietary As research in freshwater fish species, adult lake whitefish (*Coregonus clupeaformis*) were fed As contaminated diets at nominal concentrations of 0, 1, 10, and 100 μg As/g food (d.w.) for 10, 30, and 64 days. Liver, kidney, stomach, intestine, pyloric caeca, gallbladder, skin and scales were analyzed for As content. The pattern of As accumulation in fish tissues was influenced by reduced feed consumption beginning on day 45 by fish fed the 100 μg As/g food. Significant As accumulation occurred in all tissues examined from fish exposed to the 100 μg As/g food for 30 days, with the exception of gallbladder. After 30 days of exposure, the highest concentration of As was observed in pyloric caeca of fish fed the 100 μg As/g food. Significant accumulation of As occurred in livers and scales of fish fed concentrations of As as low as 10 μg/g for 30 and 64 days. Muscle, gonad, spleen, gills, and bone of lake whitefish fed a control diet for 10 days and 100 μg As/g food for 10, 30, and 64 days were also analyzed for As content. As concentrations increased in gonads, spleen, and gills of fish fed the 100 μg As/g food for 30 days. Increased concentrations of As were observed in bone of fish fed the high dose food after each duration of exposure. As concentrations did not increase in muscle of fish after 10, 30, or 64 days of exposure. Analyses of As in pyloric caeca, intestine, liver and scales are recommended to evaluate the bioavailability of As to fish in environmental monitoring programs.

1. Introduction

The release of As into aquatic environments as a result of the mining, smelting, and refining of metals, as well as the burning of fossil fuels, is of great concern in both Canada and the United States (CEPA, 1993; ATSDR, 1998). The discharge of mining wastes into freshwater systems has resulted in extremely high concentrations of As in sediments and benthic invertebrates (Wagemann et al., 1978; CEPA, 1993; Azcue and Dixon, 1994; Brumbaugh et al., 1994; Woodward et al., 1994). For example, sediment As concentrations up to 3500 μg/g (d.w.) have been found in Kam Lake, Northwest Territories, due to long-term As contamination from gold mining activities. Invertebrate organisms in this lake were found to contain levels of As as high as 820 μg/g (d.w.) (Wagemann et al., 1978). Surficial sediment As concentrations exceeding 5000 μg/g (d.w) have been found in northern Saskatchewan lakes receiving effluents from uranium operations (Klaverkamp et al., 2000a), and in lakes near Red Lake, Ontario that have received gold-mining effluents for over 50 years (J.F. Klaverkamp, unpublished data).

As is biologically available to fish living in contaminated habitats. For example, rock bass (*Ambloplites rupestris*) collected from Moira Lake, Ontario, which contains up to 1000 μg As/g in sediments (Azcue and Nriagu, 1993), were found to have elevated concentrations of As in intestine and bones plus scales (Azcue and Dixon, 1994). Another example occurs in the Clark Fork River, Montana, which received large volumes of mining wastes from 1880 to 1972, resulting in high levels of As in sediments, 404 μg/g (d.w.) (Brumbaugh et al., 1994), and benthic invertebrates, 43.1 μg/g (d.w.), (Woodward et al., 1994). Brown trout (*Salmo trutta*) collected from a contaminated site on the Clark Fork River had significantly higher concentrations of As in gill, liver,

kidney, and pyloric caeca as compared to brown trout collected from a reference site (Farag et al., 1995).

The main route of As uptake by benthic-feeding fish is through ingestion of As contaminated sediments and food (Leland and Kuwabara, 1985; Clements, 1991; McIntosh, 1991; Sorenson, 1991; Handy, 1996; Bergman and Dorward-King, 1997; ATDSR, 1998). Lab experiments on direct exposure to waterborne As simulate acute-catastrophic spills of As contaminated effluents, whereas dietary exposures are more relevant to chronic conditions experienced by fish in effluent receiving waters. The majority of laboratory research on As exposure in freshwater fish has focused on uptake of waterborne As across the gills (Sorenson, 1976; 1979a; 1979b; McGeachy and Dixon, 1990; 1992), with the exception of work conducted by Cockell (1990). It is important, therefore, to conduct experiments that examine the accumulation and distribution of As in freshwater fish exposed to dietary As, in order to determine tissues that can be used as sensitive and reliable indicators of As bioavailability in environmental monitoring programs (AETE, 1999; Environment Canada, 1999). These types of experiments would also provide useful information to guide studies on As toxicity in freshwater fish.

The objective of this study was to examine the accumulation and distribution of As in lake whitefish (*Coregonus clupeaformis*) exposed by the dietary route of uptake, at environmentally relevant concentrations for three durations of exposure. Lake whitefish was chosen because it is a benthic feeder, is widely distributed across Canada, and is a very valuable commercial freshwater fish species, as well an important part of the sports fishery (Bodaly, 1986; Craig, 1997). The form of As incorporated into fish food was arsenate, as this is the most common form of As in oxic littoral zones of freshwater

habitats (Cullen and Reimer, 1989; AQUAMIN, 1996). Concentrations of As fed to lake whitefish were based on concentrations of As found in Canadian lakes and rivers and also on concentrations of As that lake whitefish would consume as determined in a preliminary experiment (Chapter 1).

2. Materials and Methods

2.1 Fish

Lake whitefish (*Coregonus clupeaformis*) were reared at the Freshwater Institute and were 4 years of age at the beginning of the experiment. Initial body weights and fork lengths were 334 ± 7.55 g and 29.2 ± 0.204 cm (mean ± SE), respectively. Fish were fed an amount of No. 3 trout pellets (Martin Feed Mills, Elmira, Ont.) equal to 0.5% of the total body weight per tank every Monday, Wednesday, and Friday of each week. Fish were acclimated to all experimental conditions for a 5-week period.

2.2 Tanks

Fish were randomly distributed, 6 fish/tank, among twelve, 200 L fiberglass tanks. Dechlorinated City of Winnipeg tap water, maintained at or below 8 μg Cl_2/L by activated charcoal filtration and ozonation (Wagemann et al., 1987), was supplied to the tanks. The tanks were individually aerated, and maintained on a one-pass water flow at 1.2 L/min, yielding 95% replacement of tank water in 9 hours (Sprague, 1973). Tank temperature, pH, and dissolved oxygen (DO) concentrations were measured every Monday, Wednesday, and Friday prior to feeding, and are expressed as mean ± SE. Temperature and pH were 10 ± 0.02 °C and 7.7 ± 0.02, respectively. DO concentration

was 11 ± 0.04 mg/L, which is 97 ± 0.40% of saturation. Concentrations of major ions, total anions and cations, organic acids, total dissolved inorganic and organic carbon, total suspended solids, conductivity, and alkalinity of water supplied to the tanks are presented in Cooley and Klaverkamp (2000). Photoperiod was held constant at 11.5 hours of light and 11.5 hours of darkness, with 30-minute periods of 'dusk' and 'dawn'.

2.3 Processing of Diets

As was obtained as disodium arsenate heptahydrate from Sigma Chemical Co. (St. Louis, MO). Aqueous disodium arsenate heptahydrate was incorporated into 3000 g of trout chow flour, containing 42% crude protein, 16% crude fat, 2% crude fiber, 5% ash, and 0.9% calcium (Martin Feed Mills, Elmira, Ont.), and 1600 mL of deionized distilled water (DDW) using a commercial electric mixer to produce nominal As concentrations of 1 μg As/g food, 10 μg As/g food, and 100 μg As/g food. Trout chow flour was also processed without the addition of As to produce a 0 μg As/g control diet. The diets were pelleted using a laboratory pellet mill, dried in a fan-ventilated chamber, and stored at -20°C until required for feeding. The measured concentrations of As in the diets were 0.84 ± 0.00, 1.8 ± 0.01, 12 ± 0.07, and 120 ± 0.40 μg As/g, in the control, low, medium, and high dose food, respectively.

2.4 Experimental Design

The treatments consisted of a unique combination of one of the four dose groups (0, 1, 10, or 100 μg As/g) and one of the three exposure periods (10, 30, or 100 days).

Each of the 12 tanks was randomly assigned to one of the treatments. The sample size was 6 fish per treatment group.

Fish from four tanks, representing each of the four dose groups were to be sampled on days 10, 30, and 100 of the exposure period. However, due to complete feed refusal by fish being fed the 100 μg As/g food after 55 days of exposure, fish were sampled after 10, 30, and 64 days of exposure. The dates of the sampling days were March 13, April 2, and May 6, 1998.

2.5 Sampling, Removal, and Preparation of Tissues

On each sampling day, fish were anesthetized with tricaine methane sulfonate (MS-222) (Sigma Chemical Co., St. Louis, MO) at a concentration of 438 mg/L. The solution was pH neutralized by addition of ammonium hydroxide. Fish were placed in the anesthetic for no more than 3 minutes and were removed when a tail pinch failed to elicit a response. Fish lengths and weights were measured and blood was withdrawn from the caudal artery and vein using a heparinized syringe.

Following removal of tissues for histological analyses (Chapter 3), tissues were removed and frozen, according to the procedures outlined in Cooley and Klaverkamp (2000), for biochemical and As analyses, with the following exceptions. The gallbladder was separated from the liver and four of the six gallbladders from each treatment group were cut in half, rinsed thoroughly with physiological saline and frozen for As analysis. The pyloric caeca and spleen were removed from the gastrointestinal (GI) tract and frozen. Prior to As analyses, the stomach and intestine were thawed and cleaned by squeezing out the contents, making a longitudinal incision, and scraping and blotting to

remove As-containing food particles and feces. Liver and kidney were stored at –90°C until biochemical analyses were complete, after which they were stored at –20°C until As analysis. All remaining tissues were stored at –20°C immediately after sampling until As analysis.

2.6 Analysis of Arsenic

Eight tissues (gallbladder, liver, pyloric caeca, stomach, intestine, kidney, skin, and scales) from fish in all treatment groups were analyzed for As. For five tissues (muscle, gonad, spleen, gill, and bone), only samples from the day 10 control group and the high dose treatment groups at each duration of exposure were analyzed for As. Tissue and food samples were analyzed for As using a borohydride reduction method (Vijan and Wood, 1974). A sub-sample of wet tissue or food was digested with a combination of nitric (4 mL), sulfuric (0.5 mL) and perchloric (1 mL) acids for 5 hours at 130°C, followed by 2 hours at 200°C. After addition of water (15 mL) and hydrochloric acid (7.5 mL), the solution was cooled and adjusted to 25 mL with water. Arsine gas was generated from this solution by the automated addition of 2% sodium borohydride (reagent grade) and 10% potassium iodide (reagent grade) solutions, and swept by an argon gas stream into an electrically heated quartz tube furnace (800°C) installed in the burner cavity of a Varian SpectrAA-20 atomic absorption spectrophotometer.

All acids used for sample digestions were trace metal analysis grade, and all water was distilled and deionized. The test tubes were washed with 10% hot nitric acid, followed by multiple rinses with distilled, deionized water prior to use. A commercial As atomic absorption standard stock solution of 1000 mg As/L was used to make a range of

standards (0, 10, 25, 50, 75, 100 μg As/L). These standards were run before and after each block of 18 samples to ensure accurate As concentrations were calculated. To verify analytical accuracy, Certified Reference Materials for Trace Metals (National Research Council of Canada, NRC) were digested and analyzed concurrently with tissue samples. Three reference materials were used, including dogfish muscle (dorm-2), dogfish liver (dolt-2), and lobster hepatopancreas (tort-2). Duplicates of each of the reference materials were run every 18 samples and measurements were accepted when concentrations were within 5% of the known concentration of As.

Water samples were collected in 250 mL acid-washed Nalgene bottles from the four tanks remaining on day 63 of the experiment. The samples were acidified to 0.5% nitric acid and were analyzed directly for As content.

The detection limit (DL) of the Varian SpectrAA-20 atomic absorption spectrophotometer is 2.0 μg/L or 0.05 μg/g based on 1.0 g tissue weight. Due to the fact that the weight of tissue used for the As analyses varied, the DL were tissue weight-dependent. The DL calculated for the 13 tissues examined are reported as mean wet weights (μg As/g) ± SE and were as follows: gallbladder 0.94 ± 0.15, liver 0.07 ± 0.00, pyloric caeca 0.08 ± 0.00, stomach 0.09 ± 0.00, intestine 0.12 ± 0.01, kidney 0.07 ± 0.00, muscle 0.07 ± 0.00, gonads 0.09 ± 0.01, spleen 0.16 ± 0.02, gill 0.07 ± 0.00, bone 0.09 ± 0.01, skin 0.05 ± 0.00, and scales 0.05 ± 0.00. The DL for fish food was 0.14 ± 0.02.

2.7 Comparison of As Concentrations to Literature Values

In order to compare concentrations of As measured in the current study as wet weight, to concentrations of As measured in previous studies as dry weight, dry weights

were converted to wet weights based on the following calculation: dry weight x 0.2 = wet weight calculated (Jarvinen and Ankley, 1999). The converted values are indicated by (w.w. calc.) in the discussion.

2.8 Statistical Analyses

Statistical analyses were performed using SPSS version 9.0 and were based on the following references, Neter et al. (1996), Stevens (1992) and SPSS (1999). For each data set, the assumptions of an analysis of variance (ANOVA), normality and equal variance, were checked. If the assumptions were not met the necessary transformations were preformed. A two-way ANOVA was used to evaluate interaction between dose and duration. Because interactions were found to occur, each data set was split into subsets. First, to test for dose effects, concentrations of As in lake whitefish were separated into subsets for each duration. After the ANOVA assumptions were checked for each subset of data, one-way ANOVAs were conducted and where significant, treatment group means were further compared to controls using the Dunnett's one-tailed multiple comparison procedure. To test for duration effects, concentrations of As in lake whitefish tissues were also separated into subsets for each dose. After the ANOVA assumptions were checked for each subset of data, one-way ANOVAs were conducted and where significant, treatment group means were further compared using the Bonferroni two-tailed multiple comparison procedure. If more than one nonnormal population was found in a data subset, concentrations of As in lake whitefish tissues were analyzed using a Kruskal-Wallis one-way ANOVA on ranks within each duration or within each dose. Treatment group means were further compared using the Bonferroni

multiple comparison procedure. All statistical analyses were conducted at an alpha level of 0.05.

3. Results

3.1 Feed Consumption and Growth

A modification of feeding behavior, characterized by a fish taking a food pellet into its mouth and then spitting it out, was observed in lake whitefish fed the highest concentration of As, 100 μg As/g, beginning on day 45 of exposure. Although this behavior occurred several times, some pellets were still being consumed between days 45 and 52. However, by day 55, complete feed refusal was occurring. Food continued to be offered until day 64 when the experiment was terminated. Even though feed refusal occurred in the high dose treatment group, growth of lake whitefish fed both uncontaminated and As contaminated food continued throughout the experiment. Growth of fish in the high dose group, however, was considerably less than in the other groups (Figure 1).

No mortalities occurred during the experiment, however, on day 15 one fish fed the 100 μg As/g food exhibited signs of overt toxicity, including impaired buoyancy control, a failure to eat, and lethargy. This fish was sampled on day 15, decreasing the sample size of the day 64, high dose treatment group to five fish.

The gonads of lake whitefish were in an advanced state of maturation on all sampling days, with the exception of two males sampled on day 10 (one male exposed to the low dose food, and one male exposed to the high dose food).

3.2 Accumulation of Arsenic

Exposure of lake whitefish to the highest concentration of As resulted in significant accumulation in all tissues examined from the GI tract, with the exception of gallbladder (Figure 2a, b, c, and d). Significant As accumulation occurred in stomachs of fish fed the high dose food after both 10 and 30 days of exposure (Figure 2a). Pyloric caeca of fish fed the highest concentration of As had significant accumulation after each duration of exposure (Figure 2b), and there was significant accumulation of As in intestines of fish fed the high dose food after both 30 and 64 days of exposure (Figure 2c). Although As accumulation did occur in gallbladders of fish fed the highest concentration of As for both 10 and 30 days, these results were not significant due to a high degree of variability between fish and/or the small sample size of 4 fish per group (Figure 2d).

Arsenic was absorbed internally by lake whitefish, as significant As accumulation occurred in both liver and kidney of fish fed the high dose food after each duration of exposure (Figure 2e, and f). Significant accumulation of As also occurred in livers of fish fed the medium dose food, 10 μg As/g, after both 30 and 64 days of exposure.

Significant As accumulation occurred in both skin and scales of fish fed the highest concentration of As (Figure 2g, and h). There was significant accumulation of As in skin of fish fed the 100 μg As/g food for 30 and 64 days. The results for day 10 are difficult to interpret, due to the apparent abnormally high concentration of As in the skin of fish fed the control food at this duration. Significant As accumulation occurred in the scales of fish fed the high dose food for each duration of exposure. After 30 and 64 days

of exposure, significant accumulation also occurred in scales of fish fed the 10 μg As/g food.

The distribution of As in eight tissues of lake whitefish fed 100 μg As/g food for 10, 30, and 64 days is presented in Figure 3. The accumulation of As in fish tissues was not duration-dependent, with the greatest accumulation of As occurring after 30 days of exposure in all tissues, except gallbladder. The descending order of mean As concentrations measured at day 30 was as follows: pyloric caeca (5.1) > liver (4.4) > intestine (4.0) > gallbladder (2.5) > kidney (0.76) > stomach (0.75) > scales (0.48) > skin (0.29).

As concentrations in muscle, gonads, spleen, gill, and bone of lake whitefish fed a control diet for 10 days and 100 μg As/g food for 10, 30, and 64 days are shown in Table 1. Due to the fact that As analyses were not conducted for all treatment groups, statistical analyses could not be conducted for these tissues. However, statements about the accumulation of As in these tissues can still be made. As concentrations did not increase in lake whitefish muscle over 10, 30, or 64 days of exposure. As concentrations increased in gonads, spleen, and gill of lake whitefish fed the 100 μg As/g food for 30 days. Increased As concentrations were observed in bone of lake whitefish fed the high dose food after each duration of exposure.

As exposure rates for each treatment group were calculated using both initial and final (at sampling) wet body weights in order to determine reduction in As exposure due to growth dilution throughout the experiment. Cumulative totals of ingested As were also calculated (Table 2). A decrease in exposure rate occurred in each treatment group due to fish growth over each duration of exposure.

4. Discussion

Decreased consumption of As contaminated diets and modified feeding behaviors have been observed in a number of studies that examined exposure of rainbow trout (*Oncorhynchus mykiss*) to arsenate (Cockell and Hilton, 1988; Cockell et al., 1991; Cockell et al., 1992; Cockell and Bettger, 1993). However, in these experiments, alteration of feeding behavior began as early as the first or second day of feeding, as compared to after 45 days of exposure in the current study. The mechanism that mediates decreased feed consumption in fish exposed to As is not well understood. Both the type of feed refusal, where a food pellet is taken into the mouth and then rejected, and the early onset of the behavior observed in rainbow trout exposed to dietary As, indicates chemoreceptory detection of As in the food. Although the characteristics of feed refusal were similar in the current study, the response was not immediate; indicating that chemoreceptory detection of As in the food was not likely to be the sole reason for decreased feed consumption by lake whitefish.

An alternative explanation for the altered feeding behavior of lake whitefish may be elucidated from the results of a study that exposed rainbow trout (*O. mykiss*) to waterborne As (Rankin and Dixon, 1994). In the study, an alteration in feeding response of fish exposed to aqueous arsenite was observed within 1 day of exposure. The food offered to rainbow trout was not contaminated with As, suggesting that the depression of feeding activity was not mediated through chemoreceptory detection of the toxicant, but through some other mechanism(s). Erosion of the mandibular and olfactory regions in rainbow trout exposed to 9.64 mg arsenite/L was also observed by Rankin and Dixon

(1994) and it was postulated that decreased feed consumption may be associated with a loss of chemoreception, resulting in the inability of fish to smell and taste food. Although similar lesions were not observed in lake whitefish, the altered feeding behavior observed in the current study may be consistent with impaired chemoreception. For example, lake whitefish approached the food pellets and took them into their mouths when pellets were initially dropped into the tank water. An inability to taste the pellet and discern it was food may have caused the fish to spit out the pellet. Therefore, a loss of chemoreception is a plausible explanation for the altered feeding behavior observed in this study. In addition, gastrointestinal distress, indicated by mucosal sloughing of the GI tract, of lake whitefish fed the high dose food may have also contributed to the decreased feed consumption observed. Additional research, which examines the effects of As on chemoreception in freshwater fish, is needed.

The distribution of As in lake whitefish tissues is consistent with previous studies that have examined As distribution in other freshwater fish species following both aqueous and dietary As exposures. In this study on lake whitefish, significant accumulation of As occurred in stomach, pyloric caeca, intestine, liver, kidney, skin, and scales. Gallbladder, gonads, spleen, gill, and bone also showed increased As concentrations. Sorenson (1979a) conducted an aqueous exposure of green sunfish (*Lepomis cyanellus*) to 60 µg As/g as sodium arsenate for 2, 4, and 6 days. Increased As accumulation occurred in combined gallbladder and bile tissues, liver, spleen, and kidney. Similarities were observed in the current study, with the exception of gallbladder and bile. Sorenson (1979a) suggested that the high concentrations of As found in the gallbladder and bile were likely associated with the bile and not the gallbladder, however,

this is disputed by the results of the current study in which elevated As concentrations were measured in the gallbladder tissue with the bile removed. Cockell et al. (1992) and Cockell and Bettger (1993) also analyzed gallbladder and bile samples separately from juvenile rainbow trout (*O. mykiss*) exposed to dietary arsenate and elevated As concentrations were found in both. Cockell et al. (1991; 1992), and Cockell and Bettger (1993) also found increased As concentrations in liver and kidney of rainbow trout.

Although the distribution of As in tissues of freshwater fish is similar for both aqueous and dietary As exposures, the accumulation of As in fish tissues is much higher for aqueous exposures. Concentrations of As measured by Sorenson (1979a) in tissues of green sunfish (*L. cyanellus*) exposed to 60 μg/g sodium arsenate in the water for 2, 4, and 6 days were one to two orders of magnitude greater than concentrations of As measured in lake whitefish tissues in the current study. For example, concentrations of As in liver, kidney, spleen, and gill of green sunfish after 6 days of exposure were 47.7, 14.2, 18.9, and 3.8 μg/g (w.w.), respectively (Sorenson, 1979a), whereas concentrations of As in liver, kidney, spleen, and gill of lake whitefish after 30 days of exposure to 100 μg As/g food were 4.4, 0.76, 0.76, and 0.64 μg/g (w.w.), respectively.

The accumulation of As in tissues of rainbow trout exposed to dietary As is similar to the accumulation of As measured in tissues of lake whitefish and is lower than the concentrations of As measured in aqueous exposures (Cockell and Hilton, 1988; Cockell et al., 1991; Cockell et al., 1992; Cockell and Bettger, 1993). For example, juvenile rainbow trout fed 58 μg As/g food accumulated 5.91, 6.09, and 3.05 μg/g (w.w. calc.) in liver and 6.92, 7.44, and 3.92 μg/g (w.w. calc.) in gallbladder after 7, 28, and 56 days of exposure, respectively (Cockell and Bettger, 1993). Similarly, lake whitefish fed

100 μg As/g food accumulated 1.6, 4.4, and 0.88 μg/g (w.w.) in liver and 8.4, 2.5, and 0.45 μg/g (w.w.) in gallbladder, after 10, 30, and 64 days of exposure, respectively. Modified feeding behavior of juvenile rainbow trout and lake whitefish contributed to the decrease in As concentration with continued exposure in both studies.

A comparison of the results of the current study and field studies examining As accumulation shows that uptake of As through the diet results in a similar pattern of As tissue distribution, independent of whether fish are exposed to As contaminated commercial food, or As-contaminated natural diet items and sediment. The degree of accumulation of As in freshwater fish tissues does, however, differ between fish exposed to As-contaminated diets in the lab and fish collected from As-contaminated lakes and rivers. For example, brown trout (*Salmo trutta*) collected from the Clark Fork River, Montana, were found to have significantly higher As concentrations in pyloric caeca (0.29 μg/g, w.w. calc.), liver (0.32 μg/g, w.w. calc.), kidney (2.26 μg/g, w.w. calc.), and gill (0.29 μg/g, w.w. calc.), as compared to fish collected from a reference site (Farag et al., 1995). Concentrations of As were not measured in the stomach or intestine. The site in the Clark Fork River where the brown trout were collected contained 102 μg As/g (d.w.) in the sediments (Brumbaugh et al., 1994), which is similar to the concentration of As in the high dose food used in the current study. Although As did accumulate in the same tissues in lake whitefish, the pattern of As accumulation was different. The highest concentration of As in these four tissues of lake whitefish was measured in pyloric caeca (5.1 μg/g, w.w.), followed by liver (4.4 μg/g, w.w.), kidney (0.76 μg/g, w.w.), and gill (0.64 μg/g, w.w.). In another study, Azcue and Dixon (1994) found increased As concentrations in bone plus scales (0.274 μg/g w.w.) and intestine with contents (0.406

μg/g w.w.) of rock bass (*Ambloplites rupestris*) collected form Moira Lake, Ontario. Although the concentration of As measured in the sediments of Moira Lake (1000 μg As/g d.w.) was an order of magnitude higher than the concentration of As in the high dose food used in the current study, As accumulated to a greater degree in bone (0.57 μg/g, w.w.), scales (0.48 μg/g, w.w.), and intestine (4.0 μg/g, w.w.) of lake whitefish. Azcue and Dixon (1994) could not distinguish between the As in the intestine itself and the As in the contents. The results of the current study, however, show that As is in fact accumulated in the intestinal wall of lake whitefish, as well as other areas of the GI tract, including the stomach and pyloric caeca. Azcue and Dixon (1994) also measured 0.21 μg/g (w.w) of As in the liver of rock bass, which is much lower than the 4.4 μg/g (w.w.) of As measured in the liver of lake whitefish.

The above comparisons indicate that although the distribution of As in the tissues of freshwater fish fed As contaminated commercial diets in the laboratory and fish collected from As contaminated habitats is similar, there are substantial differences in the amount of As accumulated in the various tissues. These differences may be due to one or more of the following factors: 1) The bioavailability and absorption of As from natural diet items and sediments may be different from the absorption of As from an As-contaminated commercial diet; 2) Fish living in contaminated habitats consume both inorganic and organic forms of As, whereas only an inorganic form of As was used in the current study; and/or 3) As may be absorbed and excreted differently by different species of freshwater fish. Additional research that examines the differences in accumulation between fish exposed to dietary As in the lab and fish exposed to As in the field is

needed. Field studies, which examine the accumulation and distribution of As in lake whitefish collected from As contaminated habitats, would also be useful.

The mechanisms of accumulation of As in lake whitefish tissues were not investigated in this study. Once ingested by mammals and fish, As is absorbed from the GI tract and enters the circulation. As rapidly leaves the blood and is distributed to other tissues (Klaassen, 1974; Cockell et al., 1992; Fowle et al., 1992). The liver is a major site of As accumulation, and has been found to readily bind As. Studies involving both mammals and fish have shown that As is deposited primarily in the nuclei, and to a lesser degree in the cytoplasm of hepatocytes (Klaassen, 1974; Sorenson, 1979b). Elevated liver As concentrations may also be the result of deposition in the mitochondria of hepatocytes. Mammalian studies have indicated that arsenate is actively taken up by rat liver mitochondria and an As-binding component within mitochondria has been isolated (Fowler, 1977). As accumulation in lake whitefish liver also reflects the essential role this organ plays in detoxification (Sorenson, 1991). Metabolism of As in the liver involves the reduction of arsenate to arsenite, and the subsequent methylation to the mono- and di-methyl forms in both mammals and fish (Penrose, 1975; Oladimeji et al., 1979; Fowle et al., 1992; Shiomi et al., 1996). Methylation is thought to be detoxifying and to aid in the excretion of As (Fowle et al., 1992). Arsenite and the two organic forms of As are actively excreted by the liver into the bile (Cockell and Bettger, 1993). It is thought that the excretion of organic As into the bile involves an active transport system (Klaassen, 1974; Penrose, 1975; Gregus and Klaassen, 1986).

High concentrations of As in fish gallbladder are the result of absorption of arsenite from the lumen (Cockell and Bettger, 1993). Elevated As concentrations cause

alterations in the cells of the gallbladder wall, resulting in macroscopically visible lesions (Cockell and Bettger, 1993; Chapters 1 and 3). As absorbed by the blood across the gallbladder wall is returned to the liver, as the cystic vein drains the vascular plexus of the gallbladder wall and joins the portal vein as it enters the liver (Cockell and Bettger, 1993). Additionally, As which enters the GI tract through excretion in the bile is returned to the liver via enterohepatic circulation (Klaassen, 1974; Gregus and Klaassen, 1986). Cockell and Bettger (1993) postulated that substantial accumulation of As in the liver may be the result of continued re-exposure through portal circulation. In salmonids, the bile enters the GI tract at the end of the pyloric stomach (Yasutake and Wales, 1983). The continued exposure of As to the GI tract may also be a factor in the higher concentrations of As observed in the pyloric caeca, and intestine, as compared to the stomach of lake whitefish in the current study.

As, which initially enters the circulation after absorption from the GI tract, is distributed to a number of other tissues in addition to the liver. The accumulation of As in the kidney and gill may reflect the excretory roles of these organs (Oladimeji et al., 1984a). As is transported across the proximal tubule in the kidney (Ginsburgh and Lotspeich, 1963; Ginsburgh, 1965). Brown et al. (1976) found that exposure of rats to sodium arsenate in drinking water resulted in As accumulation in the mitochondria of the proximal tubular cells of the kidney and caused decreased state 3 respiration and respiratory controls by uncoupling oxidative phosphorylation. The mechanism by which oxidative phosphorylation becomes uncoupled is related to the competitive arsenate substitution for inorganic phosphate, resulting in the formation of an unstable arsenate ester that spontaneously decomposes (Fowler, 1977; Hindmarsh and McCurdly, 1986).

The elevated As concentrations measured in the spleen of lake whitefish exposed to the high dose food may be a result of filtration of blood through the spleen, as well as the blood storage function of this organ (Sorenson, 1991).

As accumulation in bone, skin, and scales is due to the affinity of As to bind to the high number of sulfhydryl groups in these tissues (Cullen et al., 1984, Hindmarsh and McCurdly, 1986; Li et al., 1991; Fowle et al., 1992), and to the analogous chemical behavior of arsenate and phosphate (Squibb and Fowler, 1983; Fowle et al., 1992). Bone and skin are known sites of As deposition in mammals (Luckey and Venugopal, 1977; Fowle et al., 1992). There is increasing evidence of As deposition in bone, skin, and scales of freshwater fish. For example, rock bass collected from an As contaminated habitat had elevated concentrations of As in bone and scales (Azcue and Dixon, 1994). Increased concentrations of As were also measured in skin of rainbow trout (*Salmo gairdneri*) fed diets containing 10, 20, and 30 μg As/g as sodium arsenite for 2, 4, 6, and 8 weeks (Oladimeji et al., 1984b). The elevated As concentrations measured in the skin of lake whitefish and rainbow trout may also indicate that this organ acts as an additional route of excretion (Heath, 1987).

Several factors may have contributed to the absence of duration-dependent As accumulation in lake whitefish, including feed refusal, growth of fish, and/or an increased excretory efficiency over the course of the experiment. These factors would lead to a decrease in the accumulation of As in fish tissues especially at day 64, as compared to day 30. The combined effects of feed refusal and growth of fish resulted in decreased exposure to As over the course of the experiment. A similar pattern of accumulation, with initially high concentrations, followed by lower concentrations with continued

exposure, has been reported for long-term exposures of rainbow trout (*O. mykiss*) to waterborne arsenate (McGeachy and Dixon, 1990) as well as both waterborne (Rankin and Dixon, 1994) and dietary (Oladimeji et al., 1984b) arsenite. This indicates that the decreased accumulation of As observed in lake whitefish may not only be due to decreased exposure to As, but also to more efficient excretion of As with continued exposure. Dixon and Sprague (1981) found that preexposed fish accumulated less As during a subsequent exposure than naive fish, and they concluded that improved excretory efficiency due to preexposure to As was occurring. Rankin and Dixon (1994) have suggested that the mechanism of improved excretory efficiency likely involves the enhancement of the biochemical pathways necessary for the conversion of inorganic As to the major As excretion products, the organoarsenicals.

5. Conclusions

The results of this experiment can be used to guide environmental monitoring programs that assess the bioavailability of dietary As to freshwater fish in areas where As contamination is a concern. Analyses of As in liver, pyloric caeca, intestine, and scales are recommended for evaluating the bioavailability of As to freshwater fish. Liver is particularly important because it is vulnerable to As toxicity at the molecular, tissue, and organ levels of biological organization as described in Chapter 3. Liver and scales appear to be the most sensitive indicators of dietary As exposure, as significant accumulation of As occurred in fish fed concentrations as low as 10 μg As/g. Scales may also be particularly useful for monitoring As bioavailability in environmental monitoring

programs because samples could be obtained from fish without sacrificing the animal. Analysis of As in muscle is not recommended for determining As bioavailability to fish.

There are a number of relevant issues, which require further research with respect to As accumulation and distribution in freshwater fish. It is important to determine how the bioavailability of As in natural diet items, such as zooplankton and benthic invertebrates, compares to the bioavailability of As in contaminated commercial food used in this experiment. It is also necessary to link tissue As concentrations to effects of dietary As exposure in both laboratory and field studies. Studies that examine the mechanisms and kinetics of accumulation, distribution, and the depuration of As in freshwater fish exposed to dietary As are needed. For example, an experiment which determines whether preexposure to dietary As results in lower As accumulation in fish tissues would provide further evidence for more efficient excretion of As with continued exposure. The mechanism by which this occurs also needs to be investigated. In addition, the mechanism that mediates decreased feed consumption in fish exposed to both dietary and waterborne As also needs to be examined further. It would also be useful to determine whether bone and scales serve as long-term storage sites of As and whether As is remobilized from these tissues after exposure ceases.

Table 1. The accumulation of As in muscle, gonads, spleen, gill, and bone of lake whitefish fed a control diet for 10 days and a 100 μg As/g diet for 10, 30, and 64 days. Concentrations of As are expressed as mean ± (SE).

Treatment Group		Tissue				
		Muscle	Gonads	Spleen	Gill	Bone
Day	Dose	(μg As/g)	(μg As/g)	(μg As/g)	(μg As/g)	(μg As/g)
10	control	0.48 (0.05)	0.18 (0.03)	0.39 (0.13)	0.11 (0.01)	0.08 (0.02)
10	high	0.46 (0.05)	0.28 (0.04)	0.50 (0.10)	0.26 (0.05)	0.30 (0.05)
30	high	0.56 (0.08)	0.45 (0.09)	0.76 (0.25)	0.64 (0.27)	0.57 (0.09)
64	high	0.38 (0.04)	0.23 (0.04)	0.45 (0.13)	0.19 (0.03)	0.52 (0.14)

Table 2. Exposure rates and cumulative totals of ingested As in lake whitefish fed contaminated diets for 10, 30, and 64 days. Exposure rates were calculated using both initial and final wet body weights (IBW and FBW, respectively). The reduction in exposure due to fish growth is expressed as a percentage decrease from the initial exposure rate.

Treatment Group		Exposure Rate[a] (μg As/g body wt/day)		Reduction in Exposure Due to Fish Growth[b] (% decrease from initial exposure)	Cumulative Total of Ingested As[a] (μg As/g body wt)	
Day	Dose	Using IBW	Using FBW		Using IBW	Using FBW
10	control	0.00	0.00	0.00	0.02	0.02
	low	0.00	0.00	0.00	0.04	0.03
	medium	0.02	0.02	0.00	0.23	0.22
	high	0.23	0.22	4.3	2.3	2.2
30	control	0.00	0.00	0.00	0.06	0.05
	low	0.00	0.00	0.00	0.12	0.11
	medium	0.03	0.02	33	0.76	0.69
	high	0.25	0.24	4.0	7.5	7.2
64	control	0.00	0.00	0.00	0.11	0.10
	low	0.00	0.00	0.00	0.24	0.22
	medium	0.03	0.02	33	1.6	1.4
	high	0.21	0.19	9.5	13	12

[a]Calculations of exposure rates and cumulative totals of ingested As were made as follows:

(1) Exposure rate = [feed consumption per tank of fish per exposure period (g) / IBW or FBW (g)] * 1/ exposure period (days) * measured dietary As concentration (μg As/g diet)

(2) Cumulative total of ingested As = [feed consumption per tank of fish per exposure period (g) / IBW or FBW (g)] * measured dietary As concentration (μg As/g diet)

[b]Reduction in exposure (%) = (exposure $rate_{(based\ on\ IBW)}$ − exposure $rate_{(based\ on\ FBW)}$ / exposure $rate_{(based\ on\ IBW)}$) * 100

Figure 1. The weight gain of lake whitefish fed a control diet and three As contaminated diets for 10, 30, and 64 days. Data are expressed as mean final body wet weight – mean initial wet body weight for each treatment group.

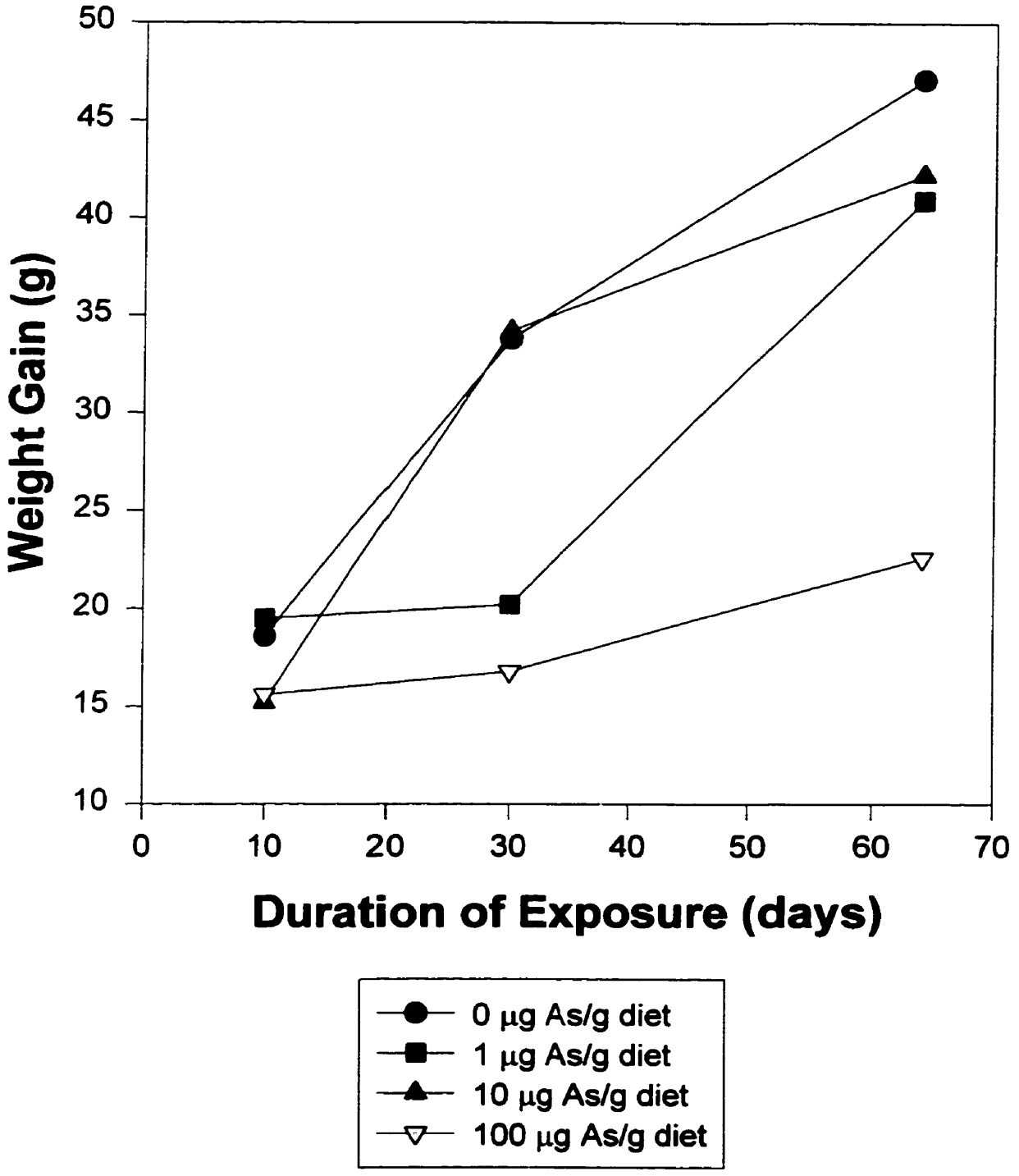
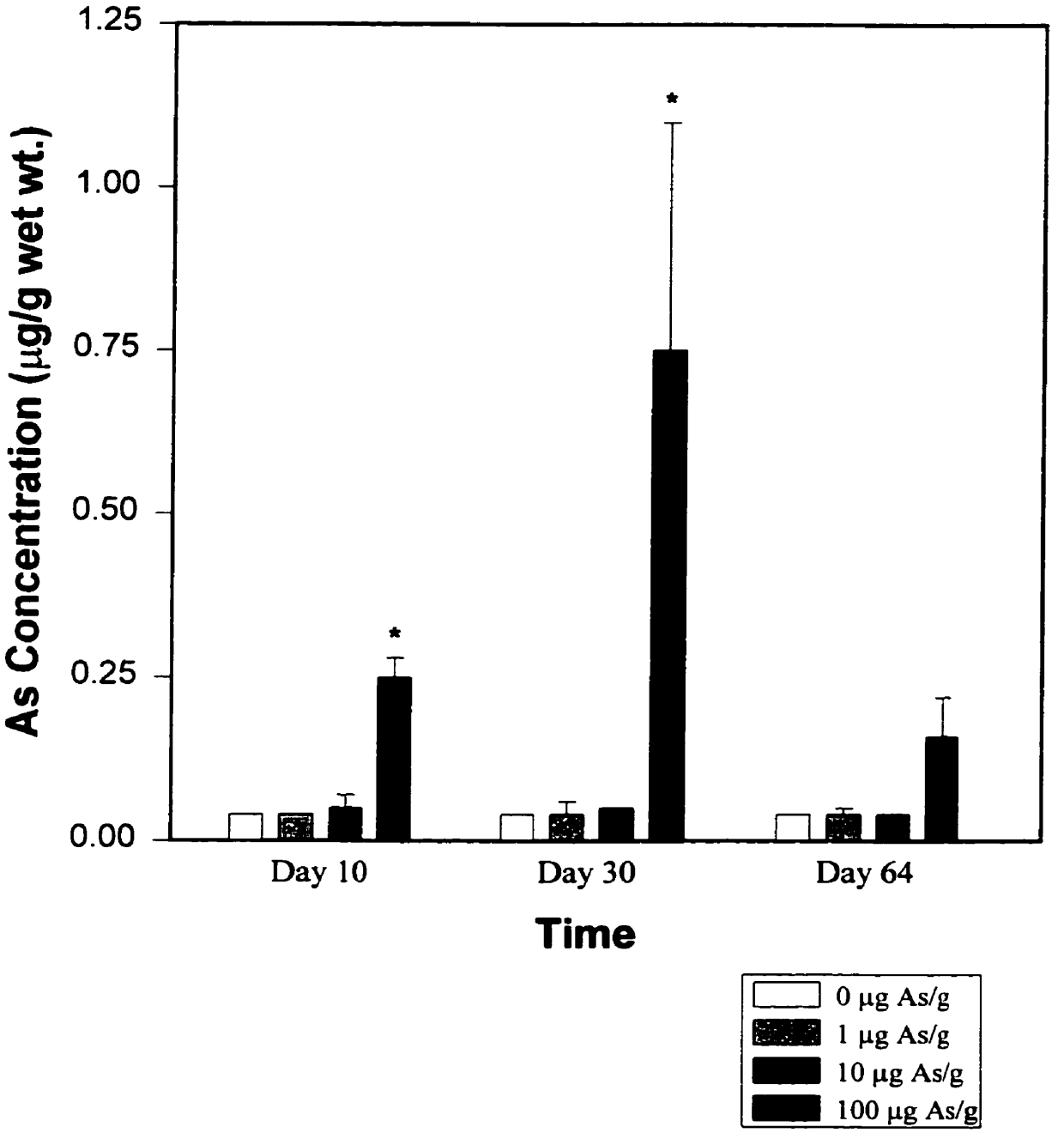
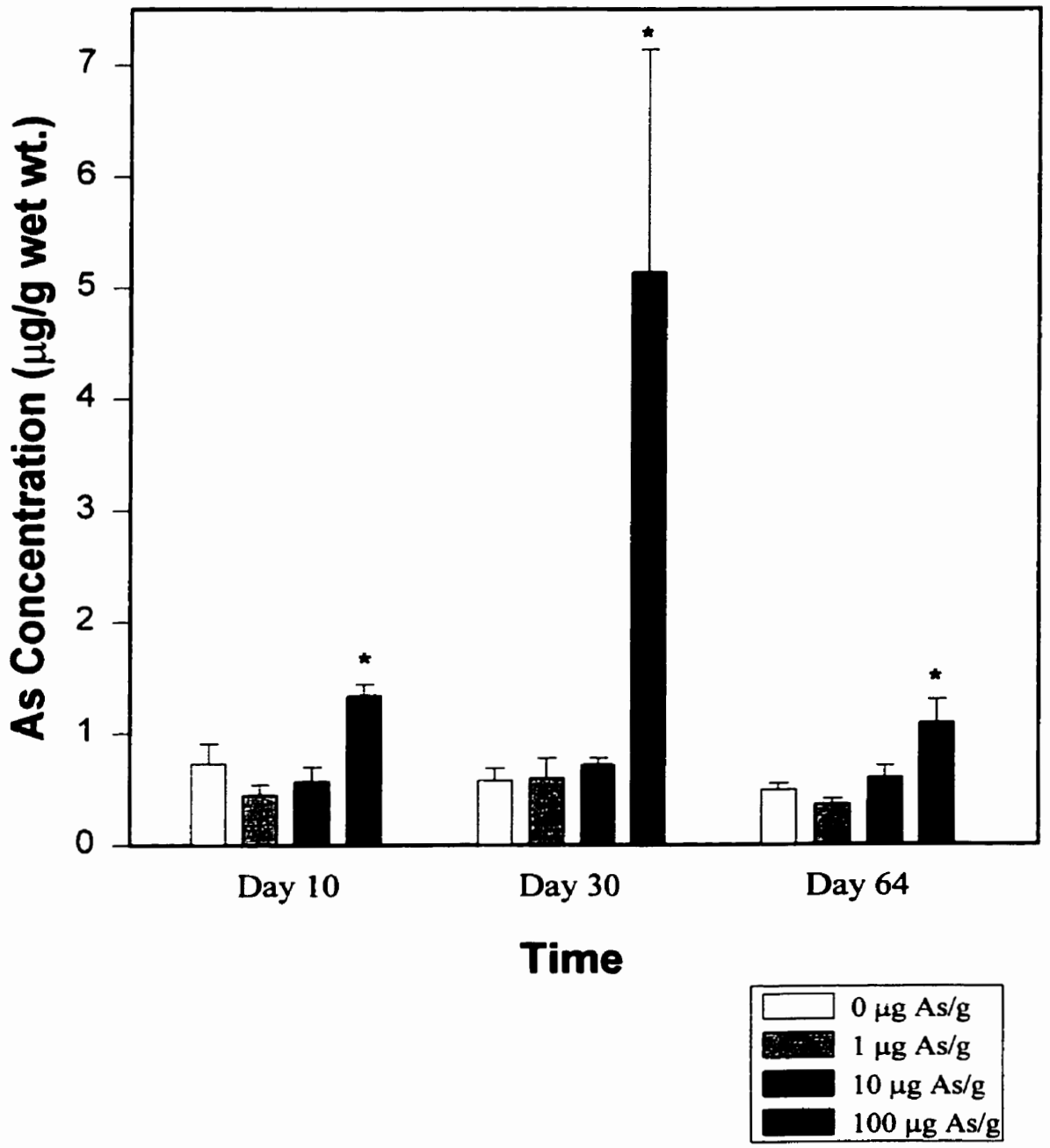

Weight Gain (g)
50
45
40
35
30
25
20
15
10
0
10
20
30
40
50
60
70
Duration of Exposure (days)
0 µg As/g diet
1 µg As/g diet
10 µg As/g diet
100 µg As/g diet

Figure 2. The accumulation of As in eight tissues of lake whitefish fed a control diet and three As contaminated diets for 10, 30, and 64 days: (a) stomach, (b) pyloric caeca, (c) intestine, (d) gallbladder, (e) liver, (f) kidney, (g) skin, and (h) scales. Data are expressed as mean (± SE). Asterisks denote means are significantly different from the control at that duration ($P < 0.05$).

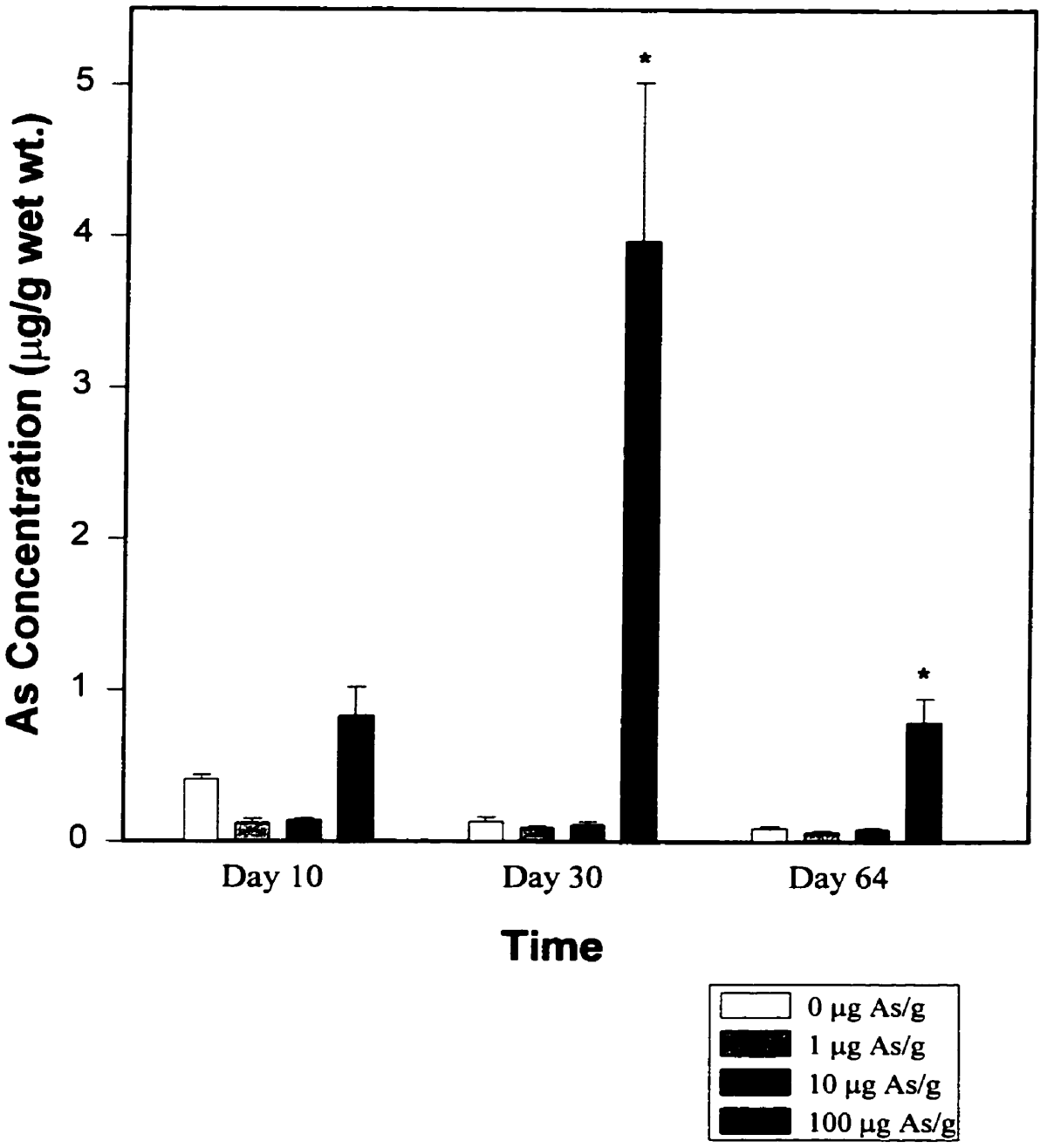
(a) stomach


1.25
1.00
0.75
0.50
0.25
0.00
As Concentration (µg/g wet wt.)
*
*
Day 10
Day 30
Day 64
Time
0 µg As/g
1 µg As/g
10 µg As/g
100 µg As/g

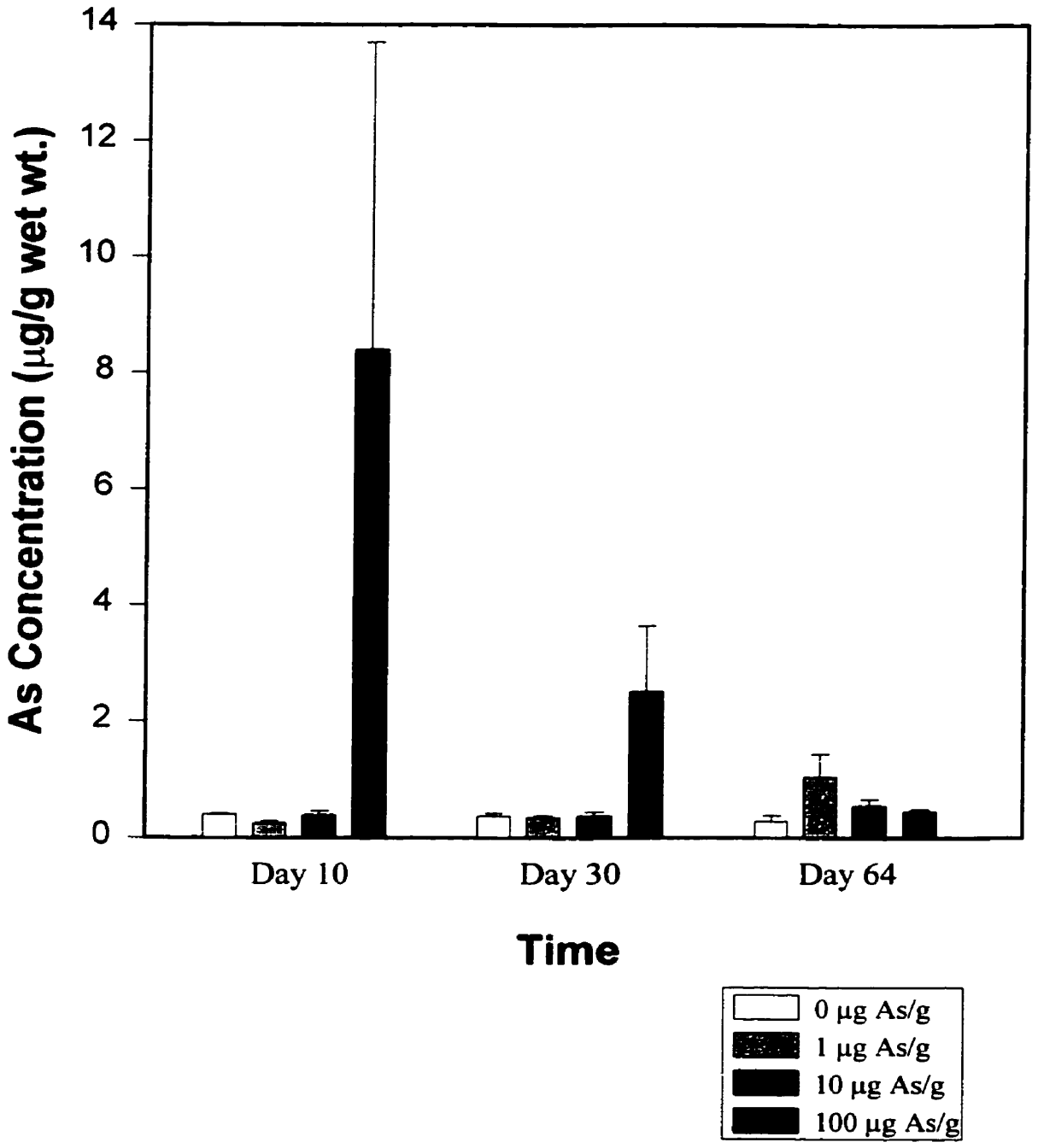
(b) pyloric caeca



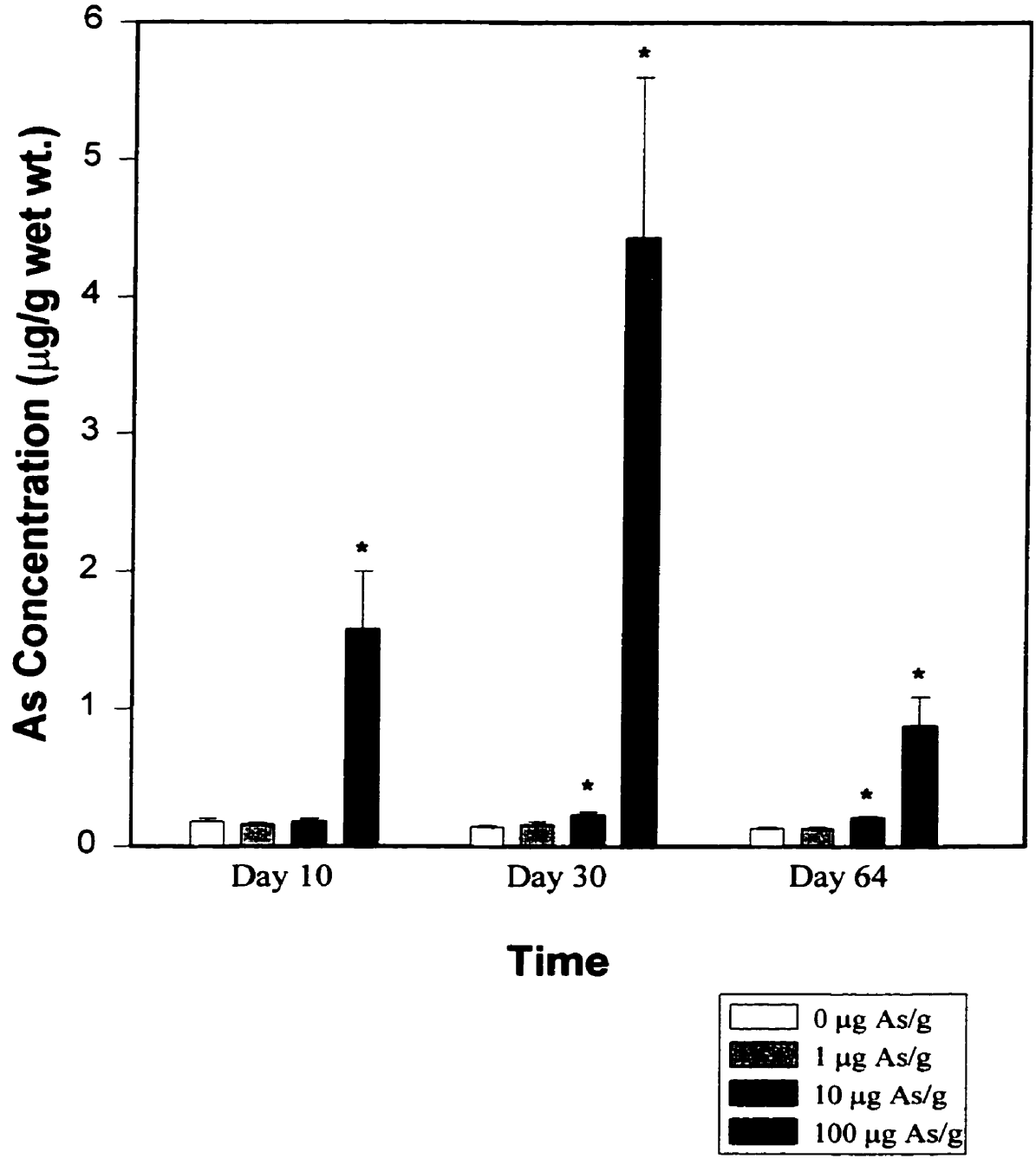
(c) intestine


As Concentration (µg/g wet wt.)
0
1
2
3
4
5
*
*
Day 10
Day 30
Day 64
Time
0 µg As/g
1 µg As/g
10 µg As/g
100 µg As/g

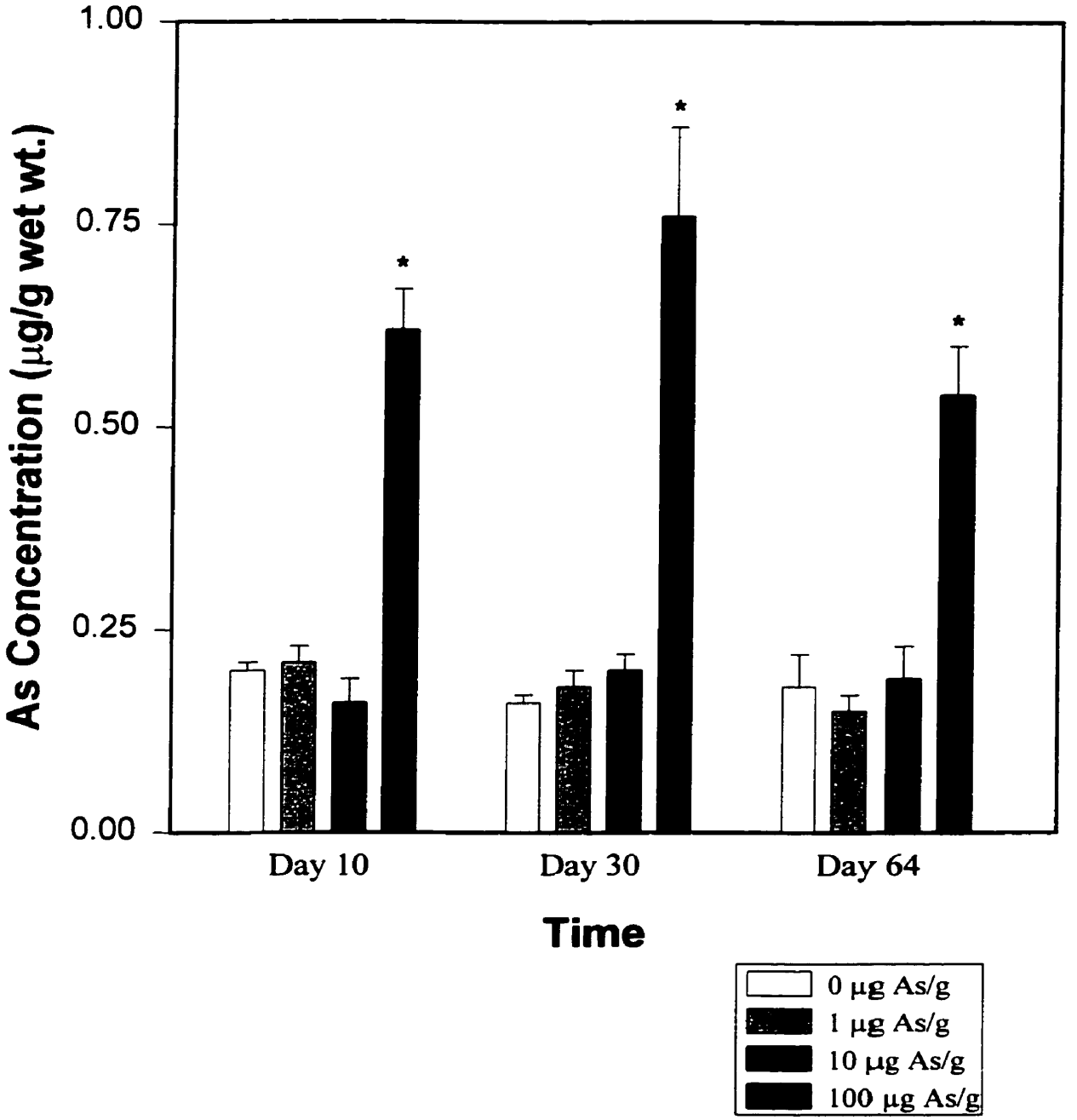
(d) gallbladder



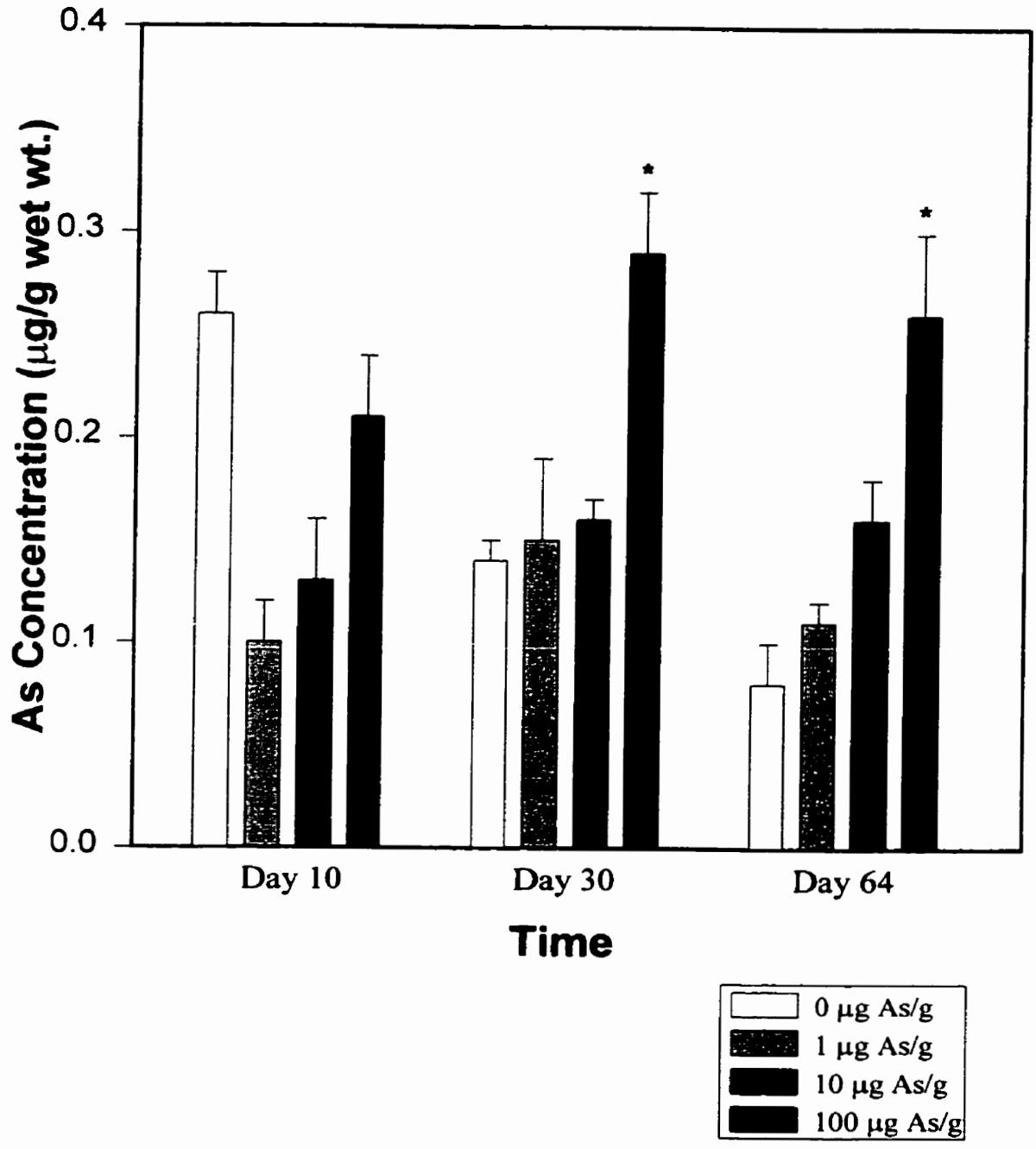
(e) liver


As Concentration (µg/g wet wt.)
6
5
4
3
2
1
0
Day 10
Day 30
Day 64
Time
0 µg As/g
1 µg As/g
10 µg As/g
100 µg As/g

(f) kidney


1.00
0.75
0.50
0.25
0.00
As Concentration (µg/g wet wt.)
*
*
*
Day 10
Day 30
Day 64
Time
0 µg As/g
1 µg As/g
10 µg As/g
100 µg As/g

(g) skin


0.4
0.3
0.2
0.1
0.0
As Concentration (µg/g wet wt.)
*
*
Day 10
Day 30
Day 64
Time
0 µg As/g
1 µg As/g
10 µg As/g
100 µg As/g

(h) scales

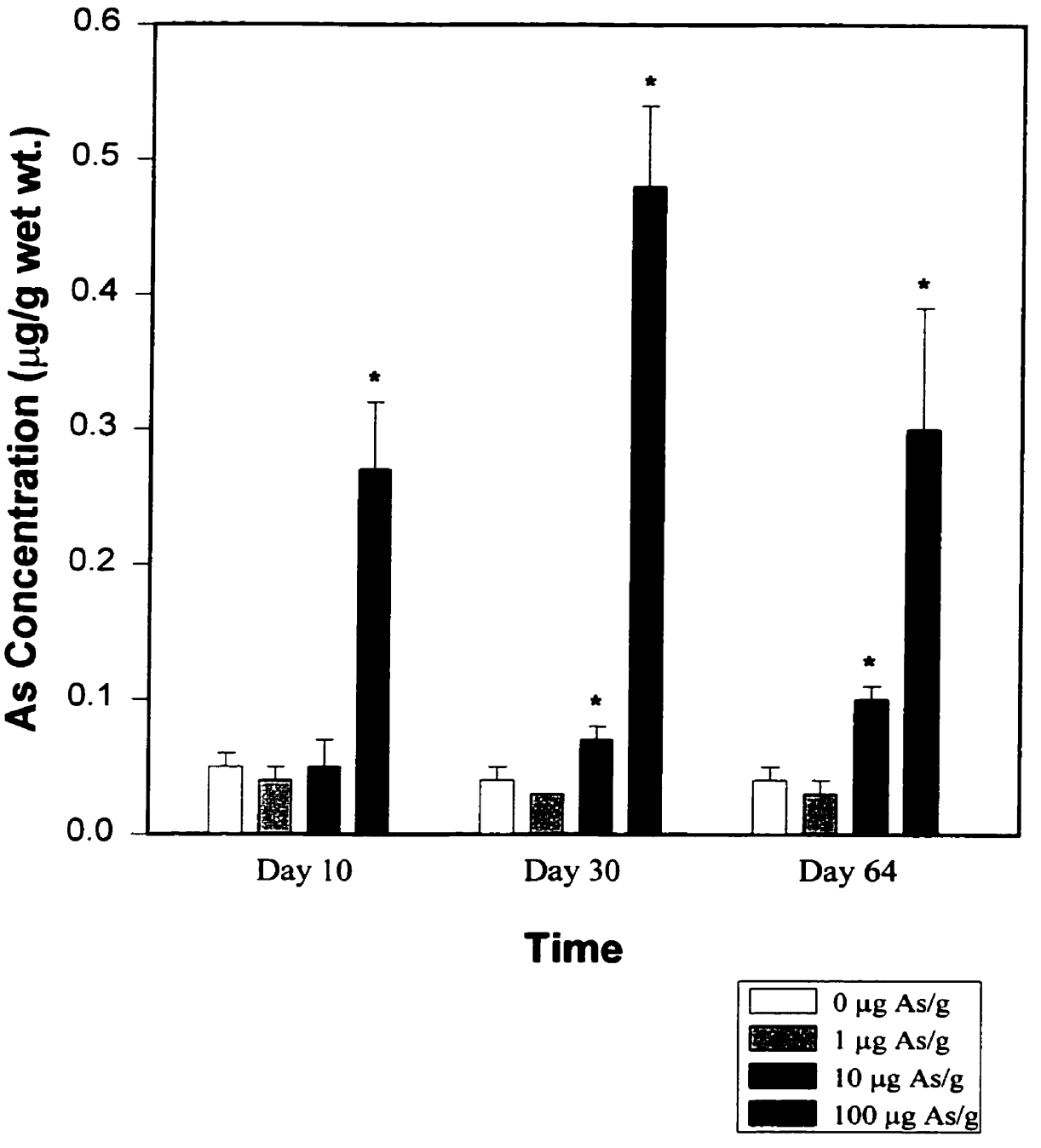
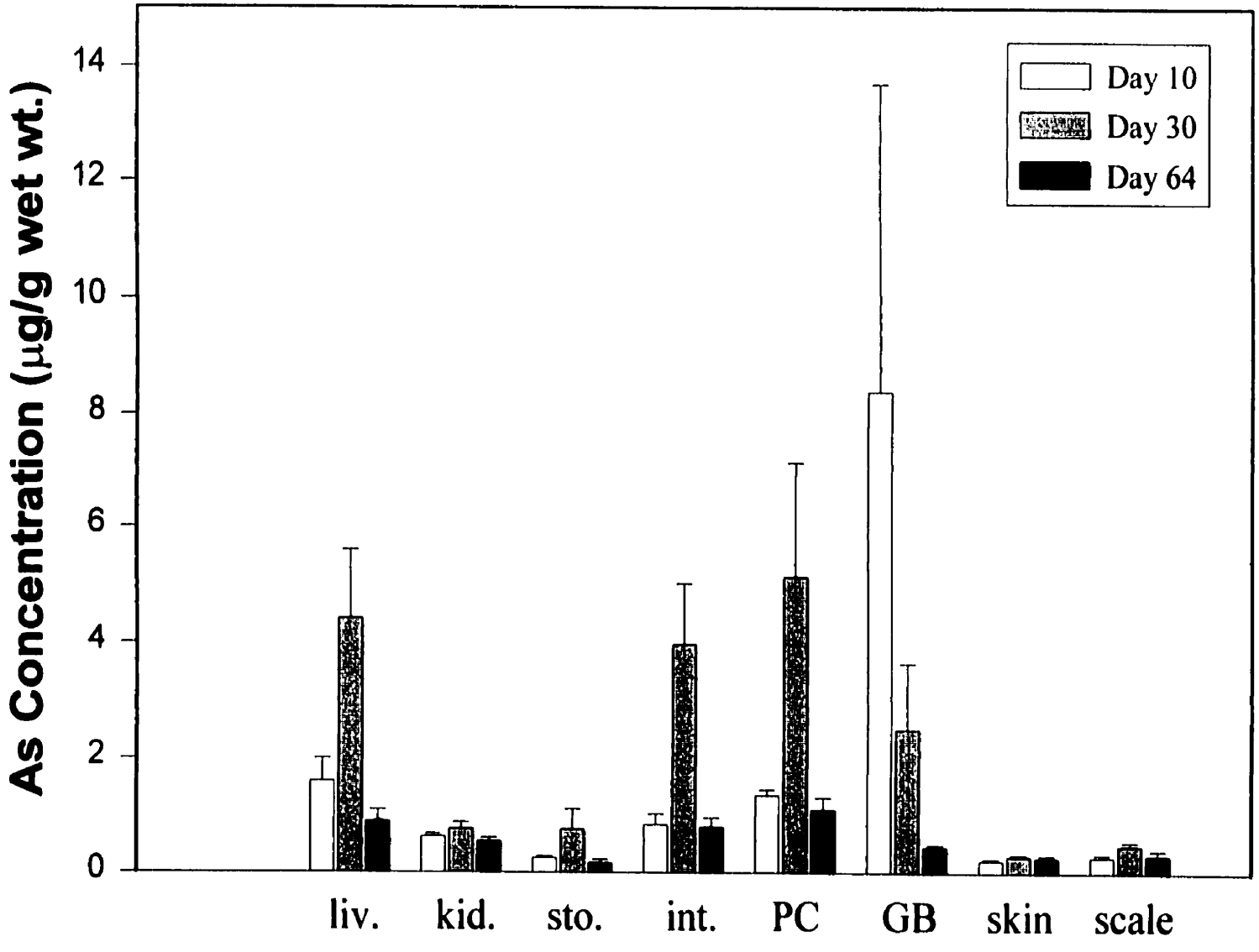

0.6
0.5
0.4
0.3
0.2
0.1
0.0
As Concentration (µg/g wet wt.)
Day 10
Day 30
Day 64
Time
0 µg As/g
1 µg As/g
10 µg As/g
100 µg As/g

Figure 3. The distribution of As in eight tissues, liver (liv.), kidney (kid.), stomach (sto.), intestine (int.), pyloric caeca (PC), gallbladder (GB), skin, and scale, of lake whitefish fed 100 μg As/g food for 10, 30, and 64 days. Data are expressed as mean (± SE).


As Concentration (µg/g wet wt.)
14
12
10
8
6
4
2
0
Day 10
Day 30
Day 64
liv.
kid.
sto.
int.
PC
GB
skin
scale

Chapter Three

Toxicological effects of arsenic in lake whitefish (*Coregonus clupeaformis*) exposed by the dietary route of uptake.

Abstract

Due to the need for research that examines the effects of dietary As exposure in freshwater fish, the toxicological effects of dietary As exposure in lake whitefish (*Coregonus clupeaformis*) were assessed at the molecular, tissue, organ, and organism levels of biological organization. Adult lake whitefish were fed As contaminated diets at nominal concentrations of 0, 1, 10, and 100 μg As/g food (d.w.) for 10, 30, and 64 days. Reduced feed consumption was observed in lake whitefish fed the 100 μg As/g food, beginning on day 45 of exposure. At the molecular level of organization, metallothionein (MT) induction occurred in lake whitefish fed the 100 μg As/g food after 10 and 30 days, and in fish fed the 1 and 10 μg As/g diets for 64 days. Dietary As exposure did not have a significant effect on lipid peroxide (LPO) concentrations in plasma of lake whitefish fed As contaminated diets within the 64 days of exposure. At the tissue and organ level, the mean liver somatic index decreased significantly in lake whitefish fed the 100 μg As/g food for 64 days, however, blood parameters were not affected by exposure to As contaminated diets. Liver and gallbladder histopathology was observed in lake whitefish fed all As contaminated diets after each duration of exposure. Histopathology observed in liver included nuclear, architectural and structural alterations, areas of inflammation, and focal necrosis. Sloughing of the epithelium, dilation of vascular elements, inflammation, edema, fibrosis, and increased width of the submucosa, were some of the alterations observed in gallbladders of lake whitefish fed As contaminated diets. Both organs were sensitive to As toxicity, as damage occurred with exposure to dietary concentrations of As as low as 1 μg/g. Whole organism parameters were unaltered by dietary As exposure. Based on the results of this study, histopathological alterations in

liver and gallbladder, and hepatic MT induction are recommended as sensitive and reliable indicators of As toxicity for use in environmental monitoring programs.

1. Introduction

The release of As into aquatic environments as a result of anthropogenic activities, including the mining, smelting, and refining of metals, and the combustion of fossil fuels, has led to high concentrations of As in sediments and benthic invertebrates (CEPA, 1993; Rowe et al., 1996; ATSDR, 1998; Farag et al., 1998). This is of great concern in both Canada and the United States, as As is biologically available to aquatic organisms, including fish, living in contaminated habitats (Azcue and Dixon, 1994, Farag et al., 1995, Hopkins et al., 1999). Exposure of aquatic organisms to As has also resulted in a number of toxicological effects (Cockell, 1990; Sorenson, 1991; CEPA, 1993).

Although the main route of As uptake by benthic-feeding fish is dietary (Sorenson, 1991; Handy, 1996; ATSDR, 1998), the majority of studies on As toxicity in freshwater fish have examined the effects associated with uptake of waterborne As across the gills (Sorenson, 1976; 1979a; 1979b; McGeachy and Dixon, 1990; 1992; Rankin and Dixon, 1994). An exception is the recent work conducted by Cockell (1990) on juvenile rainbow trout (*O. mykiss*). It is important, therefore, to conduct experiments that examine the toxicological effects of As in freshwater fish exposed by the dietary route of uptake, in order to determine parameters that can be used as sensitive and reliable indicators of As toxicity in environmental monitoring programs (Abernathy, et al., 1999; AETE, 1999; Environment Canada, 1999).

The results reported in the previous manuscript (Chapter 2) established that As was absorbed by the gastrointestinal (GI) tract of lake whitefish (*Coregonus clupeaformis*) and accumulated in liver, kidney, stomach, pyloric caeca, intestine, skin, and scales. The objective of the current study was to examine the toxicological effects of

As in lake whitefish exposed by the dietary route of uptake, at environmentally relevant concentrations, for three durations of exposure. To provide a thorough evaluation of fish health, the effects of As were assessed at several levels of biological organization (Adams et al., 1999). At the molecular level, metallothionein (MT) concentrations in liver and kidney, as well as lipid peroxides (LPO) in plasma were measured. At the tissue and organ levels, hematological parameters, liver somatic indices (LSI), and histopathology of liver, and gallbladder were evaluated. At the whole organism level, wet weights, fork lengths, growth rates and condition factor were assessed. The chemical species of As incorporated into the diet was arsenate because this is the most common form of As in oxic littoral zones of freshwater habitats (Cullen and Reimer, 1989; AQUAMIN, 1996).

2. Materials and Methods

2.1 Fish and Experimental Design

Lake whitefish were reared at the Freshwater Institute and were 4 years of age at the beginning of the experiment. Initial body weights and fork lengths were 334 ± 7.55 g and 29.2 ± 0.204 cm (mean ± SE), respectively. Lake whitefish were fed a commercial diet containing one of four doses of As (0, 1, 10, or 100 μg As/g) for one of three exposure periods (10, 30, or 64) days. The composition, processing, and source of the food are described in Chapter 2. There were a total of 12 treatment groups with a sample size of 6 fish per group. Originally, the third exposure period was to be 100 days, however, due to complete feed refusal exhibited by lake whitefish fed the 100 μg As/g food after 55 days of exposure, the experiment was terminated on day 64. Descriptions of tank parameters, fish holding, and fish sampling and dissection are given in Chapter 2.

2.2 Blood Sampling and Analyses

On each sampling day, fish were anesthetized in a pH-neutralized tricaine methane sulfonate (MS-222) (Sigma Chemical Co., St. Louis, MO) solution (438 mg/L). After anaesthetization, fish lengths and weights were measured, and a sample of blood was drawn from the caudal artery and vein using a 20-gauge needle and a 5 cc syringe rinsed with ammonium heparin. The blood sample was transferred to vacutainers containing EDTA. For each fish, a portion of blood was diluted 1:200 in a standard red blood cell (RBC) diluting pipette with Hendrick's diluting solution. The RBC diluting pipette was shaken and placed on ice. After 12 fish were sampled, RBC counts were performed following the method described in Schreck and Moyle (1990). A portion of blood was also transferred to hematocrit capillary tubes, sealed, and centrifuged at 8000 g for 5 min using a Damon/IEC Division Clinical Centrifuge (Needham, MA). Hematocrit was measured using a Damon/IEC Division microcapillary hematocrit reader (Needham, MA). Next, 20 μL of whole blood was pipetted into test tubes containing Drabkin's solution for measurement of hemoglobin concentrations. After all fish were sampled, hemoglobin measurements were made using the Sigma Diagnostics Total Hemoglobin Procedure No. 525 (St. Louis, MO). The vacutainers containing the remainder of whole blood for each fish were centrifuged for 3 min at 10 000 g. The plasma was pipetted into 1.5 mL microcentrifuge tubes and frozen at –90°C until lipid peroxide analysis was conducted using the K-Assay LPO-CC kit (Kamiya Biomedical Company, Seattle, WA). The detection limit of this kit was 2.0 nmol/mL of plasma. Mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration

(MCHC) were determined from RBC counts, hemoglobin concentrations and hemoglobin values by standard equations (Schreck and Moyle 1990).

2.3 Tissue Sampling

Following blood withdrawal, tissues were removed for MT, and histological analyses according to the following procedures. The gallbladder was separated from the liver and the bile was removed with a 23-gauge needle and 3 cc syringe. Bouin's fixative was injected into the gallbladder of two of the six fish from each treatment group before placing it into a vial to complete fixation. The gallbladders of the other four fish in the treatment group were processed for As analysis (Chapter 2). The liver was weighed, and a sub sample (approximately 5 mm^3) was taken and placed in Bouin's fixative. Following removal of the swim bladder, the kidney was excised. The kidney and the remainder of the liver were placed in WhirlPac bags, frozen on dry ice and stored at -90^0C until MT analysis.

2.4 Morphometric Parameters

Morphometric parameters were calculated using the following formulae: (a) Percent change in fork length = post-treatment length (cm) – pre-treatment length (cm) / pre-treatment length (cm) x 100, (b) Percent change in wet weight = post-treatment weight (g) – pre-treatment weight (g) / pre-treatment weight (g) x 100, (c) Condition factor = weight (g) x 10^5 / length (cm) x 10^3, and (d) Liver somatic index = liver weight (g) / body weight (g) – liver weight (g) x 100.

2.5 Metallothionein Analysis

Liver and kidney samples from lake whitefish were prepared and analyzed for MT by the mercury saturation assay of Dutton et al. (1993) as modified by Klaverkamp et al. (2000b).

2.6 Histopathological Analysis of Tissues

Gallbladders, and samples of liver were fixed in freshly prepared Bouin's fixative for 48 h, washed in 3 changes of 70% ethanol over the next 72 h, and stored in 70% ethanol until processing. Tissues were processed in an automated tissue processor (IL MVP Tissue Processor) using an ethanol/butanol series. Following processing, tissues were embedded in paraffin (Tissue Prep II), sectioned at 7 μm, and mounted on glass slides. The tissue sections were stained with Harris' haematoxylin and eosin, following the method described in Edwards (1967). All chemicals were obtained from the Fisher Scientific Company (Fair Lawn, NJ).

Liver histopathology was examined using both qualitative and semi-quantitative approaches. An area equal to 4 mm x 4 mm was examined from the liver of each fish. Alterations were examined semi-quantitatively by ranking the severity of tissue lesions using a method similar to Schwaiger et al. (1997). Ranking was: grade 0 = no pathological alterations, grade 1 = mild alterations, grade 2 = moderate alterations, and grade 3 = severe alterations. An overall assessment value of the alterations observed in the liver of each individual fish was determined from the ranking. These data were used to calculate mean assessment values (MAV) of liver alterations for each treatment group. Alterations to cellular morphology and structure, nuclear appearance, cytoplasm

appearance, staining, sinusoids, and presence of vacuolation, inflammation, and regeneration were all considered when assessing liver histopathology as recommended by Takashima and Hibiya (1995) and Bernet et al. (1999).

Gallbladder histopathology was assessed using both qualitative and quantitative approaches. The gallbladder wall was examined for alterations, including sloughing of the epithelium, folding of the epithelium and lamina propria, dilation of vascular elements, inflammation, edema, and fibrosis in the submucosa. The quantitative method was used to determine the width of the epithelium of the gallbladder wall and the total width of the submucosa, which includes the lamina propria, stratum compactum and stratum granulosum, lamina muscularis, subserosa, and serosa. The stratum compactum and stratum granulosum are considered as one layer, because they are difficult to distinguish using light microscopy. Measurements were made on projected microscopic images with a digitizer (Summagraphics Bit Pad, Fairfield, CT) using SigmaScan (version 3.90) software (Jandel Scientific, Corte Madera, CA). For each gallbladder, the widths of the epithelium and submucosa were measured at 4 points around the wall. The centermost coordinates of each gallbladder section were determined using a vernier scale, and widths were measured at points on the wall directly north, south, east, and west of the center. From these measurements, the mean ratio of the width of the submucosa to the width of epithelium (submucosa (μm) / epithelium (μm)) (SM/E) was calculated for each treatment group. Statistical analyses were not conducted on these data as only two of the six gallbladders of lake whitefish from each treatment group were examined for histopathology.

Histopathological examinations of tissues were completed using a Zeiss Photomicroscope III. Photomicrographs were taken with a Kodak DC120 digital camera using the Kodak Digital Science Microscopy Documentation 120 System (MDS120) and Adobe PhotoDeluxe (version 2.0) software (Adobe Systems, Inc.).

2.7 Statistical Analyses

Statistical analyses were performed using SPSS version 9.0 and were based on the following references, Neter et al. (1996), Stevens (1992) and SPSS (1999). For each data set, the assumptions of an analysis of variance (ANOVA), normality and equal variance, were checked. If the assumptions were not met the necessary transformations were preformed. A two-way ANOVA was used to evaluate interaction between dose and duration. Because interactions were found to occur, each data set was split into subsets. First, data sets were separated into subsets for each duration, to test for dose effects. After the ANOVA assumptions were checked for each subset of data at each duration, one-way ANOVAs were conducted and where significant, treatment group means were further compared to controls using the Dunnett's two-tailed multiple comparison procedure. Data sets were also separated into subsets for each dose, to test for duration effects. After the ANOVA assumptions were checked for each subset of data for each dose, one-way ANOVAs were conducted and where significant, treatment group means were further compared using the Bonferroni multiple comparison procedure. If more than one nonnormal population was found in a data subset, the Kruskal-Wallis one-way ANOVA was conducted on ranked data within each duration or each dose. Where differences were significant, treatment group means were further compared using the

Bonferroni multiple comparison procedure. All statistical analyses were conducted at an alpha level of 0.05.

3. Results

3.1 Feed Consumption and Morphological Indices

Lake whitefish fed the 100 μg As/g food exhibited modified feeding behavior after 45 days of exposure. The behavior was characterized by a fish taking a food pellet into its mouth and then spitting it out, however, some pellets of food continued to be consumed between days 45 and 52. After 55 days of exposure, complete feed refusal occurred in all fish in the treatment group until the experiment was terminated on day 64. Growth rates, expressed as the mean increase in wet weight from the initial body weight per day, of lake whitefish fed As contaminated diets were not significantly different from the controls on any sampling day. However, trends of decreasing growth rates were observed in all treatment groups (Figure 1). Consumption of As contaminated diets for 10, 30, or 64 days did not have significant effects on percent change in wet weights, percent change in lengths, or condition factors of lake whitefish with respect to the control group at each duration (Tables 1 and 2). LSI of lake whitefish fed As contaminated diets for 10 and 30 days did not differ from control values. Although LSI were reduced in lake whitefish fed 1, 10, and 100 μg As/g diets for 64 days, a significant difference was only observed in lake whitefish fed the high dose food (Table 2). The sex ratios of the treatment groups are shown in Table 1. Gonads of lake whitefish were in an advanced state of maturation on all sampling days, with the exception of two males sampled on day 10 (Chapter 2).

3.2 Hematology

No significant changes were observed in the hematological variables of hematocrit, hemoglobin concentration, red blood cell count, mean cell volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration for lake whitefish fed As contaminated diets after 10, 30, or 64 days of exposure as compared to control values (Table 3).

3.3 Biochemistry

Consumption of As contaminated diets had significant effects on MT concentrations in livers of lake whitefish after each duration of exposure. Significant MT induction occurred in livers of lake whitefish fed the 100 μg As/g food after 10 and 30 days of exposure. At day 64, MT was significantly induced in livers of lake whitefish fed the 1 and 10 μg As/g food, however, significant MT induction did not continue in livers of fish fed the high dose food (Figure 2a). Significant MT induction did not occur in the kidneys of exposed lake whitefish at any duration of exposure (Figure 2b).

No significant differences in lipid peroxide (LPO) concentrations were observed in the plasma of lake whitefish fed As contaminated diets for 10, 30, or 64 days (Table 3).

3.4 Gross Pathology

At each sampling day, the only macroscopically visible lesion observed was in the gallbladders of lake whitefish exposed to the 100 μg As/g food. On sampling day 10, two of the six fish fed the highest concentration of As exhibited these lesions, which were

characterized by hemorrhaging and diffuse whitening of the gallbladder wall. After 30 days of exposure, macroscopic lesions were observed in gallbladders of four of the six fish exposed to this concentration of As. The gallbladder walls were opaque white and thickened in appearance, and the gallbladders contained very little or no bile. Macroscopic gallbladder lesions were observed in two of the five fish fed the high dose food for 64 days. The lesions were similar in appearance to those observed at day 30 and were also accompanied by a reduction in the size of the organ.

3.5 Histopathology

In a normal lake whitefish liver, hepatocytes are arranged in branching cords or lamina, typically two cells thick, which are separated by a maze of sinusoids. Hepatocyte nuclei are round, and contain a single, prominent nucleolus. The cytoplasm of hepatocytes often contain glycogen vacuoles, however, the extent of vacuolation is highly variable. (Figure 3a, and b). Histopathology observed in the livers of lake whitefish fed As contaminated diets included nuclear, architectural and structural alterations, inflammation, and focal necrotic lesions.

Grade 1 (mild) alterations, characterized by the presence of a minor amount of pyknotic nuclei, minor areas of cellular debris, and eosinophilic hepatocytes, representing necrotic cells, were observed in livers of lake whitefish fed the As contaminated diets for each duration of exposure (Figure 3c and 4). Grade 1 alterations were also observed in livers of lake whitefish in the control groups; however, alterations were observed less frequently and encompassed a smaller area than in treated fish. The alterations observed in control fish represent normal cell turnover.

Grade 2 (moderate) alterations had the same characteristics as grade 1 alterations plus a disruption of the two-cell thick cord structure, indistinguishable cell outlines, and areas of hepatocytes with eosinophilic cytoplasm (Figure 5). These alterations were observed in livers of lake whitefish fed the 10 and 100 μg As/g diets for 10 days, and lake whitefish fed all As contaminated diets for 30 and 64 days. An apparent increase in the frequency of grade 2 alterations with As dose was observed at all exposure durations (Figure 4).

Grade 3 (severe) alterations consisted of the same characteristics as grade 2 alterations, as well as the presence of scattered and/or aggregations of lymphocytes. The majority of hepatocytes in the altered area contained pyknotic nuclei (Figure 6). Grade 3 alterations were observed in livers of lake whitefish fed the 100 μg As/g food for 10 and 64 days, and lake whitefish fed the 10 and 100 μg As/g diets for 30 days. These were also the treatment groups that had considerably higher total numbers of lesions as compared to the other treatment groups at each duration of exposure (Figure 4).

Mean assessment values (MAV) appear to be higher for lake whitefish fed the 10 and 100 μg As/g diets for 10 and 30 days, and all As contaminated diets for 64 days, as compared to control values. However, due to the high degree of variability, the only statistically significant differences in MAV between treated and control groups occurred in lake whitefish fed the 100 μg As/g food for 10 days and lake whitefish fed the 10 μg As/g food for 30 days (Table 4).

The lake whitefish gallbladder is a thin membranous sac. Columnar epithelial cells line the luminal surface. In order of closest proximity to the epithelium, the submucosa consist of the lamina propria, stratum compactum and stratum granulosum,

lamina muscularis, subserosa, and serosa (Figure 7a,b). Histological alterations were observed in lake whitefish fed contaminated diets for 10, 30, and 64 days. The severity, and location of alterations within the gallbladder wall varied with dose of As. The most extensive alterations were observed in the submucosa of the gallbladder wall of lake whitefish fed the 100 μg As/g food. A summary of the alterations observed in each layer of the gallbladder walls of exposed lake whitefish is shown in Table 5.

After 10 days of exposure of lake whitefish to 1 μg/g of dietary As, sloughing of the epithelial layer into the gallbladder lumen was evident. Epithelial cells in the lumen often did not exhibit necrotic changes. Minor to moderate inflammation, characterized by dilation of vascular elements (minor to moderate) and the presence of a minor amount of lymphocytes, were apparent in the submucosal layers, with more extensive inflammation occurring in the lamina propria (Figure 7c). Lake whitefish fed the 10 μg As/g food for 10 days, also exhibited sloughing of the epithelium, and necrotic changes were apparent in some epithelial cells. Additional alterations observed included minor to moderate dilation of vascular elements and minor inflammation within the submucosa, edema in all layers with the exception of the subserosa, and moderate fibrosis of the lamina propria, as well as minor folding of the epithelium and lamina propria. Eosinophilic fibrinoid material was also present in the gallbladder lumen, indicating bleeding had occurred (Figure 8a). Gallbladders of lake whitefish fed the 100 μg As/g food for 10 days contained similar alterations, however, the extent of the damage had progressed, with extensive inflammation (many lymphocytes), edema, and fibrosis observed in all layers of the submucosa (Table 5). In addition, regenerating epithelial cells were apparent along some parts of the wall (Figure 8b).

Minor sloughing of the epithelium was the only alteration apparent in gallbladders of lake whitefish fed the 1 μg As/g diet for 30 days. Exposure of lake whitefish to 10 μg/g of dietary As for 30 days also resulted in minor epithelial sloughing, as well as moderate vascular dilation, edema, and inflammation. Gallbladders of lake whitefish fed the 100 μg As/g food for 30 days exhibited alterations similar to those observed in lake whitefish fed the same concentration for 10 days, although folding of the epithelium and lamina propria were more pronounced (Table 5 and Figure 9).

Lake whitefish fed the 1 μg As/g food for 64 days exhibited similar gallbladder alterations as those observed in lake whitefish fed the same diet for 10 days. In addition, folding of the lamina propria was apparent in gallbladders of lake whitefish in this treatment group. After 64 days of exposure to the 10 and 100 μg As/g diets, gallbladders of lake whitefish had similar alterations, including epithelial sloughing, regenerating epithelial cells, eosinophilic fibrinoid material in the lumen, folding of both the epithelium and lamina propria, vascular dilation, inflammation, and fibrosis. The severity and extent of the alterations were, however, greater in lake whitefish fed the higher dose diet, with the exception of epithelial sloughing (Table 5). Increased thickness of the submucosa caused the lumen to become congested with folds (rugae) of epithelium and supporting lamina propria in gallbladders of fish fed the high dose diet. An increase in the number of mucosal (goblet) cells in epithelium was also apparent in this treatment group (Figure 10).

There was an increase in the width of the submucosa of the gallbladder wall, due to edema and fibrosis, which is reflected in the SM/E ratio. This ratio was considerably higher in lake whitefish fed the 100 μg As/g food as compared to lake whitefish fed the

control food at each duration of exposure, indicating increased width of the submucosal tissues in these fish (Figure 11).

4. Discussion

An immediate reduction in feed intake in response to both waterborne and dietary As exposures has been previously observed in freshwater fish species (Cockell 1990; Rankin and Dixon, 1994; Chapter 1). In experiments that examined the effects of dietary As exposure in rainbow trout (*Oncorhynchus mykiss*), the immediate reduction in food consumption was thought to be mediated by chemoreceptory detection of As in the diet, indicating that As was a deterrent to feeding (Cockell et al., 1991). In the current study, however, reduced feed consumption by lake whitefish was not observed until after 45 days of exposure to the 100 μg As/g food. The delayed onset of modified feeding behavior indicates that As was not an immediate deterrent to feeding in lake whitefish, but instead may have gradually led to an impairment of chemoreception with continued exposure. Impaired chemoreception could result in the inability of the fish to discern the pellet was food, resulting in the fish spitting the pellet back out, as was observed in the current study. This was also postulated to be the mechanism behind the reduced feed intake observed in rainbow trout (*O. mykiss*) exposed to waterborne arsenite (Rankin and Dixon, 1994). In addition, over the course of the experiment, the fish may have associated impaired health with consumption of the As contaminated food and modified its feeding behavior accordingly.

Whole organism parameters, including percent change in wet weight, percent change in fork length, growth, and condition factor, of lake whitefish were not

significantly affected by exposure to As contaminated diets within the 64 days of exposure. Previous studies that have examined the effects of waterborne and dietary As exposures on whole-body morphometric parameters in freshwater fish have also reported that wet weights, lengths, and condition factors of fish were not significantly altered (Sorenson, 1976; Farag et al., 1994; Schlenk et al., 1997), with one exception (Pandey and Shukla, 1982). Fingerlings of *Colisa fasciatus* were exposed to aqueous sodium arsenic (III) oxide at a concentration of 5.0 mg/L for 32 days, and parameters were measured every 4 days of exposure. Significant decreases in weights and lengths of fingerlings were observed after 8 and 12 days of exposure, respectively, and continued for the entire duration of exposure (Pandey and Shukla, 1982). In the current study, effects of As, including liver and gallbladder histopathology, observed at lower levels of biological organization may lead to effects at the whole organism level if fish are exposed for longer periods in the laboratory, or with continued exposure in an As contaminated habitat.

Although growth rates of lake whitefish fed the high dose food were apparently lower after 30 and 64 days of exposure, significant reductions in growth rates compared to control values were not detected. The absence of a significant effect on growth in the current study was unexpected, as reduced growth in response to both aqueous and dietary As exposure has been previously observed in a number of organisms, including freshwater fish, and mallards (Gilderhaus, 1966; Pandey and Shukla, 1982; Oladimeji et al., 1984b; Camardese et al., 1990; Cockell, 1990; McGeachy and Dixon, 1990; Hoffman et al., 1992; Rankin and Dixon, 1994; Stanley et al., 1994). The lack of significant differences in the current study may be due to inherent differences between species and/

or the length of exposure. For example, decreases in growth were evident in the majority of lake whitefish and lake trout (*Salvelinus namaycush*) fed diets contaminated with 100 and 1000 μg As/g as disodium arsenate after 20 days of exposure. However, a significant reduction in growth only occurred in lake trout fed the 1000 μg As/g diet (Chapter 1). In addition, a significant decrease in growth of rainbow trout (*O. mykiss*) exposed to 36 mg/L of sodium arsenate only became apparent after 77 days of exposure (McGeachy and Dixon, 1990). The mechanisms behind the direct and indirect effects of dietary As exposure on growth are further discussed in Chapter 2.

Exposure of freshwater fish to As has resulted in moderate anemia (Oladimeji et al., 1984b; Goel and Sharma, 1987; Cockell et al., 1991; 1992), however, no significant alterations in blood parameters of lake whitefish were observed within the 64 days of exposure. The absence of an effect of dietary As exposure on the blood parameters of lake whitefish may be due to one or more of the following reasons: (1) time of sampling, (2) length of exposure, and/or (3) reduced feed consumption. First, the effect of As exposure on fish blood parameters changes with continued exposure. For example, juvenile rainbow trout (*O. mykiss*) fed a 55 μg As/g diet for 8 weeks had reduced hemoglobin, hematocrit, RBC counts, MCH, and MCHC, while MCV was not altered. This pattern of responses was classified as normocytic, hypochromic anemia. However, after 12 weeks of exposure to a 60 μg As/g diet, juvenile rainbow trout had reduced hematocrit, hemoglobin concentration, MCV, and MCH, while RBC counts and MCHC were not altered. This state of anemia was classified as microcytic, normochromic anemia (Cockell et al., 1992). These results indicate that the effect of As on blood parameters varies with length of exposure. In addition, the majority of previous studies

that have examined the effect of dietary As exposure on fish blood parameters have been at least 8 weeks in duration (Oladimeji et al., 1984; Cockell et al., 1991; 1992). The results of the current study are consistent with the results of a previous study that examined the effect of dietary As on the blood parameters of lake whitefish exposed for a shorter duration. After 20 days of exposure to both 100 and 1000 μg As/g diets, blood parameters were not significantly affected (Chapter 1). Also, hemoglobin concentration and hematocrit in mallard (*Anas platyrhynchos*) ducklings fed a diet containing 200 μg As/g, as sodium arsenate, were not significantly altered after 4 weeks of exposure. Therefore, it is not surprising that significant effects on the blood parameters of lake whitefish were not observed after 10 and 30 days of exposure to As contaminated diets. Although the longest duration of exposure in the current study was over 8 weeks in duration, decreased As exposure as a result of reduced feed consumption by lake whitefish fed the high dose food may have allowed abnormal blood parameters to return to normal. Furthermore, starvation of fish is reported to cause an initial rise in RBC count, hemoglobin content, and hematocrit due to water loss (Steffens, 1989). The blood parameters of lake whitefish may have been rising as a result of starvation when sampled on day 64, so that the effect of As could not be detected.

The short-term nutritional status and metabolic energy demands of organisms are reflected in LSI measurements (Adams and McLean, 1985). The effect of dietary As exposure on LSI of freshwater fish has not been previously measured (Oladimeji et al., 1984b; Cockell and Hilton, 1988; Cockell et al., 1991; Cockell et al., 1992; Cockell and Bettger, 1993), with one exception (Chapter 1). LSI of lake whitefish exposed to diets containing 100 or 1000 μg As/g were unchanged after 20 days of exposure (Chapter 1).

Similarly, LSI of lake whitefish were not altered after 10 and 30 days of exposure to As contaminated diets in the current study. After 64 days of exposure, however, a significant reduction of LSI in lake whitefish fed the 100 μg As/g food was observed. A decrease in LSI was also observed in adult mallards (*A. platyrhynchos)* and ducklings fed diets containing 200 and 400 μg As/g, respectively, for 4 weeks. The effect of dietary As exposure on LSI is likely due to both reduced feed consumption and the severe and frequent liver lesions observed in lake whitefish fed the high dose diet. For example, a significant reduction in LSI occurred in squirrelfish (*Holocentrus marianus*) after 4 and 12 days of food deprivation (Hogstrand et al., 1996). Lake whitefish fed the 100 μg As/g food began to consume less food 19 days prior to sampling, and stopped feeding completely 9 days before the experiment was terminated.

Histopathological alterations in fish tissues are useful biomarkers of effects from exposure to environmental contaminants, as they are more sensitive than changes at higher levels of organization, and generally provide a better estimation of organism health than biochemical parameters (Hinton et al., 1992; Hinton, 1993; Teh et al, 1997). The use of hepatic histopathological biomarkers has been well studied (Hinton and Lauren, 1990; Hinton, 1993), whereas, gallbladder alterations have not yet been proposed as a biomarker candidate.

The liver is considered the critical target organ for As toxicity in fish and mammals due to the role it plays in detoxification (Sorenson, 1991). This theory was supported by the results of the current study, as livers of lake whitefish exposed to dietary As were a significant site of As accumulation (Chapter 2) and contained nuclear, architectural and structural alterations, as well as areas of inflammation, and focal

necrosis. Similar alterations have been observed in fish exposed to As in both the laboratory and the field (Gilderhaus, 1966; Sorenson et al., 1979a; 1979b; Sorenson et al., 1980; Chang et al., 1998; Kotsanis and Iliopoulou-Georgudaki, 1999; Chapter 1). For example, livers of green sunfish (*L. cyanellus*) collected from Finfeather Lake, which had a mean sediment As concentration of 4700 μg/g, d.w., contained abnormally shaped parenchymal hepatocytes arranged in a disorganized fashion, such that the two-cell thick cord structure was disrupted, as well as exhibiting pronounced focal necrosis (Sorenson et al., 1980). Other hepatic alterations, including fatty infiltration, cirrhosis, cytoplasmic vacuolation, hemosiderin granules, necrotic and fibrous bodies, and nuclear and cytoplasmic inclusions, have been observed in green sunfish exposed to waterborne As (Sorenson, 1991). Interestingly, previous studies that have examined the effects of both aqueous (McGeachy and Dixon, 1990; Rankin and Dixon, 1994) and dietary (Cockell, 1990; Chapter 1) As exposures in rainbow trout (*O. mykiss*) and lake trout (*S. namaycush*) have reported no histological changes in liver. Lake whitefish, therefore, appears to be more sensitive to dietary As exposure, with respect to histological effects in liver, than either lake trout or rainbow trout.

In liver, the primary site of As metabolism, arsenate is reduced to arsenite (Yamauchi and Fowler, 1994; Shiomi, et al., 1996; Styblo et al., 1999), so that toxic effects may be caused by both compounds. The mechanisms through which As results in hepatic alterations remain to be elucidated, however; they may involve uncoupling of oxidative phosphorylation due to arsenate substitution for phosphate, enzyme inhibition as a result of arsenite interaction with thiol-containing proteins, and/or elevated lipid

peroxidation (Hindmarsh and McCurdly, 1986; Fowle et al., 1992; Okada and Yamanaka, 1994).

Gallbladders are a significant site of As accumulation and histpathological alterations in fish exposed to both waterborne and dietary As (McGeachy and Dixon, 1990; Cockell et al., 1991; 1992; Cockell and Bettger, 1993; Rankin and Dixon, 1994; Chapter 1), indicating the importance of the biliary-fecal route in As excretion, regardless of the route of exposure (Gregus and Klaassen, 1986; Gyurasics et al., 1991). These overt, macroscopic lesions observed in gallbladders of lake whitefish exposed to the high dose food had the same characteristics, including diffuse whitening, thickened walls, and reduced organ size, as lesions previously described in studies that examined the effects of waterborne and dietary As exposure in rainbow trout (*O. mykiss*) (McGeachy and Dixon, 1990; Cockell et al., 1991; 1992; Cockell and Bettger, 1993; Rankin and Dixon, 1994). Histological alterations, including sloughing of the epithelium, dilation of vascular elements, inflammation, edema, fibrosis, and increased width of the submucosa, observed in gallbladders of lake whitefish also occurred in juvenile rainbow trout (*O. mykiss*) exposed to concentrations of dietary arsenate ranging from 33 to 182 μg As/g diet for 8 to 24 weeks (Cockell et al., 1991; 1992; Cockell and Bettger, 1993). The severity of the gallbladder histopathology observed in rainbow trout increased with dose of As and duration of exposure, as in the current study. Similar lesions were also observed in lake whitefish fed 100 and 1000 μg As/g diets for 20 days (Chapter 1), indicating that gallbladder damage as a result of As exposure is consistent between rainbow trout and lake whitefish.

Cockell et al. (1991) suggested that the gallbladder lesion associated with dietary As exposure resembles chronic acalculous cholecystitis. The molecular mechanisms through which As causes gallbladder pathology have not been investigated. Cockell and Bettger (1993) postulated, however, that chemical mediators of inflammation, such as prostaglandins, may be released as a result of As induced damage to epithelial cells. This, in turn, results in inflammation of the adjacent submucosa, and once the epithelial layer is removed, the bile can act directly on the submucosal layers of the gallbladder wall, exacerbating the irritation already occurring. With continued exposure to dietary As, epithelial cells must undergo an adaptation in order to allow them to regenerate. Mechanisms through which this adaptation occurs may include increased metabolic transformation of As to a less toxic form, reduction of net accumulation by decreasing uptake or increasing excretion of As, binding of As to proteins, such as MT, or storage of As in intracellular granules (Cockell, 1990). Once the epithelial layer regenerated in gallbladders of lake whitefish, fibrosis of the submucosal layers was extensive, such that the lesion may only be slowly reversible when As exposure has ceased (Cockell and Bettger, 1993). Scarring of the gallbladder may, therefore, be a useful indicator of both current and prior exposure to As in environmental monitoring programs.

MT is a low molecular weight, cysteine-rich, metal-binding protein that has been identified in vertebrates, invertebrates, and microorganisms (Mason and Jenkins, 1995). It functions in resistance to non-essential metal toxicity and in the homeostasis of essential metals, Zn and Cu (Roesijadi, 1992). The use of MT induction to monitor the effects of metals on aquatic organisms in environmental monitoring programs has been promoted (Benson et al., 1990; Couillard, 1997; AETE, 1999). The induction of MT in

livers of lake whitefish fed As contaminated diets at all durations of exposure is consistent with results of previous studies that have observed the induction of hepatic MT in freshwater fish as a result of subcutaneous and waterborne As exposure (Schlenk et al., 1997; Das et al., 1998; Eller-Jessen and Crivello, 1998a,b). For example, channel catfish (*Ictalurus punctatus*) were treated with aqueous concentrations of monosodium methyl arsonate (MSMA), sodium arsenite, and sodium arsenate. Fish were exposed to 0.01, 0.1, and 1.0 mg/L of each compound for 1 week. Exposure to all compounds resulted in dose-dependent induction of hepatic MT, however, significant induction only occurred in fish exposed to 1.0 mg/L of MSMA and sodium arsenite (Schlenk et al., 1997). It is important to note that the current study is the first to examine the effect of dietary As exposure on MT induction in a freshwater fish, with the exception of a preliminary experiment conducted in our laboratory (Chapter 1). It is also the first to demonstrate a significant effect of As exposure on hepatic MT in fish when inorganic As was administered as the pentavalent form.

The effect of As exposure on MT induction in kidney has not been previously measured in freshwater fish. The results of the current study, in which a significant induction of renal MT in response to dietary As exposure was not observed, are in agreement with the results of previous studies that examined the effects of As exposure in mammals (Albores et al. 1992; Flora and Tripathi, 1998). For example, male Spraque-Dawley rats were injected subcutaneously with sodium arsenite in water at 25, 50, 75, or 90 μmol/kg, and sodium arsenate in water at 50, 100, 150, or 200 μmol/kg. Kidney levels of MT were not affected by sodium arsenite or sodium arsenate exposure after 24 hours, whereas, hepatic concentrations of MT were significantly induced at all exposure

concentrations of sodium arsenite and at the two highest exposure concentrations of sodium arsenate (Albores et al., 1992).

Although hepatic MT was significantly induced in lake whitefish fed the high dose diet after 10 and 30 days of exposure, significant induction did not occur in fish fed this diet after 64 days of exposure. However, MT was induced in the livers of lake whitefish fed the 1 and 10 μg As/g diets for 64 days. The absence of significant MT induction with continued exposure to the high dose diet may be due to one or more of the following reasons: (1) a decline in As dose, (2) overt As toxicity, and/or (3) a biphasic pattern of MT induction with continued exposure. First, a decline in As dose occurred in this treatment group as a result of reduced feed consumption beginning on day 45 of exposure. If a critical minimum concentration of As is necessary to cause induction of MT, it may not have been maintained for 64 days. It is not likely that starvation of lake whitefish directly caused the reduction in MT concentration, as food deprivation results in elevated hepatic MT induction in mammals (Shinogi et al., 1999). The second explanation could be overt As toxicity in the livers of lake whitefish, resulting in decreased capability of MT induction. Toxicity was indicated by both the decreased LSI and severe liver histopathology observed in fish fed the high dose diet for 64 days. Finally, the biphasic pattern of hepatic MT induction observed may be typical of continued As exposure, as a similar pattern of induction has been previously reported. Climbing perch (*Anadas testudineus*) exposed to 0.75 mg As/L as As_2O_3 had significant increases in MT induction of 81%, 294%, and 98%, after exposure for 2, 15, and 30 days, respectively (Das et al., 1998).

The mechanism of hepatic MT induction by As compounds has not been well studied, however, the results of two studies indicate that As indirectly induces MT in liver (Albores et al., 1992; Schlenk et al., 1997). Possible mechanisms of indirect MT induction as a result of As exposure include alteration of Zn homeostasis, activation of an antioxidant response, and/or the release of an unknown endogenous factor(s) that induces hepatic MT. For example, dose-related increases in MT, Zn, and As concentrations were observed in livers of rats 24 hours after subcutaneous injection with 25, 50, 75, and 90 μmol/kg of sodium arsenite. It was also observed that in liver cytosols, a major portion of Zn, but not As, was associated with MT, indicating As did not bind to MT under the experimental conditions. Therefore, As may induce hepatic MT indirectly by causing increased Zn concentrations, which in turn results in elevated MT induction (Cousins, 1985). In addition, significant hepatic MT induction occurred in channel catfish (*I. punctatus*) exposed to 1.0 mg As/L as MSMA and sodium arsenite without significant As accumulation in the liver, and As was not present as a ligand in the induced hepatic protein (Schlenk et al., 1997). It was suggested that MT induction may have been through activation of various antioxidant response elements located in the 5' promoting region of the MT gene, or alternatively, through the release of an unknown endogenous factor(s) at an extrahepatic site, as As did not accumulate in liver (Schlenk et al., 1997).

LPO refers to the peroxidation of cellular lipids by free radicals, such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals. This process can alter the balance of fluidity and structure of subcellular and cellular membranes by damaging polyunsaturated fatty acids located in the membrane, eventually resulting in tissue damage and cell death (Winston and Di Giulio, 1991; Kehrer, 1993). Metals that exist in

more than one valence state and that undergo intracellular oxidative-reduction reactions can initiate lipid peroxidation (Wills, 1985). For example, a correlation was observed between increased LPO concentrations in plasma and histopathology in liver and kidney of lake whitefish exposed to dietary uranium (Cooley et al., 2000). Although these characteristics apply to As, results from previous studies that have examined the effect of As exposure on LPO in fish, birds, and mammals have not been consistent. For example, the majority of mammalian studies have reported that As exposure results in increased LPO concentrations in tissues, such as liver, kidney, lung and heart (Yanez et al., 1991, Yamanaka et al., 1991, Okada and Yamanaka, 1994, and Ramos et al., 1995). On the other hand, studies that examined the effects of As in mallards (*A. platyrhynchos*) reported both unchanged and decreased hepatic LPO after dietary As exposure for 4 and 10 weeks, respectively (Camardese et al., 1990, and Hoffman et al., 1992). Furthermore, aqueous As exposure did not alter hepatic lipid peroxidation in channel catfish (*I. punctatus*) (Schlenk et al., 1997) and resulted in transient LPO induction in livers of climbing perch (*A. testudineus*) (Das et al., 1998).

Although As produces increased LPO in mammals and climbing perch (*A. testudineus*), significant increases in LPO were not observed in the current study in the plasma of lake whitefish fed As contaminated diets for 10, 30 or 64 days. The absence of LPO induction in lake whitefish may be due to one or more of the following reasons: (1) route of exposure, (2) length of exposure, (3) induction of LPO in other tissues, and/or (4) scavenging of free radicals by MT. First, mammalian studies in which significant increases in LPO were observed, administered the As dose by intraperitoneal injection (Yanez et al., 1991 Yamanaka et al., 1991, Okada and Yamanaka, 1994, and Ramos et

al., 1995). For example, Yanez et al., (1991) found an intraperitoneal injection of 10 μg/g of As to significantly induce LPO in heart tissue of rats after 24 hours, whereas Schinella et al. (1996) exposed rats to the same concentration of As orally in tap water for 2 days and induction of LPO in liver was not observed. LPO induction was also not observed in mallards (Camardese et al., 1990, and Hoffman et al., 1992) and lake whitefish (Chapter 1) when As was administered in a contaminated commercial diet. In addition, mammalian studies have generally examined the effects of acute As exposure, with animals being sampled between 1 hour and 1 day after a single As dose was given (Yanez et al., 1991 Yamanaka et al., 1991, Okada and Yamanaka, 1994, and Ramos et al., 1995). Therefore, LPO may not be induced in organisms when As uptake occurs through the dietary route of exposure and/or may not remain elevated with continued exposure. For example, hepatic LPO was induced in climbing perch (*A. testudineus*) exposed to 1.5 mg/L of As for 48 hr; however, significant induction was not present after 30 days of exposure to 0.75 mg/L (Das et al., 1998). In the current study, LPO may have initially been induced in lake whitefish, but was no longer elevated when fish were sampled. LPO may have also been induced in other tissues of lake whitefish, but concentrations present in plasma were not high enough to be detected.

Although the primary role of MT is to detoxify and regulate metals, MT can also act as a free radical scavenger when induced in response to a number of contaminants, including As (Sato and Bremner, 1993; Guzzo et al., 1994). For example, the absence of LPO induction in liver of climbing perch after 30 days of exposure was correlated with a significant increase in hepatic MT concentration at this time period (Das et al., 1998). In

the current study, elevated hepatic MT in lake whitefish may have provided adequate protection against LPO induction as a result of dietary As exposure.

5. Conclusions

The results of this research identify a number of parameters that should be thoroughly evaluated as sensitive and reliable indicators of As toxicity in freshwater fish for use in environmental monitoring programs. At the tissue and organ levels, histopathological examinations of liver and gallbladder are recommended, as both were sensitive to As toxicity, with damage occurring in fish fed concentrations as low as 1 μg As/g. Macroscopic gallbladder lesions may be particularly useful indicators in monitoring programs in which thorough histological examinations are not possible. An assessment of hepatic MT induction is recommended at the molecular level of organization, as this parameter was very sensitive to As exposure, with elevated MT concentrations occurring in livers of lake whitefish fed the 1 μg As/g diet after 64 days. Parameters that are not recommended as indicators of As exposure include blood parameters, and measurement of LPO concentrations in plasma.

As indicated by the results of this study, there are a number of relevant issues, with respect to As toxicity in freshwater fish, which require further research. It is necessary to determine the mechanisms behind reduced feed consumption exhibited by lake whitefish, both the delayed response observed in the current study and the immediate response observed in previous work (Chapter 1). It is also important to investigate if effects observed at lower levels of biological organization would result in effects at the whole organism level of organization with exposure to dietary As beyond 64 days. The

mechanisms through which liver and gallbladder alterations are mediated in freshwater fish remain to be elucidated. More research on MT, including the mechanism of induction due to As exposure, in freshwater fish is also needed. For example, a study, which examines if hepatic MT concentrations remain elevated with, continued exposure to lower concentrations of As when feed refusal is not a factor, would be useful. In addition, it is necessary to examine if LPO concentrations are increased in tissues, such as liver and gallbladder, of freshwater fish as a result of dietary As exposure. The relationship between MT induction and oxidative stress due to As exposure remains to be determined. It would also be valuable to collect fish from a freshwater habitat, in which As contamination is a problem, in order to determine if the results of this study are applicable to fish exposed to As in the field.

Table 1. Sex ratios and morphometrics (percent change in wet weight and percent change in fork length) of lake whitefish after 10, 30, and 64 days of exposure to a control diet and three As contaminated diets.

Diet (µg As/g)	Sex Ratio (female:male)			% Change in Wet Weight[a]			% Change in Fork Length[a]		
	Day 10	Day 30	Day 64	Day 10	Day 30	Day 64	Day 10	Day 30	Day 64
0	3:3	2:4	5:1	6.13 (1.59)	10.1 (2.99)	16.1 (4.61)	2.10 (0.264)	2.74 (0.382)	6.37 (0.838)
1	1:5	2:4	3:3	6.93 (2.19)	6.48 (8.41)	12.4 (5.67)	2.84 (0.589)	3.01 (0.791)	4.06 (0.248)
10	2:4	4:2	3:3	4.34 (2.94)	10.0 (1.28)	12.7 (4.04)	1.62 (1.09)	4.15 (0.489)	4.62 (0.782)
100	2:4	2:4	3:2	4.83 (1.54)	4.72 (2.01)	8.50 (3.03)	1.98 (0.300)	2.93 (0.620)	4.50 (0.514)

[a]Data are expressed as mean (± SE). No significant differences were observed in the treated groups relative to the control group at each duration of exposure ($P < 0.05$).

Table 2. Condition factors and liver somatic indices of lake whitefish fed a control diet and three As contaminated diets for 10, 30, and 64 days. Data are expressed as mean (± SE). Asterisk denotes mean is significantly different from the control at that duration ($P < 0.05$).

Diet (µg As/g)	Condition Factor			Liver Somatic Index (%)		
	Day 10	Day 30	Day 64	Day 10	Day 30	Day 64
0	1.3 (0.02)	1.3 (0.03)	1.3 (0.03)	0.71 (0.04)	0.77 (0.04)	0.96 (0.14)
1	1.3 (0.04)	1.3 (0.05)	1.3 (0.07)	0.67 (0.02)	0.67 (0.05)	0.82 (0.06)
10	1.3 (0.02)	1.3 (0.04)	1.3 (0.03)	0.72 (0.07)	0.76 (0.03)	0.66 (0.05)
100	1.4 (0.03)	1.3 (0.02)	1.2 (0.02)	0.67 (0.06)	0.77 (0.03)	0.57 • (0.11)

Table 3. The hematological parameters, hematocrit, hemoglobin concentration, red blood cell count (RBC #), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), and lipid peroxide (LPO) concentrations in plasma of lake whitefish fed a control diet and three As contaminated diets for 10, 30, and 64 days. Data are expressed as mean (± SE). No significant differences were observed in the treated groups relative to the control group at each duration of exposure ($P < 0.05$).

Parameter	Day 10 Diet (µg As/g)				Day 30 Diet (µg As/g)				Day 64 Diet (µg As/g)			
	0	1	10	100	0	1	10	100	0	1	10	100
RBC (10^6/mL)	0.82	0.95	0.98	0.89	0.97	1.1	0.96	0.80	0.95	1.0	0.94	0.91
	(0.06)	(0.02)	(0.05)	(0.05)	(0.04)	(0.10)	(0.06)	(0.08)	(0.07)	(0.06)	(0.11)	(0.05)
Hematocrit (%)	36.7	38.2	39.4	36.1	39.2	36.8	40.1	34.2	42.3	38.8	37.1	36.7
	(1.33)	(1.65)	(2.87)	(1.42)	(1.14)	(1.74)	(2.91)	(3.61)	(2.22)	(2.22)	(2.40)	(1.31)
Hemoglobin (g/dL)	9.6	10	11	9.5	11	11	11	9.8	10	9.8	9.6	9.2
	(0.54)	(0.22)	(0.85)	(0.35)	(0.52)	(0.51)	(0.70)	(1.1)	(0.31)	(0.71)	(0.50)	(0.46)
MCH (pg)	120	110	110	110	110	100	120	120	110	97	110	100
	(5.8)	(1.7)	(4.7)	(7.4)	(6.4)	(5.9)	(4.5)	(7.8)	(5.3)	(3.9)	(11)	(5.1)
MCHC (%)	26	27	27	26	27	29	28	29	25	25	26	25
	(1.3)	(0.87)	(1.3)	(1.2)	(0.96)	(0.69)	(1.0)	(1.1)	(0.68)	(1.1)	(0.98)	(1.4)
MCV (fl)	460	400	400	410	410	370	420	430	450	400	410	410
	(31)	(11)	(22)	(12)	(20)	(19)	(15)	(27)	(25)	(14)	(33)	(16)
LPO	1.8	1.2	1.8	1.4	2.2	1.0	1.2	1.4	1.7	1.2	1.0	1.4
	(0.64)	(0.21)	(0.37)	(0.35)	(0.85)	(0.00)	(0.17)	(0.26)	(0.44)	(0.23)	(0.00)	(0.36)

Table 4. Mean assessment values (MAV) of liver alterations observed in lake whitefish fed a control diet and three As contaminated diets for 10, 30, and 64 days. Data are expressed as mean (± SE). Asterisk denotes mean is significantly different from the control at that duration ($P < 0.05$).

Diet (μg As/g)	Mean Assessment Value (MAV)		
	Day 10	Day 30	Day 64
0	0.83 (0.17)	0.67 (0.21)	0.33 (0.21)
1	0.33 (0.21)	0.50 (0.34)	1.3 (0.33)
10	1.4 (0.20)	1.8* (0.12)	1.4 (0.33)
100	2.1* (0.08)	1.5 (0.37)	1.4 (0.49)

Table 5. Alterations observed in the layers of the gallbladder wall of lake whitefish fed a control diet and three As contaminated diets for 10, 30, and 64 days. The degree of damage is represented as 0 (none), 1 (minor), 2 (moderate), and 3 (extensive). A dashed line indicates that the alteration is not applicable to the tissue layer. Sc and sg represents the stratum compactum and stratum granulosum.

Layer of Gallbladder Wall	Sloughing			Folding			Dilation of vascular elements			Inflammation			Edema			Fibrosis		
	10d	30d	64d	10d	30d	64d	10d	30d	64d	10d	30d	64d	10d	30d	64d	10d	30d	64d
1 µg As/g diet																		
epithelium	2	1	2	0	0	0	-	-	-	-	-	-	-	-	-	-	-	-
lamina propria	-	-	-	0	0	1	1	0	2	2	0	2	0	0	0	0	0	0
sc and sg	-	-	-	-	-	-	2	0	1	1	0	1	0	0	0	0	0	0
lamina muscularis	-	-	-	-	-	-	2	0	0	1	0	2	0	0	0	0	0	0
subserosa	-	-	-	-	-	-	2	0	1	1	0	0	0	0	0	0	0	0
10 µg As/g diet																		
epithelium	2	1	3	1	1	3	-		-	-	-	-	-	-	-	-	-	-
lamina propria	-	-	-	1	1	3	1	2	3	1	2	3	1	2	0	2	0	2
sc and sg	-	-	-	-	-	-	1	2	3	1	0	2	1	0	2	0	0	2
lamina muscularis	-	-	-	-	-	-	2	0	3	1	0	1	1	1	2	0	0	2
subserosa	-	-	-	-	-	-	2	2	2	1	0	2	0	0	2	0	0	2
100 µg As/g diet																		
epithelium	3	3	1	1	2	2	-	-	-	-	-	-	-	-	-	-	-	-
lamina propria	-	-	-	1	2	3	3	3	2	3	3	3	3	3	2	3	2	3
sc and sg	-	-	-	-	-	-	2	3	2	3	2	2	3	3	2	3	0	3
lamina muscularis	-	-	-	-	-	-	2	3	3	3	3	2	3	2	2	3	3	3
subserosa	-	-	-	-	-	-	3	3	3	3	3	2	3	2	3	3	2	2

Figure 1. The growth rates, calculated as mean final body wet weight – mean initial wet body weight / duration of exposure, of lake whitefish fed a control diet and three As contaminated diets for 10, 30, and 64 days. Data are expressed as mean (± SE). No significant differences were observed in the treated groups relative to the control group at each duration of exposure ($P < 0.05$).

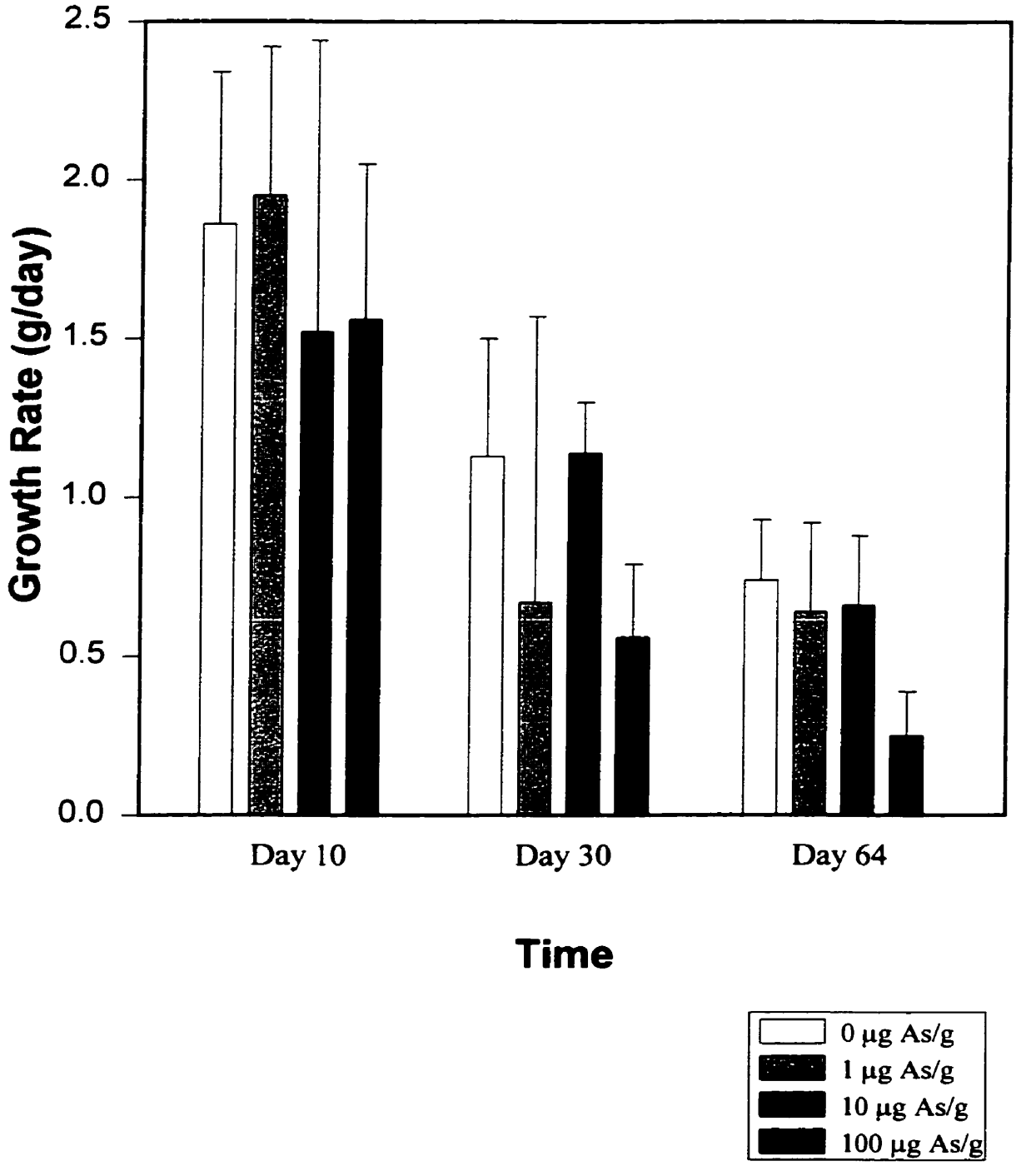

2.5
2.0
1.5
1.0
0.5
0.0
Growth Rate (g/day)
Day 10
Day 30
Day 64
Time
0 µg As/g
1 µg As/g
10 µg As/g
100 µg As/g

Figure 2. Metallothionein (MT) concentrations in the (a) livers and (b) kidneys of lake whitefish fed a control diet and three As contaminated diets for 10, 30, and 64 days. Data are expressed as mean (± SE). Asterisk denotes mean is significantly different from the control at that duration ($P < 0.05$).

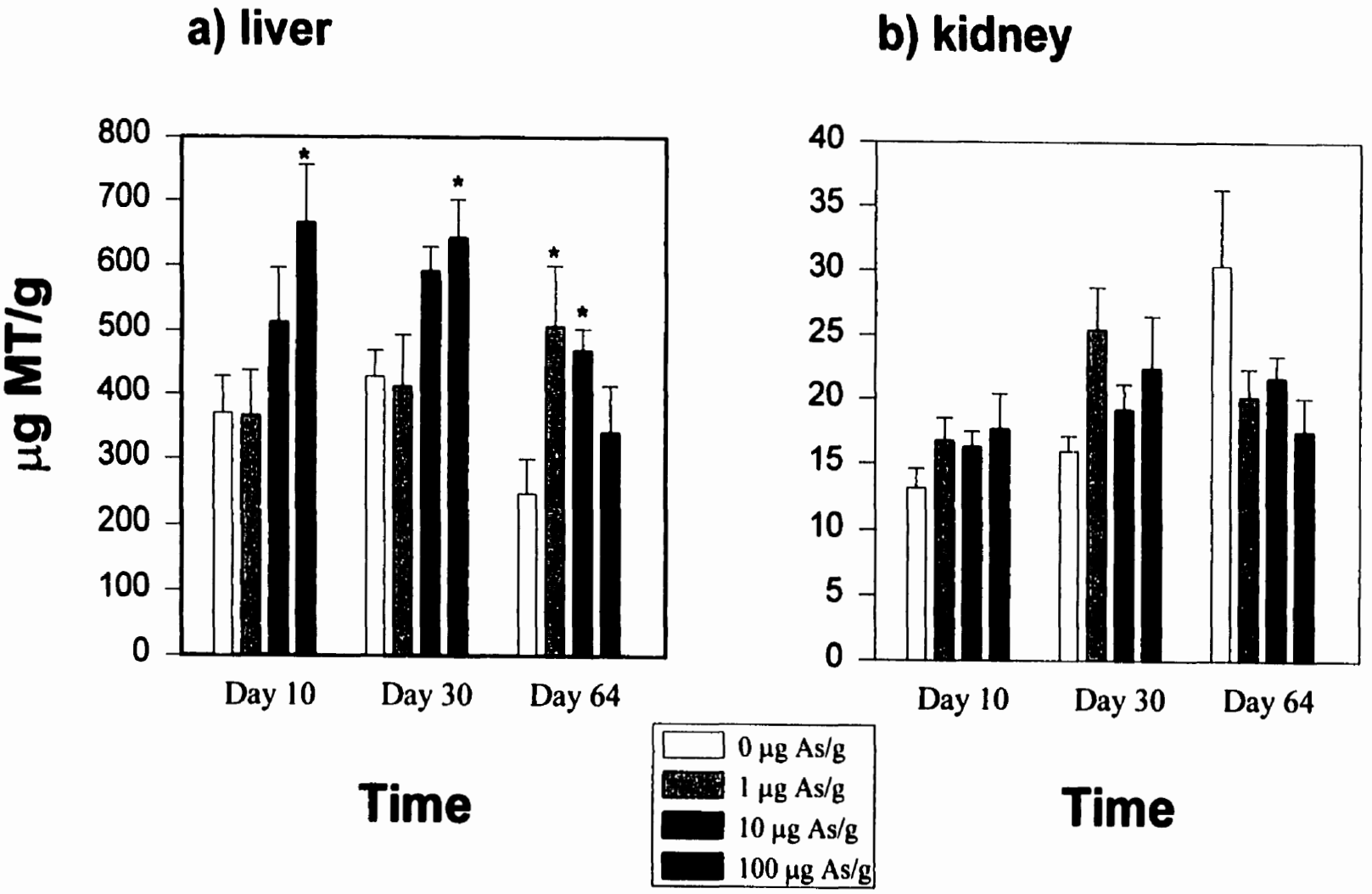
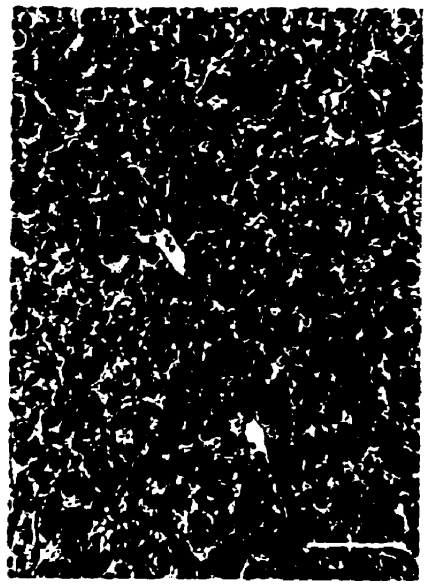
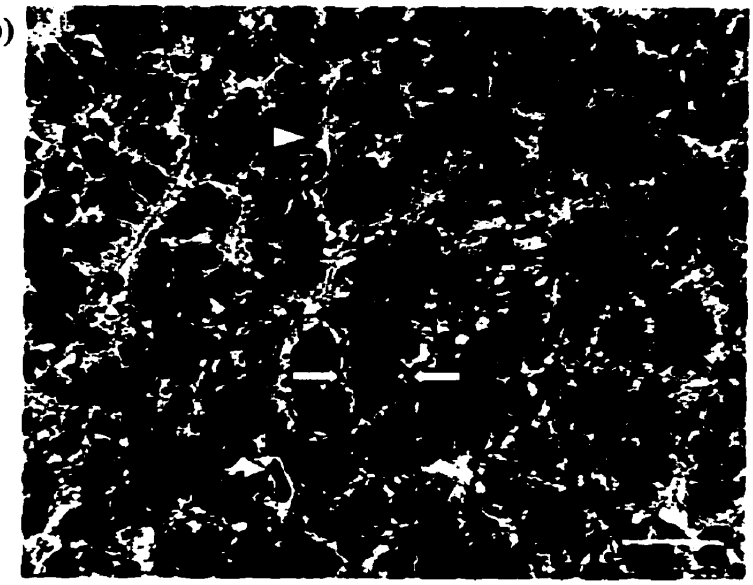

a) liver
b) kidney
µg MT/g
800
700
600
500
400
300
200
100
0
40
35
30
25
20
15
10
5
0
Day 10
Day 30
Day 64
Time
0 µg As/g
1 µg As/g
10 µg As/g
100 µg As/g

Figure 3. a) Low magnification micrograph of a liver from lake whitefish fed an uncontaminated diet. Bar = 50 μm. b) Higher magnification micrograph of the same histological section demonstrating the arrangement of hepatocytes in two-cell thick cords (between the block arrows), separated by liver sinusoids (arrowhead), with circulating erythrocytes (small arrow). Note that there are few glycogen vacuoles present (G). Bar = 30 μm. c) High magnification micrograph of a liver from lake whitefish fed the 1 μg As/g food for 10 days demonstrating the characteristics of grade 1 alterations. Note the pyknotic nuclei (arrows), cellular debris (CD) from degenerating hepatocytes and eosinophilic hepatocytes (E). Bar = 30 μm. H&E stain.

a)



b)



c)

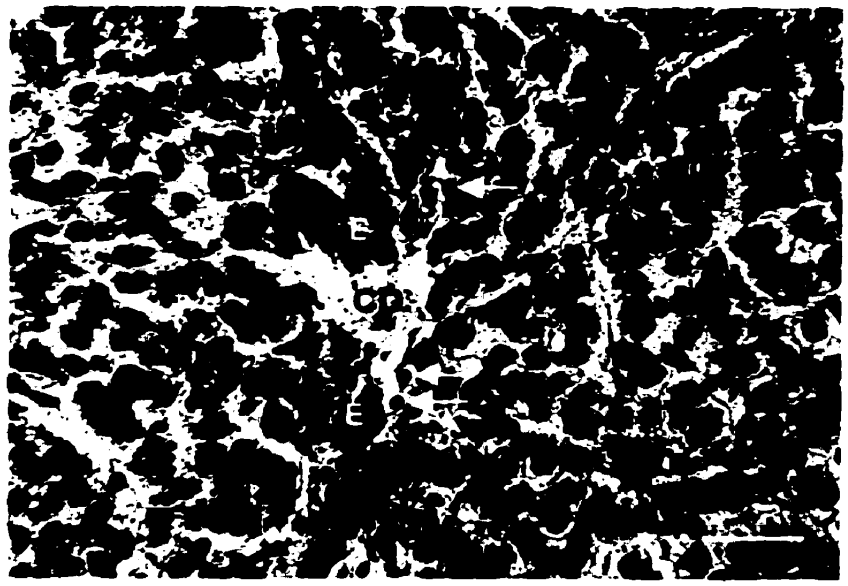


Figure 4. The total number of lesions observed in a 4 mm x 4 mm area in livers of lake whitefish fed a control diet (0 μg As/g) and three As contaminated diets (1, 10, and 100 μg As/g) for 10, 30, and 64 days. The sample size of each treatment group was 6 fish. The histological appearance of each lesion was categorized according to the degree of alteration observed. Grades 1, 2, and 3 represent mild, moderate, and severe alterations, respectively.

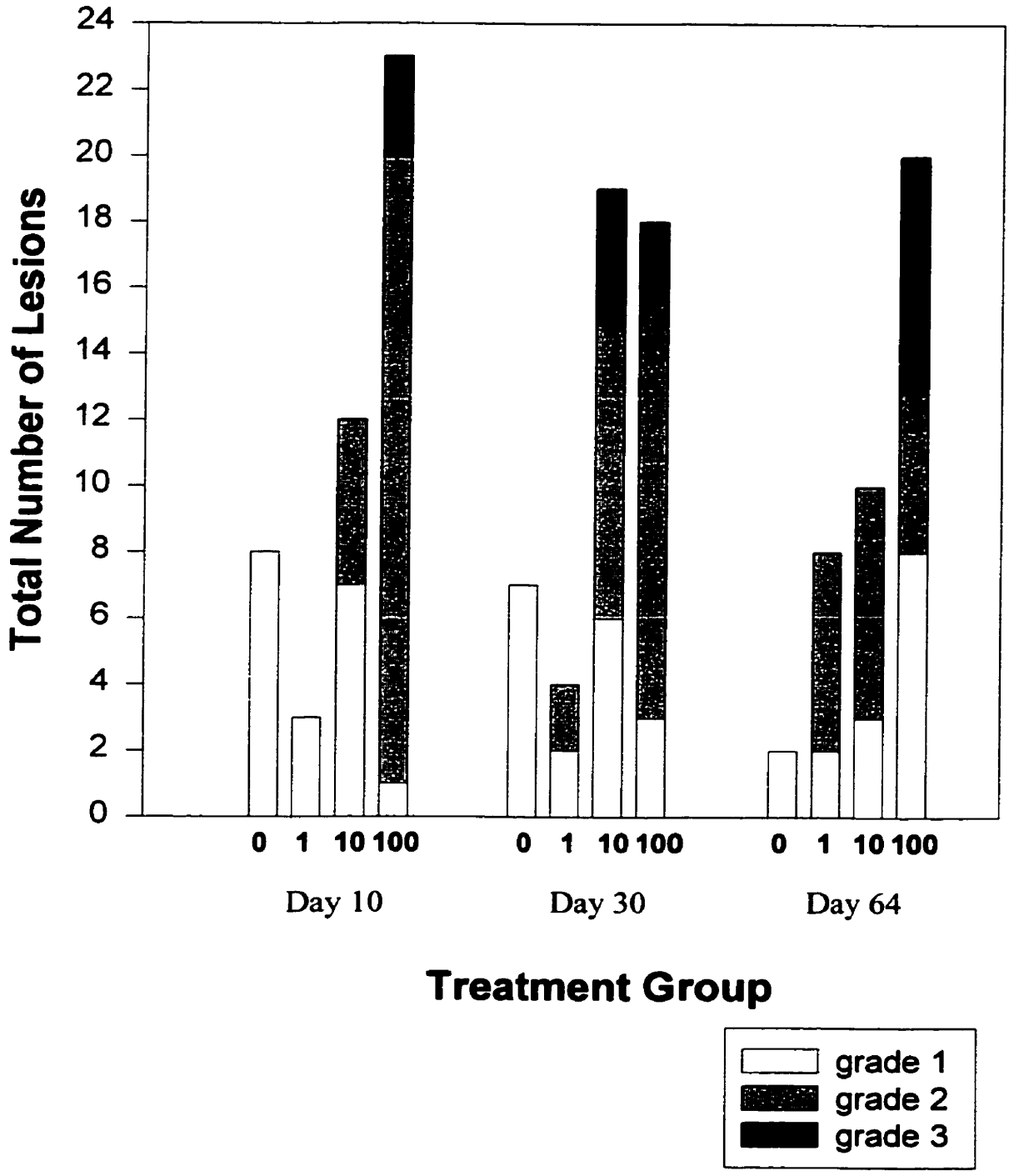
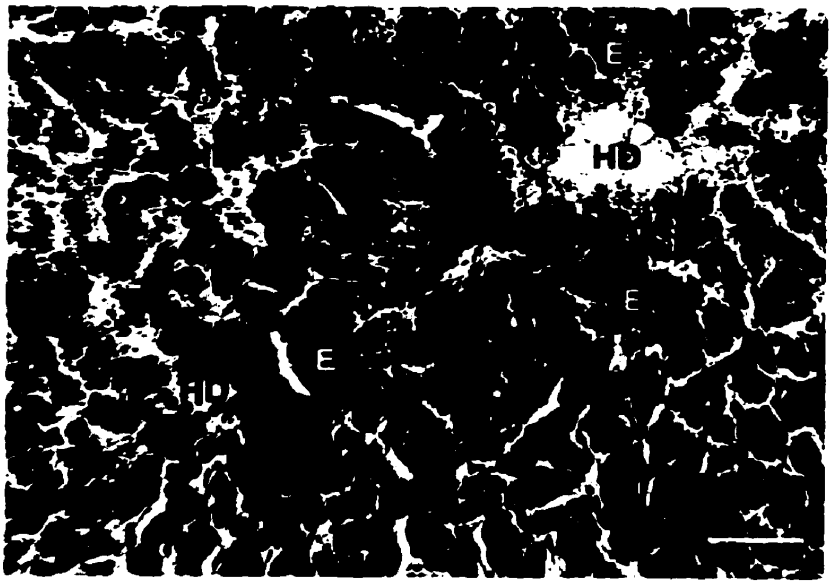

Total Number of Lesions
24
22
20
18
16
14
12
10
8
6
4
2
0
0 1 10 100
0 1 10 100
0 1 10 100
Day 10
Day 30
Day 64
Treatment Group
grade 1
grade 2
grade 3

Figure 5. a) Low magnification micrograph of a liver from lake whitefish fed the 10 μg As/g food for 30 days demonstrating the characteristics of grade 2 alterations. Note the hepatocyte degeneration (HD) and disruption of cord structure, as well as the areas of eosinophilic hepatocytes (E). Bar = 50 μm. b) Higher magnification micrograph of the same histological section demonstrating hepatocytes with pyknotic nuclei and indistinguishable cell outlines (between arrows), as well as a large area of hepatocyte degeneration (HD), eosinophilic hepatocytes (E), and disruption of cord structure. Bar = 30 μm. H&E stain.

a)



b)

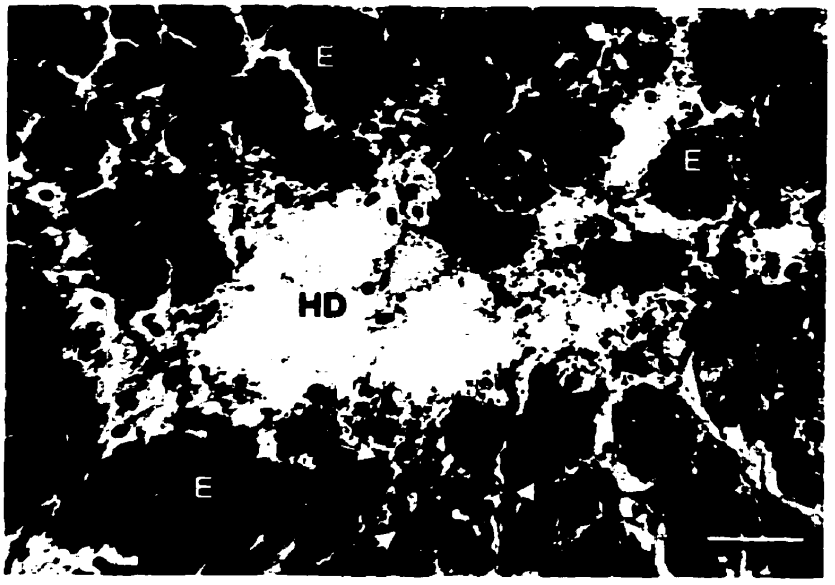
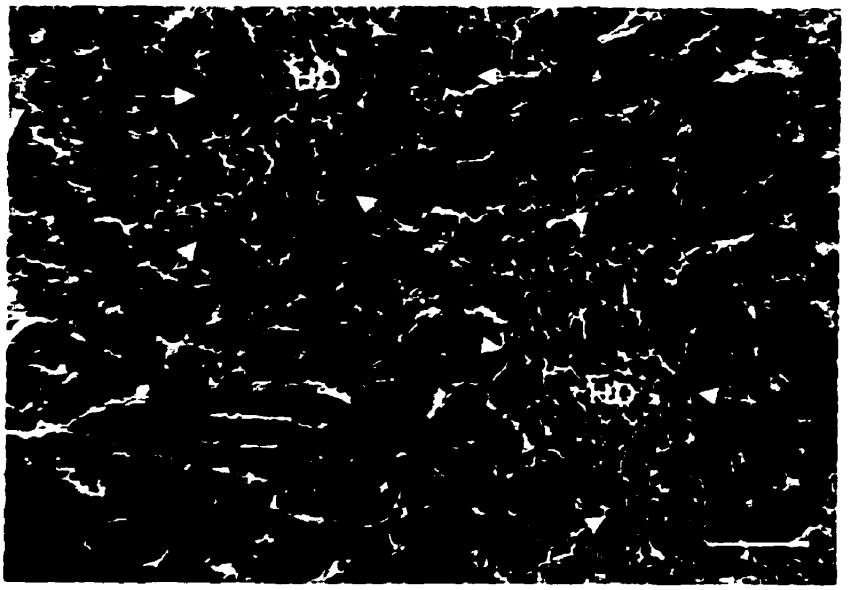


Figure 6. a) Low magnification micrograph of a liver from lake whitefish fed the 100 μg As/g food demonstrating the characteristics of grade 3 alterations. Note the inflammation, characterized by the aggregation of lymphocytes (between arrows), associated with the areas of hepatocyte degeneration (HD). Bar = 50 μm. b) Higher magnification micrograph of the same histological section showing lymphocyte aggregation and focal necrosis. Pyknotic nuclei in necrotic hepatocytes are indicated (arrows). Bar = 30 μm. H&E stain.

a)



b)

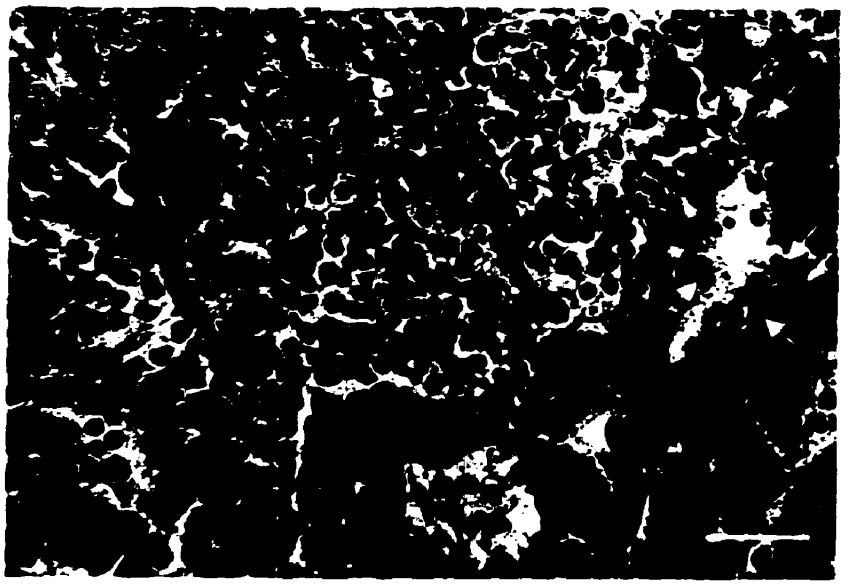
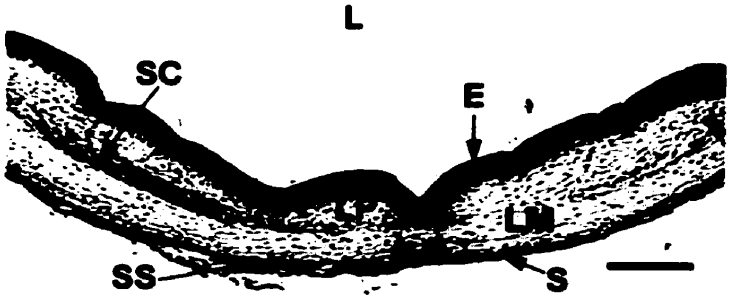
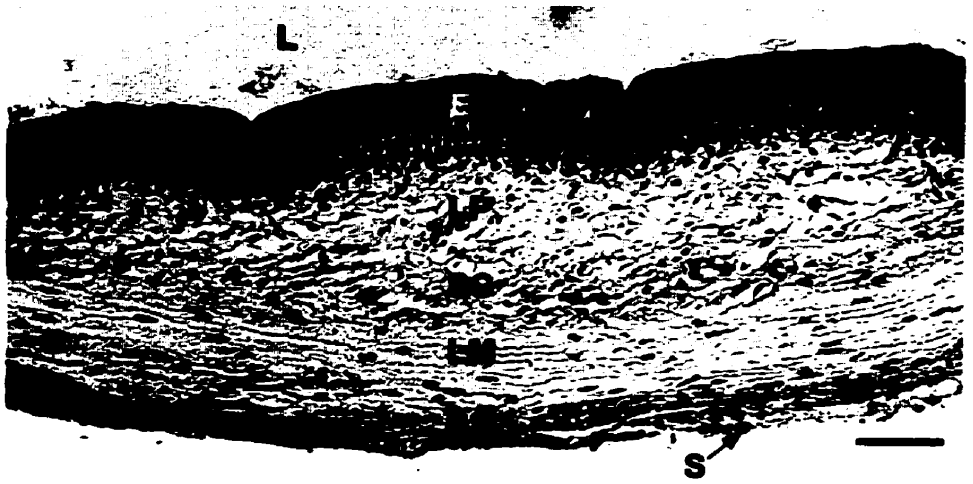


Figure 7. a) Low (bar = 200 μm) and b) high (bar = 60 μm) magnification micrographs of a gallbladder from lake whitefish fed an uncontaminated diet demonstrating the layers of the gallbladder wall. Closest to the lumen (L) is the epithelium (E), followed by the lamina propria (LP), stratum compactum and stratum granulosum (SC), lamina muscularis (LM), subserosa (SS), and serosa (S). The stratum compactum and stratum granulosum are considered as one layer because they are difficult to distinguish. c) High magnification micrograph of a gallbladder from lake whitefish fed the 1 μg As/g food for 10 days. Note sloughing of the epithelium, and minor inflammation (I), characterized by minor to moderate dilation of vascular elements (VD) and presence of lymphocytes, apparent in the submucosa. Bar = 50 mm. H&E stain.

a)



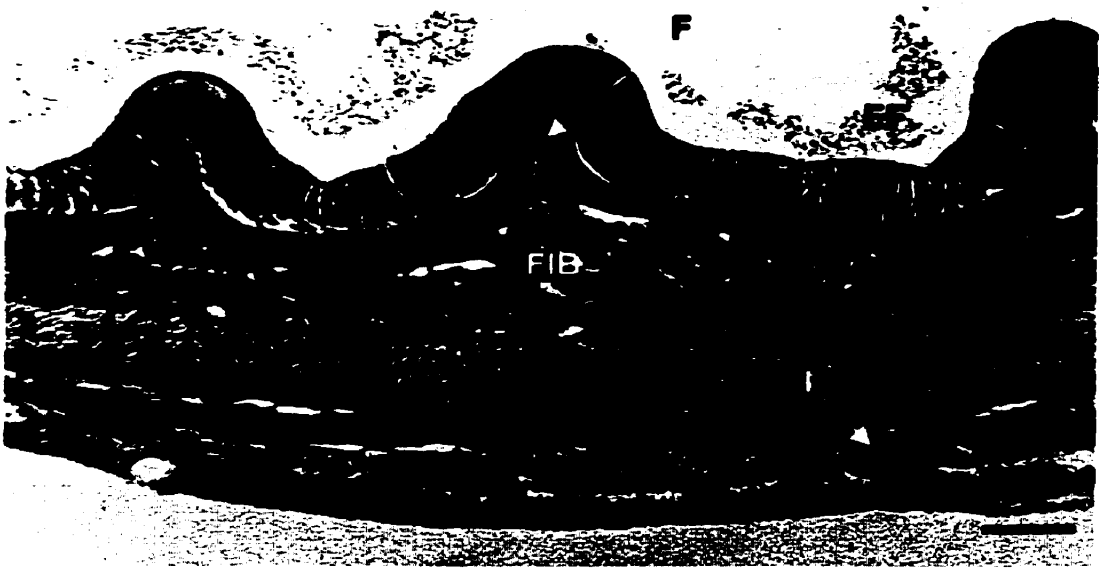
b)



c)

Figure 8. a) High magnification micrograph of a gallbladder from lake whitefish fed the 10 μg As/g diet for 10 days. Note minor inflammation (I) within the submucosa, edema in all layers with the exception of the subserosa, moderate fibrosis (FIB) of the lamina propria, and eosinophilic fibrinoid (EF) material present in the lumen. Minor folding (F) of the epithelium and lamina propria are also apparent. Bar = 40 μm. b) High magnification micrograph of a gallbladder from lake whitefish fed the 100 μg As/g food for 10 days. Note the extensive inflammation (I), characterized by moderate to extensive dilation of vascular elements (VD) and presence of lymphocytes, as well as the extensive edema and fibrosis in all layers of the submucosa. Bar = 70 μm. H&E stain.

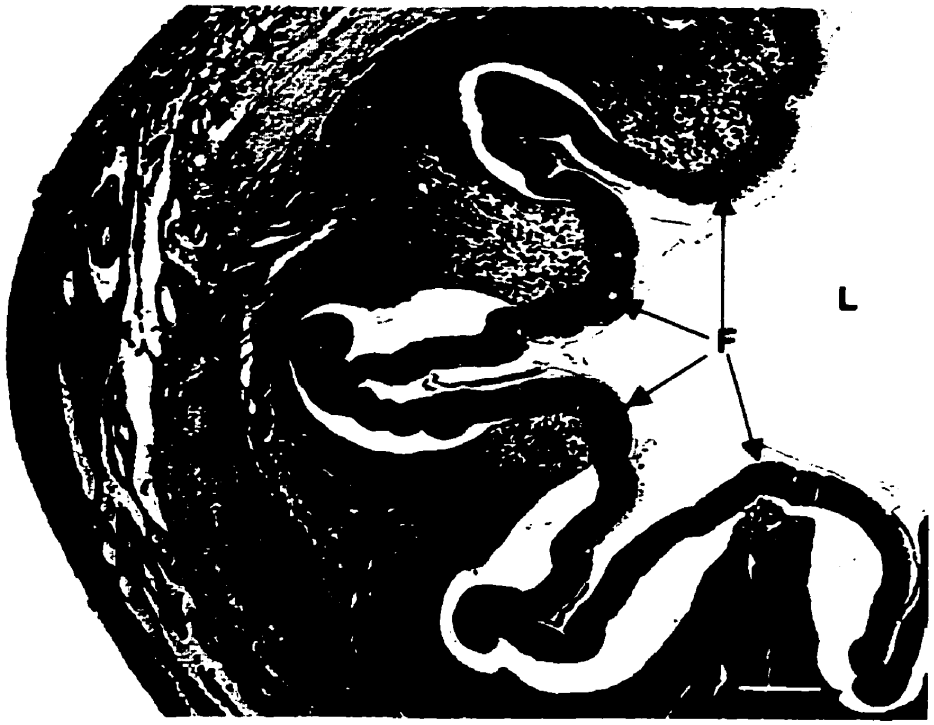
a)



b)

Figure 9. a) Low magnification micrograph of a gallbladder from lake whitefish fed the 100 μg As/g diet for 30 days. Note the moderate to extensive folding (F) of the epithelium and lamina propria into the lumen (L), as a result of extensive inflammation, edema, and fibrosis in the submucosa. The space between the epithelium and the lamina propria is a fixation/processing artifact. Bar = 200 μm. b) Higher magnification micrograph of the same histological section demonstrating increased width of the submucosa, epithelial sloughing, inflammation, edema, and fibrosis (FIB), particularly in the lamina propria (LP). The layers have become difficult to distinguish, as a result of these alterations. The serosa is indicated (S). Bar = 60 μm. H&E stain.

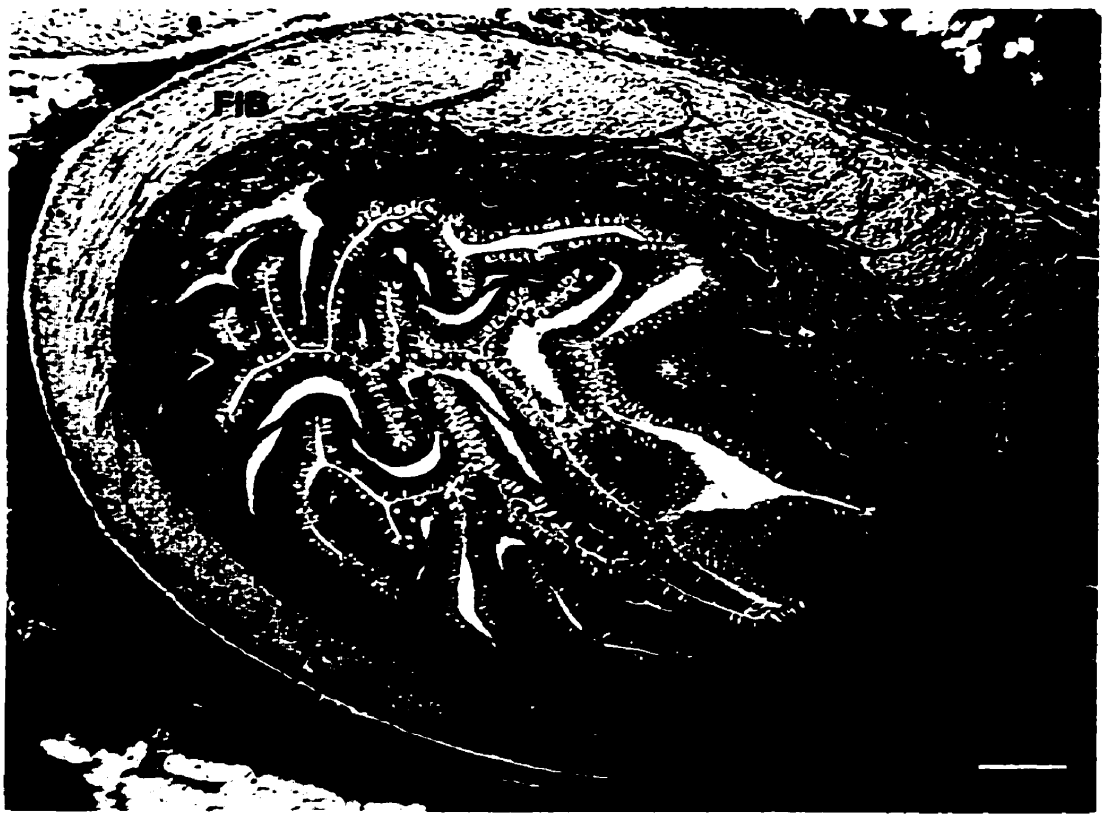
a)



b)

Figure 10. a) Low magnification micrograph of a gallbladder of lake whitefish fed the 100 μg As/g food for 64 days. Note the extensive thickening of the gallbladder wall as a result of edema and fibrosis (FIB) of the submucosa. The lumen is congested with folds of epithelium (E) and lamina propria (LP). Bar = 120 μm. b) Higher magnification micrograph of the same histological section, in which the layers have been indicated: epithelium (E), lamina propria (LP), stratum compactum and stratum granulosum (SC), lamina muscularis (LM), and subserosa (SS). Note the increased amount of mucous (M) cells in the epithelium. Bar = 50 μm. H&E stain.

a)



b)

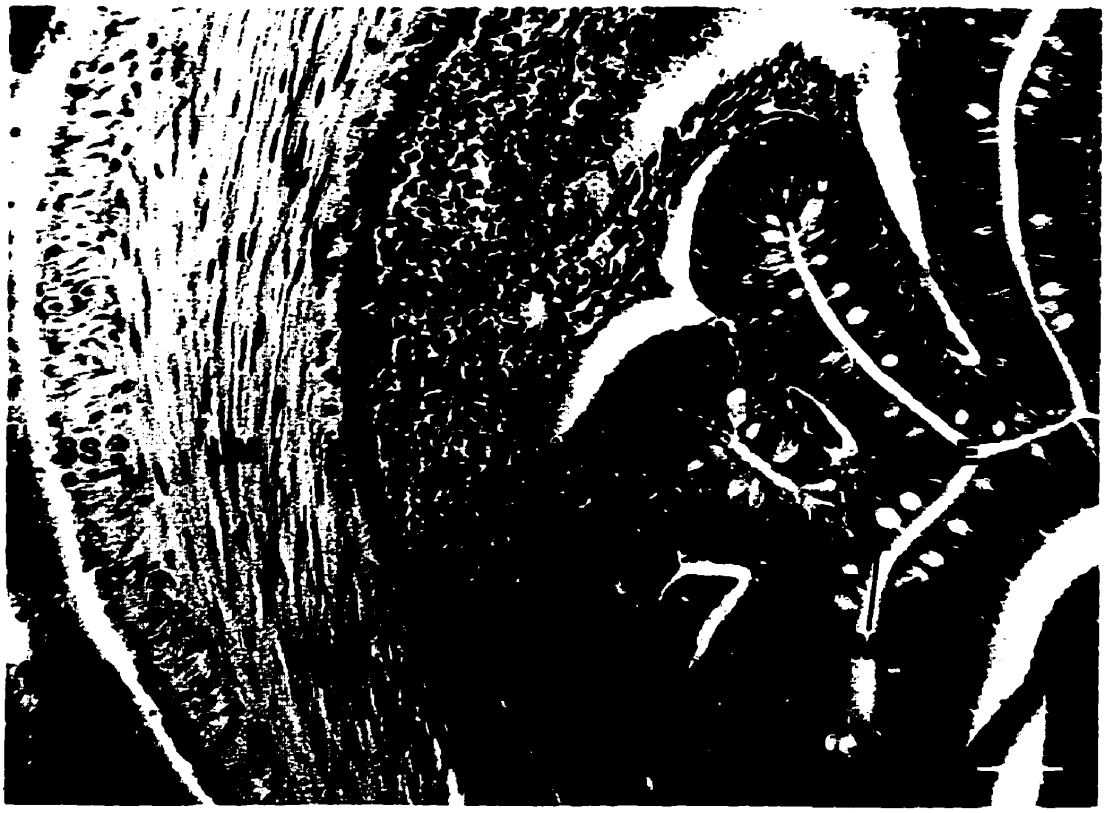
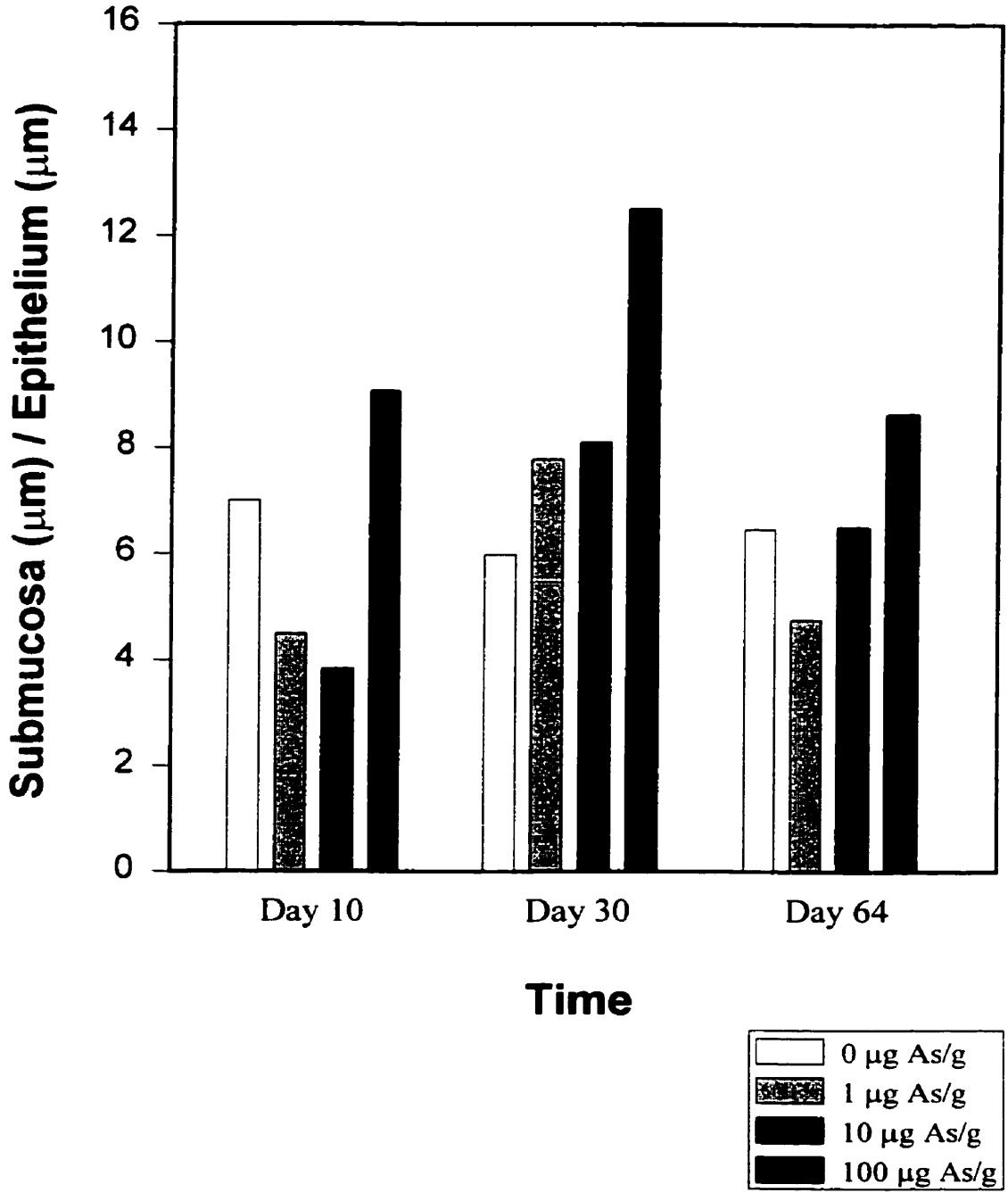


Figure 11. Ratios of the width of the submucosa to the epithelium of gallbladders of lake whitefish fed a control diet and three As contaminated diets for 10, 30, and 64 days. Statistical analyses were not conducted as only two of the six gallbladders of lake whitefish from each treatment group were examined for histopathology.


16
14
12
10
8
6
4
2
0
Submucosa (μm) / Epithelium (μm)
Day 10
Day 30
Day 64
Time
0 μg As/g
1 μg As/g
10 μg As/g
100 μg As/g

Conclusions

Two experiments were conducted to investigate the uptake and toxicity of dietary As exposure in freshwater fish species. The following results were observed in the preliminary short-term experiment that examined the accumulation, distribution, and toxicological effects of As in lake whitefish and lake trout fed 0, 100, and 1000 μg As/g diets with and without the addition of brine shrimp for 20 days:

- Reduced feed consumption occurred in lake whitefish and lake trout fed all As contaminated diets, except the 100 μg As/g NS (no shrimp) food.
- Addition of brine shrimp did not alter the consumption of As contaminated diets.
- The pattern of As tissue distribution differed between the two species.

At the molecular level of organization:

- LPO (lipid peroxide) concentrations significantly increased in plasma of lake trout fed the 1000 μg As/g WS food.
- MT (metallothionein) concentrations in liver and kidney were not altered by As exposure.

At the tissue and organ levels or organization:

- Blood parameters were not affected by As exposure.
- LSI (liver somatic index) were significantly decreased in both species.
- Histological alterations were not observed in liver, anterior and posterior kidney, pyloric caeca, or spleen examined from lake trout exposed to dietary As.

- Histological alterations were observed in gallbladder, liver, kidney, pyloric caeca, and intestine examined from lake whitefish exposed to dietary As.

At the whole organism level of organization:

- Growth was significantly decreased in lake trout fed the 1000 μg As/g food.
- Condition factors were unaltered by As exposure.

Based on the results of the preliminary experiment, lake whitefish were selected for use in a longer-term experiment. To decrease the chances that feed refusal would occur, concentrations of As chosen for the second study were 0, 1, 10, and 100 μg As/g.

The longer-term experiment was conducted to examine the accumulation, distribution, and toxicological effects of As in lake whitefish exposed by the dietary route of uptake for 10, 30, and 64 days. The following results, with respect to the accumulation and distribution of As, were observed in lake whitefish:

- The pattern of accumulation in lake whitefish tissues was influenced by reduced feed consumption beginning on day 45 in fish fed the 100 μg As/g food.
- Significant accumulation occurred in liver, kidney, stomach, pyloric caeca, intestine, skin, and scales of lake whitefish fed the 100 μg As/g food for 30 days.
- Significant accumulation occurred in liver and scales of lake whitefish fed the 10 μg As/g food for 30 and 64 days.

The following results, with respect to the toxicological effects of As, were observed in lake whitefish:

At the molecular level of organization:

- MT induction occurred in livers of lake whitefish fed the 100 μg As/g food after 10 and 30 days, and in fish fed the 1 and 10 μg As/g food for 64 days.
- LPO concentrations in plasma were not altered by As exposure.

At the tissue and organ levels of organization:

- LSI was significantly decreased in lake whitefish fed the 100 μg As/g food for 64 days.
- Blood parameters were not affected by As exposure.
- Liver and gallbladder histopathology was observed in lake whitefish fed all As contaminated diets after each duration of exposure.

At the whole organism level of organization:

- Wet weights, fork lengths, growth rates, and condition factors were unaltered by As exposure.

Based on the results of this experiment, analyses of As in liver, pyloric caeca, intestine, and scales are recommended for use as indicators of As bioavailability in EEM programs. In addition, measurement of hepatic MT concentrations and histopathological examinations of liver and gallbladder should be evaluated for use as early, sensitive and reliable indicators of As toxicity.

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