The physiological effects of a biologically incorporated silver diet on rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Silver was biologically incorporated into a diet by exposing rainbow trout for 7 days to 100 mg/l of waterborne silver as silver thiosulphate. These fish were processed into a fine powder (trout meal) and pelleted to form a nutritionally balanced feed which was then fed to juvenile rainbow trout (*Oncorhynchus mykiss*). Fish were fed either a diet containing 3.1 µg/g biologically incorporated silver (an environmentally relevant concentration), or one of three control diets containing approximately 0.05 µg/g Ag for 128 days. All dietary treatments were fed to satiation once daily. Dietary silver did not significantly affect mortality, growth, food consumption, or food conversion efficiency. Furthermore, ion regulation (plasma Na^+ levels and Na^+ influx rates), hematological parameters (hematocrit, plasma protein, hemoglobin levels), plasma glucose, metabolism (oxygen consumption, ammonia and urea excretion rates) and intestinal Na/K-ATPase and amylase activities were all unaffected. Based on the physiological parameters investigated here, this dietary silver exposure appeared to be physiologically benign to rainbow trout. However, silver concentrations in the livers of the silver-fed fish were significantly elevated at day 16, and reached a steady-state level of ~ 20 µg/g Ag by day 36. The concentration specific accumulation rate in the livers of fish fed biologically incorporated silver was about 4.6 orders of magnitude greater than when fed dietary silver sulfide, indicating much greater bioavailability. Despite this increase, hepatic metallothionein concentrations remained unchanged, in contrast to waterborne exposures, indicating that bioaccumulated silver behaves differently depending on whether it is taken up from the diet or from the water. Apart from a significant reduction in hepatic Cu at day 16, liver concentrations of Cu and Zn were not affected by dietary silver. Silver concentrations were also significantly elevated (relative to control fish) in the kidneys of the silver-treated fish on days 88 and 126, and in the gills and plasma at day 126. © 2001 Elsevier Science B.V. All rights reserved.

1. Introduction

Since the early 1990s, a concerted effort has been made to understand the physiological mechanisms associated with acute silver toxicity to aquatic organisms. This research demonstrates that the free Ag^+ ion probably is the primary toxic moiety to freshwater fish (Bury et al., 1999a; Galvez and Wood, 1997; Wood et al., 1996a). During waterborne exposures, Ag^+ acts as a surface-active toxicant, eliciting its primary effect at the gill epithelium (Hogstrand and Wood, 1998; Janes and Playle, 1995; Morgan et al., 1997; Wood et al., 1999). Nevertheless, various forms of silver, although seemingly non-toxic in an acute sense, are able to accumulate readily in freshwater fish (Hogstrand and Wood, 1998; Hogstrand et al., 1996; Wood et al., 1996b). The most explicit examples are seen in freshwater fish exposed to Ag thiosulphate complexes. On the one hand, waterborne Ag thiosulphate was found to be at least four–five orders of magnitude less toxic than $AgNO_3$ during routine acute toxicity tests. On the other hand, when presented as Ag thiosulphate, Ag accumulated readily in the gills and internal tissues such as the liver. In fact, rainbow trout exposed to ~ 100 mg/l Ag (as $Ag(S_2O_3)_n$) accumulated ~ 900 μg/g Ag in the liver, resulting in a 300% increase in hepatic metallothionein (MT) concentration (Hogstrand et al., 1996).

Bioaccumulation of waterborne silver thiosulphate is environmentally relevant because up to 99% of the photographic facilities in the US discharge their effluents into municipal sewers as soluble silver thiosulphate complexes, accounting for up to 44% of the total anthropogenic discharge to the aquatic environment (Lytle, 1984; Purcell and Peters, 1998). However, concentrations of silver in the water column near US urban centers are extremely low, ranging from 0.01–0.1 μg/l Ag (Shafer et al., 1998). At these low concentrations of silver, acute toxicity to aquatic organisms is not expected, but silver might enter the food chain. The majority of the silver accumulates in sediment either as silver sulfide or bound to particulate or colloidal materials (Purcell and Peters, 1998). Silver bound within sediments potentially can be taken up by benthic invertebrates and then be passed to higher trophic levels (Ratte, 1999). The concentrations of silver in tissues of aquatic plants and animals collected near industrialized sites are typically elevated, implying that environmental silver is bioavailable to aquatic organisms (reviewed in Eisler, 1996). Silver concentrations in bivalves living in contaminated environments may exceed 100 μg/g Ag dry weight (~ 20 μg/g Ag wet weight) (Luoma and Phillips, 1988), whereas silver burdens in freshwater fish have been reported up to 1.9 μg/g Ag wet weight (US Public Health Service (PHS), 1990).

A recent interdisciplinary workshop recommended that criteria to protect aquatic ecosystems from metal pollution must in future incorporate a food chain component to account for all routes of exposure to aquatic organisms (Bergman and Dorward-King, 1997). However at present, only a few studies have addressed the trophic transfer of silver to freshwater fish under laboratory conditions (Galvez and Wood, 1999; Garnier and Baudin, 1990; Terhaar et al., 1977). Using mathematical simulations on a simplified 'water–carp–trout' food chain, Garnier and Baudin (1990) concluded that the trophic route would represent the primary source of metal uptake into the organism after only 13 days of continuous exposure to both waterborne and dietary silver in the form of ^{110m}Ag labelled AgCN at 4 μg/l in the original water to which both species were exposed. In contrast, Galvez and Wood (1999) found that food spiked with silver sulfide to levels up to 3000 μg/g Ag did not result in significant silver accumulation by rainbow trout and produced no deleterious physiological effects over 58 days of exposure. Only the latter study has attempted to address the physiological effects (other than metal bioavailability) of food chain transfer of silver.

The primary objective of this study was to assess the growth, metabolism and physiology of

juvenile rainbow trout fed a diet containing biologically incorporated silver over 126 days. Silver was incorporated into the feed by initially exposing trout (destined to become food) to silver thiosulphate via the water, and then processing these 'prey' trout into a commercial grade feed. This is in contrast to the majority of dietary studies of metals on fish performed to date, which have instead relied on direct addition of metal salts to yield contaminated diets. Harrison and Curtis (1992) point out that naturally contaminated foods tend to have higher absorption efficiencies than superficially contaminated diets. This statement is supported by the profound effects seen in rainbow trout fry fed with metal-laden invertebrates collected from the field (Woodward et al., 1994). Additional dietary treatments, in the present study were implemented to monitor any effects due to the thiosulphate ion and quality of our synthetic diets. The quality and palatability of our homemade diets (using synthetic trout meals) were tested against a control diet made with commercial herring meal. Each of the additional control dietary treatments was pair-fed at the same ration as consumed by the fish fed the silver-contaminated diet. Pair fed controls tested whether potential physiological effects were due to the dietary silver exposure, or simply a result of differences in feed intake between treatments (Lanno, 1989).

2. Materials and methods

2.1. Fish husbandry

Juvenile rainbow trout (*Oncorhynchus mykiss*) ($n \sim 1100$) weighing 5–10 g were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada) and transported to McMaster University, Hamilton, ON. Upon arrival in the laboratory, fish were placed in a 400-l tank, mixed and then randomly divided amongst five well-aerated 400-l holding tanks. Fish were held for 3 weeks before use in the experiment. Each tank was supplied with 1.5 l/min of Hamilton dechlorinated tap water. The chemical composition of the water was as follows (in mM): $Na^+ = 0.6$; $Cl^- = 0.7$;

$Ca^{2+} = 1.0$; titratable alkalinity to pH 4.0 of 1.0 mM; and pH 7.8–8.0. Water temperature was maintained at ambient conditions, which ranged from 4–11°C over the course of the study. Fish were fed commercial trout pellets (Martin's Feed, Elmira, ON) until the start of the study, at which time they were switched to one of the prepared diets (see Section 2.2). Fish were fed once daily to satiation during both the acclimation and experimental phases of the study.

In order to distinguish between treatments in tests in which animals were exposed together, each group of fish was uniquely marked with Alcian Blue dye using a Panjet injector (Wright Health Group; Dundee, Scotland). During the marking procedure, fish were lightly anaesthetized with 0.1 g/l MS-222 buffered with 0.2 g/l sodium bicarbonate, and allowed to recover in fresh water before being placed back in the holding tanks. All fish were marked two weeks before the start of the study.

2.2. Preparation of 'homemade' trout meals

Adult rainbow trout ($n = 120$) (Humber Springs Trout Hatchery; average weight ~ 300 g) were randomly selected and transferred in equal numbers (40 fish per tank) to three well-aerated 400-l tanks served with dechlorinated Hamilton tap water. Fish were allowed to acclimate to these holding tanks for 2 days. At the start of the waterborne exposures, water flow was turned off to each tank and one of three water chemistries was assigned to each. These water chemistries included:

1. Silver diet. Trout were exposed to waterborne silver at 100 mg/l Ag (as silver thiosulphate; 0.37 mmole/l). The Ag thiosulphate solution was made by reacting on a molar basis, 1 part AgCl (0.37 mmole/l) (Johnson Matthey Ltd, Brampton, ON) to 4 parts $Na_2S_2O_3$ (1.48 mmole/l) (BDH).
2. Control diet. Trout were exposed to Hamilton dechlorinated tap water.
3. Thiosulphate diet. Trout were exposed to waterborne thiosulphate (1.48 mmole/l $Na_2S_2O_3$) (the same concentration of thiosulphate used during the Ag thiosulphate exposure).

Every 24 h, 50% of the water from each tank was removed and replaced with 200-l of fresh Hamilton dechlorinated tap water, and the appropriate amount of silver thiosulphate or sodium thiosulphate stock was added to tanks 1 and 3. Waterborne exposures lasted 7 days. Following the 7th day, fish were sacrificed by a quick cephalic blow and the whole fish carcass was portioned into small pieces ($\sim 2 \times 2$ cm). Fish chunks were placed in a plastic bag and kept refrigerated until further processed at the University of Guelph the following day. In total, 10 kg of fish per diet were required to produce sufficient quantities of feed.

2.3. Formulation and steam-pelleting of diets

All experimental diets were formulated in the Department of Animal Science and Nutrition at the University of Guelph, using the ingredients listed in Table 1. Each diet was identical, except for differences in the trout meal and herring meal portions. Three of the experimental diets consisted of 45% fish meal (25% 'home-made' trout meal plus 20% commercial herring meal (Wedge-

port Enterprises Ltd, Yarmouth, NS, Canada) as filler. The remaining 55% of the mixture consisted of corn gluten meal (Martin Feed Mills), wheat middlings (Martin Feed Mills), vitamin premix (Vit-9408), mineral premix (Min-9504) and fish oil (Martin Feed Mills) (Table 1). The vitamin and mineral premixes were donated by Dr C.Y. Cho from the Department of Animal Sciences and Nutrition at the University of Guelph. In brief, fish chunks were dried in an oven at $\sim 48°C$ for 48 h. After drying, carcasses were mixed with an equal proportion (on a weight basis) of wheat middlings and ground up into a fine powder using a household blender. The remaining ingredients of the diets were added to the fine powder mixture and mixed for 30 min. Diet formulations were then steam-pelleted to produce a commercial-grade pellet. All diets were sieved using a 2 mm sieve to remove any fine particulate material. An additional diet was prepared using only commercial herring meal (herring diet) as the 45% fish meal component. This feed was incorporated into the study as a benchmark against which to evaluate the palatability of the diets made with trout meal.

Table 1
Constituents of control, thiosulphate, silver and herring diets fed to juvenile rainbow trout over a 4-month period: all diets are identical except for their proportion of trout meal and herring meal, and the nature of the trout meal.

Ingredients (as % of total) Ingredients	Control diet[b]	Thiosulphate diet[c]	Silver diet[a]	Herring diet[d]
Trout meal	25	25	25	0
Herring meal	20	20	20	45
Wheat middlings	25	25	25	25
Corn gluten meal	15	15	15	15
Vitamin premix	1	1	1	1
Mineral premix	1	1	1	1
Fish Oil	13	13	13	13
Silver conc.[e] ($\mu g/g$)	0.048 ± 0.0001	0.048 ± 0.0001	3.12 ± 0.13	0.058 ± 0.0003

[a] The silver diet was made from trout exposed to waterborne silver (as silver thiosulphate; 0.37 mmol/l Ag) in dechlorinated Hamilton tap water for 1 week.

[b] The control diet was made from trout exposed to only Hamilton dechlorinated tap water.

[c] The sodium thiosulphate diet was made from trout exposed to 1.48 mmol/l $Na_2S_2O_3$ in dechlorinated Hamilton tap water, the same concentration of thiosulphate used during the Ag thiosulphate exposure.

[d] A herring diet was formulated using commercial herring meal only.

[e] Silver concentrations of diets are given as mean $\pm$ SE ($n = 4$).

2.4. Experimental protocol

2.4.1. Feeding regime, growth and food consumption

At the start of the study, each treatment was switched to one of the experimental diets. In total, there were five experimental treatments. Two groups were fed either the control or Ag diets to satiation daily, referred to as the control satiation and Ag satiation treatments, respectively. Satiation was determined using the protocol of Wilson and Wood (1992) and Galvez et al. (1998). In brief, small amounts of food (~ 2 g) were lightly sprinkled on the water surface of each tank every minute. Food was distributed to tanks at 1-min intervals. This process was continued until food was found floating at the surface after the 1 min period. Feeding was stopped for 1 min. If the administered food had been subsequently consumed, feeding was continued as normal. Satiation was reached once food remained uneaten on the water surface for more than 2 min. On average approximately 30 min was required for fish to reach satiation. The amount of food consumed was recorded for both the control and silver satiation treatments daily. Three additional treatments were either fed the control, thiosulphate or herring diet. These groups served as pair-fed controls, fed the same ration on a per body weight basis as the dietary silver treatment. The ration of food consumed at satiation was expressed on a %/day basis, calculated by taking the average amount of food consumed per fish, divided by the mean fish weight and multiplied by 100. Mean fish weights were recorded approximately every 2 weeks by bulk-weighing all of the fish from each tank. Fish from each treatment were removed from their tanks (one tank at a time) and placed in a bucket containing water and a sieve insert. The total biomass of each tank was calculated from the difference between the mass of the bucket, water and sieve, with and without the fish. Mean fish weight in grams was calculated by dividing the number of fish in each treatment into the total biomass. Specific growth rates (SGR) were determined by least-squares linear regression (SPSS Version 8.0) through the natural logarithm (ln) of mean fish weight versus time data and expressed on a %/day basis. Food-conversion efficiency (FCE) (in %) for each treatment is the ratio of mean SGR (%/day) and daily food consumed (%/day), multiplied by 100.

Five-millilitre water samples were taken weekly from each tank for analysis of total silver.

2.4.2. Tissue sampling

On days 0, 16, 36, 88 and 126, ten fish per tank were randomly selected and immediately sacrificed by a quick cephalic blow. Blood was taken by caudal puncture using a 1-ml Hamilton syringe pre-rinsed with ammonium heparin (50 i.u/ml). Blood was centrifuged at $10\,000 \times g$ for 2 min and plasma was collected and analyzed immediately for total protein. The remaining plasma was frozen in liquid nitrogen and stored at $-70°C$, until analyzed for plasma Na^+, Ag and glucose. In addition, entire gill baskets, kidneys, livers, and intestines were excised from fish for silver analysis. Although all treatments were sampled, only the data from control satiation and silver satiation treatments were analyzed for silver and presented here. Gills were rinsed in 18 MOhm double-deionized water to remove any fine particulate matter. The intestinal tract was flushed with Cortland saline and scraped with a fine spatula to remove any undigested feed or feces. All tissues were blotted dry, frozen in liquid nitrogen, and stored at $-70°C$ until processed. Liver digests were also measured for total Cu, Zn and metallothionein concentrations to assess the impact of silver accumulation on metal metabolism, and intestines collected on day 88 were also analyzed for Na/K-ATPase and amylase activities.

2.4.3. Routine metabolism

Routine 'in tank' oxygen consumption, ammonia and urea excretions were measured on days 0, 16, 36, 55, 88 and 126. Each sampling period consisted of four cycles performed at 6-h intervals over 24 h. Sampling was begun exactly 1.5 h after fish feeding. At the beginning of each cycle, the surface of the water of each tank was sealed with a transparent, heavy plastic sheet, and water flow and aeration turned off. A small pump was used to transfer water from the bottom of each tank and reintroduce it to the top of the tank at a rate

of 10 l/min via a small connector on the plastic lid. Care was taken to ensure that no atmospheric air was introduced into the system during measurement of routine metabolism. Change in water P_{O2} was measured over a half-hour period by taking 10-ml water samples from each tank at 10-min intervals. Water was injected into a Cameron E101 oxygen electrode, maintained at the temperature of the experimental tanks, and connected to a Cameron OM-200 O_2 meter. Water P_{O2} levels were not allowed to decrease below 100 torr. In-tank oxygen consumption rate (M_{O2}) was calculated according to the following equation.

$$M_{O2} = (\Delta P_{O2} \cdot \alpha O_2 \cdot vol)/(mass \cdot time) \qquad (1)$$

where ΔP_{O2} is the average change in P_{O2} (torr) over the 30-min time period, αO_2 is the solubility constant for O_2 in water at the experimental temperature, vol is the volume of water in the experimental tanks, and mass is the biomass of the entire treatment group in grams.

Ammonia-N and urea-N excretion rates were measured at the same time as oxygen consumption, except that each cycle was extended to 1.5 h to allow greater analytical accuracy. Water flow was turned off for the entire cycle, but aeration was reinstated after the 30 min P_{O2} measurement period. Water samples (2×5 ml) were collected from each tank at the beginning and end of the 1.5 h period, and immediately frozen for later analysis of total ammonia-N and urea-N. After the terminal water samples were taken for N-waste excretion, water flow to each tank was resumed. Half the water volume of each tank was removed, and then the tank was flushed with fresh dechlorinated tap water to help return water ammonia and urea levels to normal, prior to the start of the subsequent cycle. The N-waste excretion rate for each cycle was determined from the difference in N concentration of the water samples taken at the start and end of each cycle. Mean M_{O2} and mean N-waste excretion rates were calculated for each treatment from the four replicate values per sample period. All M_{O2} and N-waste excretion rates were normalized to a weight of 1 kg using the weight exponent of 0.824 as determined for rainbow trout. The nitrogen quotient (NQ) was calculated from the ratio of moles of N produced (ammonia-N and urea-N excretion) to moles of oxygen consumed.

2.4.4. Na^+ influx and hematological indices

Unidirectional Na^+ influx rates were measured on days 0, 36 and 126. Tests were performed in a single, plastic bucket containing 40-l of aerated, dechlorinated Hamilton tap water. Two hours before the start of each flux period, eight fish per treatment were randomly selected and allowed to acclimate to the flux chamber. At time 0, 40 μCi of ^{22}Na (Mandel Scientific, Guelph, ON) was added to the water and allowed to mix for 5 min. Two 5 ml water samples were taken at 5 min, and again at 2, 4 and 6 h, for analysis of total Na^+ concentration and ^{22}Na activity. After 4 h, five fish were removed from the flux tank and placed in a 15-l bucket of 'clean' dechlorinated water to remove any superficially bound ^{22}Na from the fish. After a few minutes, fish were netted one at a time from the 'clean' water and quickly sacrificed with buffered 1.0 g/l MS-222. Blood was immediately sampled by caudal puncture using a Hamilton syringe rinsed with ammonium-heparin. Small amounts of blood were placed in ammonium-rinsed capillary tubes, spun and analyzed for blood hematocrit and plasma protein. Blood hemoglobin concentrations were measured using the remaining blood. Once the blood work was completed, fish were blotted dry and weighed, and individual fish were placed in scintillation vials for δ-counting (Canberra-Packard A5000 Minaxi). After all five fish initially removed from the flux tank had been sampled, an additional five fish were transferred into the 'clean' water. All 40 fish were sampled using the same protocol. The exact time at which each fish was removed from the radioisotopic solution was recorded and substituted into Eq. (2) below for calculation of Na^+ influx rate (absolute time ranged from 4 to 6.5 h). The unidirectional Na^+ flux in nmol/g/h was calculated as:

$$J_{in} = Fish\ CPM/(MSA \cdot W \cdot t) \qquad (2)$$

where MSA is the mean specific activity in CPM/nmol Na of the exposure water; W is the weight of fish in grams, and t is time in hours.

2.5. Analytical methods

Five millilitre water samples were acidified to 0.5% HNO_3 (trace-metal grade acid) and analyzed for total silver by atomic absorption spectroscopy (AAS) on a Varian AA 1275 unit fitted with a graphite furnace atomizer (Varian GTA-95). Total plasma protein was analyzed immediately after dissection by placing plasma on an optical refractometer and measuring the refractive index (American Optical, Buffalo, NY). Plasma Na was analyzed by flame AAS (Varian AA 1275), whereas plasma Ag was measured by graphite furnace AAS (Varian GTA-95). Plasma was diluted 1000-fold or 50-fold with 0.5% HNO_3 (trace metal grade acid; Fisher Scientific) prior to analysis of total Na and Ag, respectively. Plasma glucose was measured according to the hexokinase method using a commercial reagent kit (Sigma kit no. 16-20, St.Louis, MO). Whole-blood hemoglobin concentrations were measured using the cyanmethemoglobin method (Sigma kit 525-18). In short, 20 µl of whole blood was immediately placed in a vial containing 5 ml of Drabkin's reagent (Sigma) and measured against known standards at 540 nm (LKB, Ultraspec Plus).

Tissues were individually homogenized in 1-3 ml of 50 mM Tris–HCl buffer (Sigma), pH 8.0 at 2°C using an ice-cold glass-Teflon homogenizer (Thomas Scientific). Aliquots (100 µl) of tissue homogenate were digested with 1 ml of concentrated trace-metal grade HNO_3 in acid-washed test tubes, and the digests were slowly brought to 120°C and evaporated to dryness. Each tube was subsequently reconstituted with ~ 5 ml of 0.5% HNO_3 and analyzed for total Ag by graphite furnace AAS. The remaining ice-cold homogenate was immediately centrifuged at $16000 \times g$ for 20 min at 2°C. The resulting supernatant was collected in centrifuge tubes, frozen in liquid nitrogen, and stored at -70°C until analysis of tissue metallothionein concentration. The MT assay used a double antibody radioimmunoassay as described by Hogstrand and Haux (1990). This included rabbit antiserum raised against perch (*Perca fluviatilis*) as the first antibody, ^{125}I-labelled rainbow trout MT tracer, and goat anti-rabbit IgG as the second antibody.

Intestinal homogenates for day 88 fish were assayed for Na/K-ATPase and amylase activities. Na/K-ATPase was measured using the phosphate release method described by McGeer and Wood (1998), except that intestine was used rather than gill filaments. Amylase was measured using the method of Bernfeld (1955).

Ammonia-N concentrations in water were determined by the colorimetric salicylate–hypochlorite method of Verdouw et al. (1978). Water samples (5 ml) taken for urea-N analysis were completely lyophilized and reconstituted to 1 ml with distilled water, to increase analytical sensitivity. Urea-N analysis was performed using a modification of the colorimetric diacetyl monoxime method described by Lauff and Wood (1996).

2.6. Statistics

Specific growth rates of the various dietary exposure groups were statistically compared to the simultaneous control (satiation) group using an unpaired, two-tailed t-test. The alpha level was modified using a Bonferroni correction to allow for multiple comparisons. Plasma Na^+, Na^+ influx, blood hematocrit, plasma protein, blood hemoglobin, and plasma glucose in the exposure groups were tested for statistical significance against the control satiation treatment using a one-way analysis of variance, followed by Student–Newman–Keuls test for multiple comparisons. For all metal metabolism data, the dietary silver treatment was statistically compared to the control (satiation) group using an unpaired t-test. A P value of 0.05 was considered statistically significant throughout. No statistical comparisons could be performed on O_2 consumption and N-waste excretion measurements because the data represent the means of four time replicates from all of the fish within a tank.

3. Results

The total silver concentrations of water samples taken throughout the course of the study were always below the analytical detection limit of 0.25 µg/l Ag. As a result, any effects seen in the present

Table 2
Specific growth rates (SGR), food consumption rates, and food conversion efficiencies (FCE) of juvenile rainbow trout fed control, thiosulphate, herring, or silver diets over 126 days.

	Diet ration		Control satiation[a]	Silver satiation[a,b]	Control pair-fed[b]	Thiosulphate pair-fed[b]	Herring pair-fed[b]
SGR[c,e] (%/day)			1.03 ± 0.04	1.03 ± 0.04	1.01 ± 0.04	1.10 ± 0.04	0.99 ± 0.05
Food consumption[c,f]	Month	Temp					
(%/day)	Feb	4.0	1.16 ± 0.10	1.25 ± 0.11	1.25 ± 0.11	1.25 ± 0.11	1.25 ± 0.11
	Mar	4.7	1.14 ± 0.05	1.16 ± 0.06	1.16 ± 0.06	1.16 ± 0.06	1.16 ± 0.06
	Apr	5.6	1.45 ± 0.11	1.46 ± 0.12	1.46 ± 0.12	1.46 ± 0.12	1.46 ± 0.12
	May	11.0	1.69 ± 0.17	2.06 ± 0.19	2.06 ± 0.19	2.06 ± 0.19	2.06 ± 0.19
FCE[d]							
(%)	Feb	4.0	89	82	81	88	79
	Mar	4.7	90	89	87	95	85
	Apr	5.6	71	71	69	75	68
	May	11.0	61	50	49	53	48

[a] The 'control satiation' and 'silver satiation' treatments were fed the control and silver diets, respectively, to satiation daily.

[b] The three additional treatments were fed either the control, thiosulphate or herring diets pair-fed at the same daily ration as the 'silver satiation' treatment.

[c] SGR and food consumption are mean $\pm$ SEM.

[d] FCE are calculated by SGR/food consumption rates.

[e] SGRs were calculated by linear regression analysis of the natural logarithm (ln) of mean fish weight over time. Food consumption and FCE are reported on a monthly basis due to changes in their rates over time.

study were very probably due to dietborne silver rather than waterborne silver.

3.1. Food consumption and growth

Food consumption of the dietborne silver treatment did not differ significantly from the control satiation treatment. However, food consumption of these two groups increased approximately 46–65% between February and May (Table 2). Note that the food consumption rates of the three pair-fed treatments were the same as in the silver treatment, and all the food given was consumed. Therefore, appetite did not appear to be suppressed in these groups. Average food consumption rate increased most during the last 30 days of the study when ambient water temperatures increased from 6 to 11°C. In comparison, specific growth rates were not greatly affected by the 7°C rise in water temperature; therefore, only a single mean SGR was calculated for each treatment over the entire 126-day period. Specific growth rates did not differ significantly among any of the dietary treatments. Fish in all experimental treat-

ment groups grew well, increasing in mean fish weight approximately 3.5-fold during the study. Furthermore, dietary silver had no apparent effects on food-conversion efficiency, relative to controls, although FCE decreased from 84 to 52% in all treatments between the months of February and May. The largest decrease occurred during the last 30 days when water temperature was increasing. Food-consumption rates, food-conversion efficiencies and growth rates for fish reared on diets formulated with the synthetic trout meals were all within expected ranges for rainbow trout at the reported experimental conditions (Linton et al., 1998). Moreover, there were no significant differences between the four treatments that were fed trout meals versus the group fed the herring meal diet. These results suggest that our homemade diets were nutritionally-balanced formulations that were readily consumed by the trout.

3.2. Physiological indices of effect

Dietary silver exposure exerted no significant effect on ion regulation, based on plasma Na^{+}

concentrations (Fig. 1a) and Na^+ influx (Fig. 1b) measurements during the 4 month exposure. In addition, no significant differences in hematocrit, plasma protein, and whole blood hemoglobin concentrations were seen among any of the treatments (Fig. 2a–c), although both plasma protein and hemoglobin tended to increase with time in all groups. Plasma glucose showed no consistent response to dietary exposure (Fig. 3), nor were there any effects of dietary silver exposure apparent on oxygen consumption (Fig. 4), or ammonia-N and urea-N excretion rates (Fig. 5a and b). Accordingly, nitrogen quotients (NQ) remained
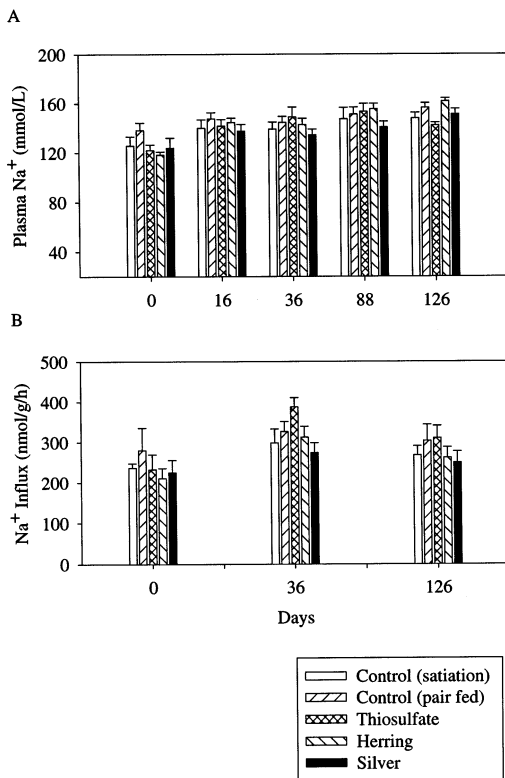
Fig. 1. The effects on (A) plasma Na^+ and (B) Na^+ influx in juvenile rainbow trout fed to satiation daily with either a control (0.05 µg/g Ag) or a silver-contaminated (3.1 µg/g Ag) diet. Additional treatments were fed once daily either the control, thiosulphate or herring diets (each ~ 0.05 µg/g Ag) at the same rate as the silver treatment. Values are means $\pm$ SEM ($n = 10$ for plasma Na^+ data and $n = 8$ for Na^+ influx data). There were no significant differences between any dietary treatment and the control satiation group.
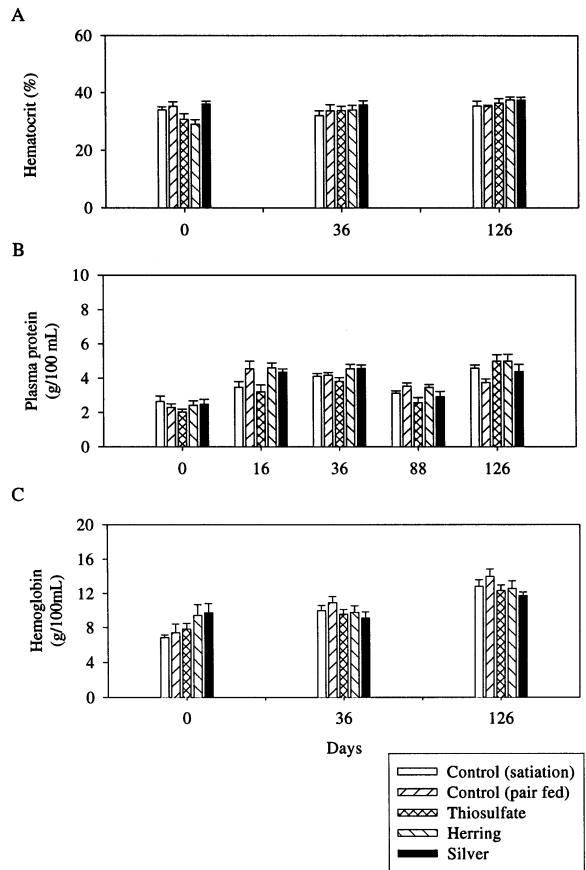

Fig. 2. The effects on (A) blood hematocrit, (B) plasma protein and (C) blood hemoglobin in juvenile rainbow trout fed to satiation daily with either a control (0.05 µg/g Ag) or a silver-contaminated (3.1 µg/g Ag) diet. Additional treatments were fed once daily either the control, thiosulphate or herring diets (each ~ 0.05 µg/g Ag) at the same rate as the silver treatment. Values are means $\pm$ SEM ($n = 10$ for plasma protein data and $n = 8$ for blood hematocrit and hemoglobin data). There were no significant differences between any dietary treatment and the control satiation group.

relatively constant (0.15–0.20) over the treatments (Fig. 5c). Similar to effects on food consumption, steady increases in oxygen consumption and ammonia-N excretion, but not urea-N excretion, were noted in all treatments as water temperature increased. The enzymatic activities of intestinal Na/K-ATPase and intestinal amylase, measured on day 88, were not significantly affected by dietary silver exposure (Table 3).

3.3. Metal metabolism

The most pronounced effect of dietary silver exposure was a 12-fold increase in liver Ag burdens after 126 days of exposure (Fig. 6a). Silver concentrations in the liver were significantly higher than controls by day 16, and appeared to saturate at approximately 20 μg/g Ag by day 36. Silver bioaccumulation had no significant effect on hepatic Cu (Fig. 6b) or Zn (Fig. 6c) concentrations, apart from a 38% reduction in the liver Cu concentration of the dietary silver treatment at day 16. Although dietary silver was readily accumulated in the livers of fish, metallothionein levels were not different between treatments. Metallothionein concentrations ranged from 51.3 and 99.2 μg/g Ag for all treatments, and did not significantly vary with time or liver weight (data not shown). Most importantly, there was no correlation between liver silver concentration and hepatic metallothionein concentrations (Fig. 7).
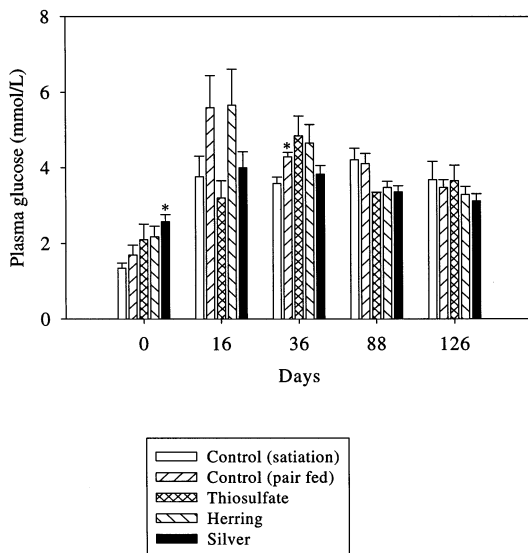

Fig. 4. The effects on routine 'in-tank' oxygen consumption in juvenile rainbow trout fed to satiation daily with either a control (0.05 μg/g Ag) or a silver-contaminated (3.1 μg/g Ag) diet. Additional treatments were fed once daily either the control, thiosulphate or herring diets (each 0.05 μg/g Ag) at the same rate as the silver treatment. Values are means $\pm$ SEM ($n = 4$ replicates of the same tank over time). Each value represents the oxygen consumption rate for an entire treatment ($n = 1$), statistical analysis could not be performed. The data have been scaled for weight using the weight exponent 0.824.

Concentrations of silver in the intestine were significantly elevated with dietary silver exposure, but only on days 36 and 88 (Fig. 8a). Interestingly, by day 126, Ag concentrations in the control and silver treatment were similar. Silver concentrations were also significantly elevated in the kidney on days 88 and 126 (Fig. 8b), and in the gills and plasma on day 126 only (Fig. 8c and d), in fish fed Ag-laden food. Interestingly, silver concentrations in the kidneys and gills of controls decreased over time. However by day 126, gill and kidney concentrations of the silver treatment were two-fold higher than the control treatment. Liver silver concentrations did not correlate with liver weight (Fig. 9a), however, kidney and gill silver concentrations appeared to decrease exponentially with increasing tissue weight (Fig. 9b and c), explaining the reduction in background Ag concentrations over time.

Fig. 3. The effects on plasma glucose in juvenile rainbow trout fed to satiation daily with either a control (0.05 μg/g Ag) or a silver-contaminated (3.1 μg/g Ag) diet. Additional treatments were fed once daily either the control, thiosulphate or herring diets (each ~ 0.05 μg/g Ag) at the same rate as the silver treatment. Values are means $\pm$ SEM ($n = 10$). An asterisk (*) denotes a significant difference in mean value for a dietary treatment relative to the control satiation group, $P < 0.05$.
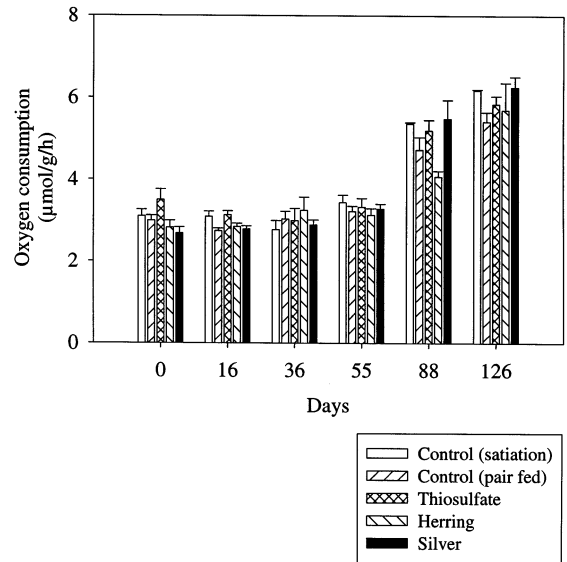
4. Discussion

4.1. Environmental relevance of experimental protocol

Environmental concentrations of waterborne silver are exceedingly low due to the tendency of
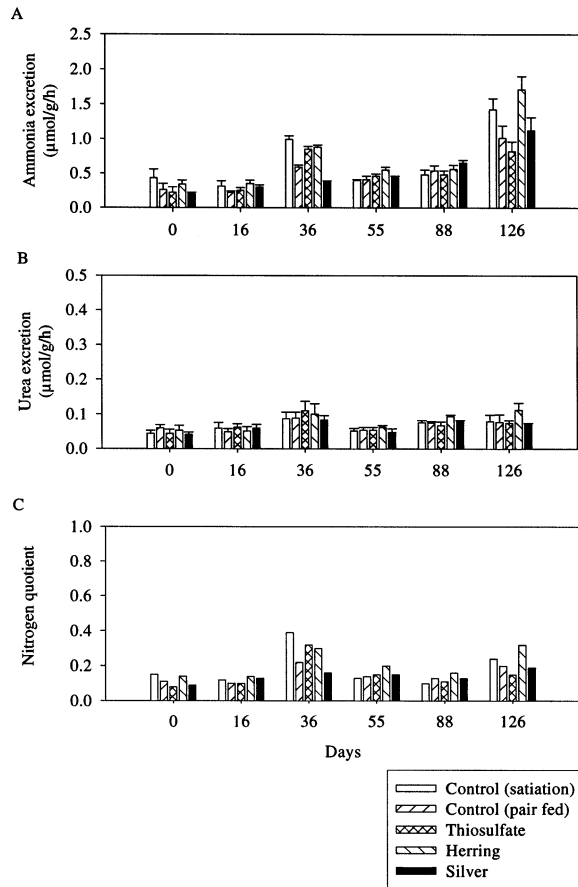

Fig. 5. The effects on routine 'in-tank' (A) ammonia-N excretion, (B) urea-N excretion and (C) nitrogen quotient in juvenile rainbow trout fed to satiation daily with either a control (0.05 μg/g Ag) or a silver-contaminated (3.1 μg/g Ag) diet. Additional treatments were fed once daily either the control, thiosulphate or herring diets (each 0.05 μg/g Ag) at the same rate as the silver treatment. Ammonia and urea excretion rates represent means ± SEM ($n = 4$ replicates of the same tank over time). Because each value represents the N-waste excretion rate for an entire treatment ($n = 1$), statistical analysis could not be performed. The data have been scaled for weight using the weight exponent 0.824. Nitrogen quotient was calculated as (ammonia-N + urea-N excretion) ÷ oxygen consumption rate.

silver to bind with colloidal material or form complexes with suspended sediments (Shafer et al., 1998). Benthic invertebrates have been shown to assimilate particle-bound silver, allowing the metal to enter the aquatic food chain (Fisher and Wang, 1998). Consequently, the majority of silver accumulated in pelagic organisms is likely derived from food, rather than via the water column (Yamazaki et al., 1996). This is consistent with the bioaccumulation found in feral aquatic organisms for other metals (i.e. Cu, Cd), despite the fact that the concentrations of these metals were low in water (Dallinger and Kautzky, 1985; Dallinger et al., 1987). Whole body silver burdens as high as 1.9 μg/g Ag have been reported for fish in contaminated sites in the United States (reviewed in Eisler, 1996). In the present study, a dietary silver burden of 3.1 μg/g Ag (60-fold above background) was used to investigate the effects of food chain transfer of silver at the upper limits of environmental relevance.

This study is unique, in that dietary silver was presented in a biologically incorporated form, achieved by exposing rainbow trout (destined to become food) to waterborne silver thiosulphate. Silver thiosulphate was chosen based on its predominance in photographic effluents. Moreover, laboratory studies have indicated that silver thiosulphate is readily accumulated in the liver, kidneys and gills of freshwater fish during aqueous exposures but does not produce acute toxicity (Hogstrand et al., 1996; Terhaar et al., 1977; Wood et al., 1996b). Consequently, it was possible in the present study to expose the 'feed' trout to relatively high concentrations of waterborne silver thiosulphate in order to maximize accumulation. At present, the importance of speciation of dietary metal on bioavailability of the metal to aquatic organisms is not fully appreciated. Hardy et al. (1987) observed that a zinc–amino acid chelate was absorbed ~50% better than $ZnSO_4$ following a single dietary dose in rainbow trout. Furthermore, several studies have observed that natural diets containing elevated concentrations of metals are much more deleterious to freshwater fish than diets artificially laden with metal salts. Metals bound to amino acids may be taken up concurrently across the intestine via amino acid

Table 3

Intestinal Na/K-ATPase and amylase activities in juvenile rainbow trout fed to satiation daily for 88 days with either a control (0.05 µg/g Ag) or a silver-contaminated (3.1 µg/g Ag) diet: additional treatments were fed once daily either the control or thiosulphate diets (each 0.05 µg/g Ag) at the same rate as the silver treatment (no data were available from the herring treatment)[a].

	Control satiation	Silver satiation	Control pair fed	Thiosulphate pair fed
Na/K-ATPase (µmol/mg protein/hour)	1.08 ± 0.20	0.89 ± 0.18	0.99 ± 0.21	1.29 ± 0.22
Amylase (µg/mg protein/hour)	97.2 ± 11.2	81.8 ± 6.4	79.9 ± 9.4	111.5 ± 11.7

[a] Values are mean $\pm$ SEM ($n = 10$); there were no significant differences among treatments.

transporters on the mucosal surface of the gut, possibly explaining the improved bioavailability of biologically incorporated diets (Storelli et al., 1989).

In order to ensure that our homemade diets were sufficiently nutritious for juvenile rainbow trout, a variety of supplements were added to the formulations prior to pelletting (Table 1), according to recommendations by Lanno (1989). Woodward et al. (1994) noted that the effects of dietary metal exposure were most pronounced when diets were prepared with field-collected invertebrates alone, but that the results may be confounded by nutritional deficiencies in such experimental diets. Considering the length of the present exposure (126 days), we felt that supplementing the experimental diets would help eliminate any such aberrations. In addition, a pair-feeding regime was used in order to account for any potential differences in food consumption rate between treatments. Several studies have demonstrated that food refusal, or regurgitation of food is often elicited in response to elevated concentrations of dietary metals- e.g., Ag (Galvez and Wood, 1999), Cu (Handy, 1992; Lanno et al., 1985) and Cd (Handy, 1992). Consequently, it is often difficult to resolve whether the effects of dietary metal exposure are due to the toxicant or simply a product of reduced food consumption (Lanno, 1989). However, this was not an issue in the present study because food consumption rates were the same in all treatments.

4.2. Physiological effects of dietary silver exposure

Dietary exposure to biologically incorporated silver had no apparent adverse effects on rainbow trout during the 126-day experiment. None of the dietary treatments produced significant mortality, and specific growth rates, food consumption rates and food conversion efficiencies were not significantly different among treatments (Table 2). All the indices of performance were within normal ranges for juvenile rainbow trout reared between 4–11°C, and fed to satiation daily (Linton et al., 1998). The lack of effect of dietary silver on growth in freshwater fish is consistent with the studies of Terhaar et al. (1977), Garnier and Baudin (1990), and Galvez and Wood, (1999). However, only Galvez and Wood specifically measured food-consumption rates and food-conversion efficiencies: food consumption in trout fed with 3 µg/g Ag as silver sulfide was reduced by 21% from control values. The fact that silver sulfide elicited such a marked reduction in food consumption at only 3 µg/g Ag, whereas the present study produced no effect at 3.1 µg/g Ag, would suggest that the reduction seen by Galvez and Wood was due to decreased palatability of the diet. Unfortunately, the former study did not include pair-fed controls and therefore it was impossible to make this conclusion definitively.

The physiological mechanism of acute silver toxicity to freshwater fish via waterborne exposure has been characterized as a severe inhibition of Na^+ uptake mechanisms at the gill epithelium (Morgan et al., 1997). In contrast, dietborne silver did not impair Na^+ influx or reduce plasma Na^+ concentration (Fig. 1). Furthermore, dietborne silver did not produce any significant effects on intestinal Na/K-ATPase even at concentrations 300-fold higher than previously shown to inhibit gill Na/K-ATPase during waterborne $AgNO_3$ exposure (Bury et al., 1999b; McGeer and Wood, 1998; Morgan et al., 1997).

The ionoregulatory impairment caused by waterborne $AgNO_3$ exposure produces a cascade of physiological perturbations including increased plasma protein, hemoglobin, hematocrit, and plasma glucose concentrations (Webb and Wood, 1998; Wood et al., 1996a). In the present study, the hematological parameters were unaffected, suggesting that hemoconcentration was not likely produced by dietary silver. Plasma glucose concentrations were also not affected by the dietary
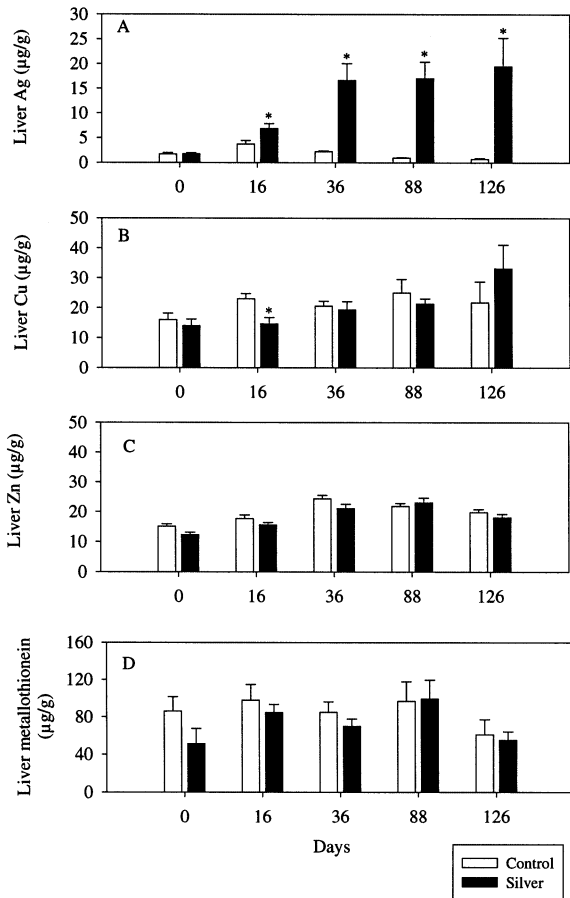

Fig. 7. Liver metallothionein concentrations and liver silver concentrations in juvenile rainbow trout fed either a control diet (0.05 μg/g Ag) or a silver-contaminated diet (3.1 μg/g Ag).

treatments in any consistent manner (Fig. 2), implying that fish were not acutely stressed as a result of the exposures (Fig. 3).

Routine 'in-tank' metabolism was measured to assess subtle effects of dietary silver exposure on metabolic demand, or energy utilization. Oxygen consumption rates were similar among all dietary treatments, suggesting that oxygen uptake at the gills and delivery of oxygen to tissues was not altered. However, oxygen consumption increased in all treatments over time (Fig. 4). Because oxygen consumption rates were size-corrected using the weight exponent, elevations were not likely due to fish growth. Instead, the increase in oxygen consumption over time generally coincided with the 7°C increase in ambient temperature and the accompanying increase in fish appetite (Table 2) (Brett, 1979).

Waterborne $AgNO_3$ is known to increase ammonia excretion, likely as a result of stress- induced mobilization of cortisol, followed by cortisol-mediated protein catabolism (Webb and Wood, 1998). In the present study, ammonia and urea excretion rates were monitored under routine 'in-tank' conditions to determine the influence of dietary silver on energy utilization and aerobic protein catabolism in fish. Ammonia excretion rates steadily increased in all dietary treatments, due likely to the elevation in water temperature

Fig. 6. The effects of dietary exposure to either a control (0.05 μg/g Ag) or a silver-contaminated (3.1 μg/g Ag) diet on the liver concentrations of (A) silver, (B) copper (C) zinc and (D) metallothionein in juvenile rainbow trout. Values are means ± SEM ($n = 10$). An asterisk (*) denotes a significant difference in mean value of the dietary silver treatment relative to the control satiation group, $P < 0.05$.
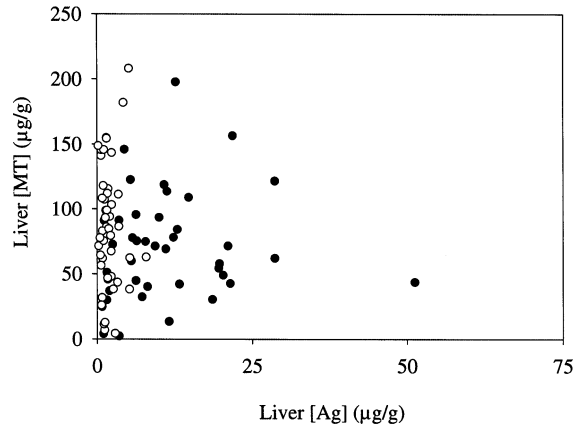
(Fig. 5a). However, dietborne silver did not appear to affect ammonia excretion. In comparison, urea excretion was not greatly affected by changes in water temperature, nor was it influenced by dietborne silver (Fig. 5b). The nitrogen quotient was not affected by dietborne silver, implying that the use of protein as a metabolic fuel (c.f. Alsop and Wood, 1997) was not affected (Fig. 5c).

In contrast to the apparent benignity of dietary silver, toxicity to other metals through the diet has routinely been noted. Both dietary Cu
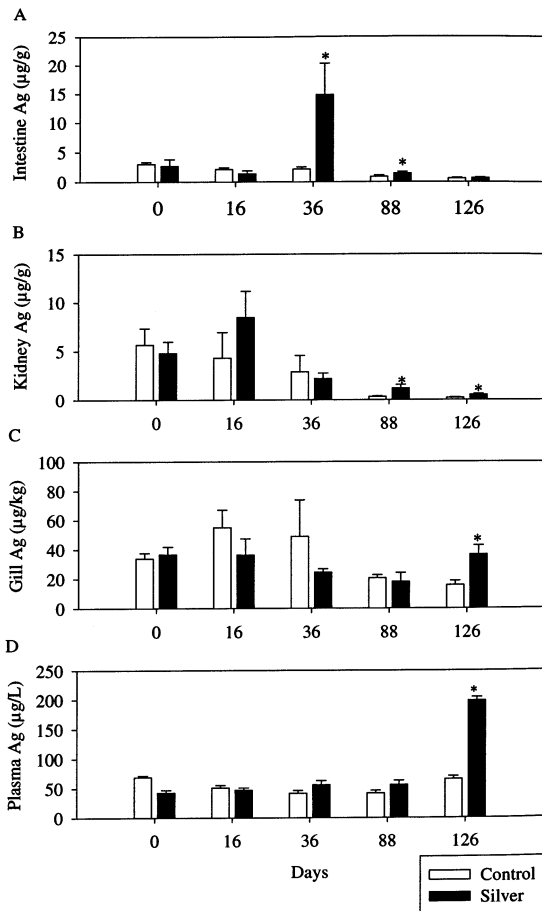
Fig. 8. The effects of dietary exposure to either a control (0.05 μg/g Ag) or a silver-contaminated (3.1 μg/g Ag) diet on the concentrations of silver in (A) intestine, (B) kidney, (C) gills and (D) plasma in juvenile rainbow trout. Values are means ± SEM ($n = 10$). An asterisk (*) denotes a significant difference in mean value of the dietary silver treatment relative to the control satiation group, $P < 0.05$.
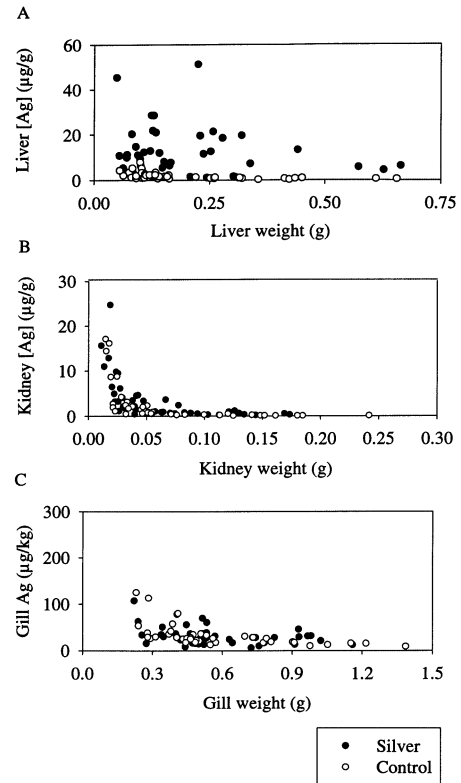

Fig. 9. (A) liver, (B) kidney and (C) gill silver concentrations versus tissue weight for juvenile rainbow trout fed either a control diet (0.05 μg/g Ag) or a silver-contaminated diet (3.1 μg/g Ag).

(Handy, 1993; Lanno et al., 1985) and Cd (Handy, 1993) have caused mortality in rainbow trout at extremely high doses. More commonly, toxicity is characterized by reduced growth and food-conversion efficiencies in fish. However, these effects are usually attributed to food refusal at elevated concentrations of dietborne metal. To our knowledge, no studies to date have used pair-fed controls in their experimental designs to address the effects of reduced food consumption. Several studies have also assessed the effects of dietary metal on various hematological parameters such as hematocrit, plasma glucose and protein, and blood hemoglobin concentrations. In most cases dietborne metal has produced no significant effects (Lanno et al., 1985), although Murai et al. (1981) and Knox et al. (1982) observed

slight reductions in hematocrit and/or hemoglobin with increasing dietary Cu concentrations. Significant reductions in gill Na/K-ATPase activity, as well as morphological alterations in the gill epithelium after only 14 days of Cd exposure, have also been noted (Pratap and Wendelaar Bonga, 1993). This effect was attributed to the distribution of dietary cadmium to the gills of fish.

4.3. Metal metabolism

Even though physiological disturbances were not produced by dietborne silver, silver accumulated in various tissues of the fish. Significant accumulation of silver was seen in the liver after only 16 days of exposure to a diet containing 3.1 µg/g of biologically incorporated silver (Fig. 6a). Hepatic silver concentrations stabilized at about 12-times control values after 36 days. In comparison, livers of rainbow trout fed 3000 µg/g dietary Ag as silver sulfide accumulated only fourfold above control concentrations after 43 days (Galvez and Wood, 1999). In the present study, silver accumulated in the liver at a rate of 0.4 µg/g Ag/day during the first 36 days. However, if accumulation is normalized for the silver concentration in the diet (see Hogstrand and Wood, 1998), a concentration specific accumulation rate (CSAR) of 0.13 ng/g/d/ppb was evident by 16 days. This CSAR is 4.6 orders of magnitude greater than the CSAR measured for dietary silver sulfide exposure, but nonetheless 2000 to 20000 times lower than during waterborne $AgNO_3$ exposure. Interestingly, the CSAR presented here (based on food originally contaminated with silver thiosulphate) is similar to the 0.34–0.38 ng/g/d/ppb seen in rainbow trout directly exposed to waterborne silver thiosulphate (Hogstrand and Wood, 1998).

In the present study, silver concentrations in the liver increased quickly up until day 36, at which point they appeared to reach a plateau of ~ 15–20 µg/g Ag (Fig. 6a). This plateau is almost identical to the maximal Ag concentration reported following exposure to waterborne $AgNO_3$ for 7 days (Hogstrand et al., 1996). During such waterborne exposures, this bioaccumulated silver is known to significantly increase hepatic MT concentrations (Galvez et al., 1998; Hogstrand et al., 1996; Wood et al., 1996b). In contrast, MT was not induced by dietary silver exposure despite the 12-fold increase in silver tissue burden. MT can bind 12 equivalents of Ag^+ per mole of MT at saturation (Li and Otvos, 1996). Accordingly, about 17.3 µg/g Ag can potentially bind to 80 µg/g MT, the approximate amount of MT in the livers of our fish (Fig. 6d). Based on these calculations, basal MT concentrations theoretically would be sufficient to bind all of the silver in the liver. It is presently unclear why bioaccumulated silver has a varying ability to induce MT depending on its route of uptake in fish; however, this observation is consistent with other metals (Miller et al., 1993). Lanno et al. (1985) and Miller et al. (1993) have argued that a functional separation exists between metals accumulated via the diet and water. One possibility is that the difference is rate-related, so that the induction of MT depends on the rate of silver accumulation in the tissue. Therefore, MT induction would only be observed during waterborne silver exposure due to the fast rate of silver accumulation in the liver. Certainly more work needs to be done to clarify this issue.

Several studies have indicated that bioaccumulated silver may interfere with the metabolism of essential metals such as copper and zinc. Coleman and Cearley (1974) noted an inverse relationship between whole-body silver and zinc concentrations in smallmouth bass and bluegills chronically exposed to silver. In comparison, whole-body copper concentrations were unaffected. This contrasts with studies showing that silver can interfere with copper metabolism in the livers of rats (Hirasawa et al., 1994). Because silver is known to bind to MT in vivo, bioaccumulated silver might be able to displace copper and zinc from MT and significantly affect the distribution of these essential metals (Cousins, 1985). In the present study, copper concentrations in the liver were significantly lower in the dietary silver treatment on day 16 only (Fig. 6b), coinciding with the initial increase in hepatic silver burden (Fig. 6a). In comparison, no effect on liver zinc concentrations was

noted (Fig. 6c). The transient reduction in liver copper concentration has several explanations. Any copper initially displaced by silver in the cytosol may have been subsequently incorporated into copper-containing enzymes (i.e., ceruloplasmin) and transported out of the liver (Cousins, 1985) and/or excreted into the bile. Alternately, silver may have inhibited copper uptake either across the gut epithelium or into the liver. Regardless, the effect would have had to be transient to explain the subsequent increase in copper concentration in the liver.

Apart from the intestine, silver did not accumulate in other tissues until day 88. Accumulation in plasma, gills, and kidney occurred only after the liver silver concentration reached a plateau (Fig. 8). Interestingly, concentrations of silver in the kidneys and gills of control fish decreased over time, with tissue Ag concentration decreasing exponentially with increasing tissue weight (Fig. 9). As such, all significant increases in the silver burdens of these two tissues were never higher than initial control values. Silver concentrations in the intestines of the dietary silver treatment were significantly elevated on days 36 and 88 only. Nonetheless, intestinal Na/K-ATPase and amylase activities were not significantly altered on day 88 (Table 3). The lack of effect of dietary silver on amylase activity is particularly interesting because the enzyme is released into the intestinal lumen to promote digestion of dietary carbohydrates, and should, therefore, be fully accessible to silver.

In hindsight, the lack of effect of dietary silver on these physiological parameters is not surprising because the acute toxic response to aqueous exposure to silver appears to be mediated by the action of Ag^+ at the gill epithelium. Aqueous concentrations of silver were not elevated at any time during the exposure. Therefore, in order for silver to act at the gills it would first need to be taken up via the gastrointestinal tract and transported via the blood. However, dietary silver was predominantly sequestered within the liver, and never accumulated substantially in any other tissue. In contrast, dietary exposure to Cu (Miller et al., 1993) and Cd (Handy, 1992) resulted in significant accumulations of these metals in tissues such as the kidney and gills. The ability for these metals to accumulate readily in the gills may explain in part why dietary exposure to these metals had toxic effects.

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