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# Bioaccumulation Potential of Dietary Arsenic, Cadmium, Lead, Mercury, and Selenium in Organs and Tissues of Rainbow Trout (*Oncorhyncus mykiss*) as a Function of Fish Growth

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The distribution and potential bioaccumulation of dietary arsenic, cadmium, lead, mercury, and selenium in organs and tissues of rainbow trout (*Oncorhyncus mykiss* Walbaum, 1792), a major aquaculture species, was studied in relation to fish growth over a period of >3 years. Fish were reared under normal farming conditions, that is, fed a standard fish food and exposed to negligible levels of waterborne trace elements. The age-related variations in the content of each trace element in gills, kidney, liver, muscle, and skin were studied through nonparametric regression analysis. A buildup of all elements in all tissues and organs was observed, but due to dilution with growth, the concentrations did not increase, except in a few cases such as cadmium and mercury in liver and kidney. In muscle tissue, the concentrations of mercury, lead, and selenium did not alter significantly with growth, whereas cadmium increased but remained at exceedingly low levels. The concentration of arsenic in muscle tissue peaked at 14 months and then decreased in adult specimens. Arsenic speciation by high-performance liquid chromatography−inductively coupled plasma mass spectrometry revealed that arsenic in muscle was almost exclusively present in the form of nontoxic arsenobetaine. Application of a mercury mass balance model gave predicted concentrations in agreement with measured ones and showed that in farmed rainbow trout the ratio of mercury concentrations in feed and in fish is about 1:1. Therefore, rainbow trout does not approach the limits established for human consumption even when reared with feed at the maximum permitted levels. These findings highlight the low bioaccumulation potential of toxic trace elements such as cadmium, lead, and mercury in rainbow trout following dietary exposure. On the other hand, selenium concentrations in muscle (about 0.2 $\mu$g $g^{-1}$ of fresh weight) show that rainbow trout may be a good source of this essential element.

**KEYWORDS: Trace elements; aquaculture; farmed fish; arsenic speciation; nonparametric regression; kinetic models; food safety**

## INTRODUCTION

Fish is an important part of a healthy diet as it provides energy and nutrients such as proteins, vitamins, selenium (Se), and omega-3 fatty acids. However, concerns about fish as a possible source of toxic trace elements led the European Union (EU) to introduce maximum levels (MLs) for cadmium (Cd), lead (Pb), and mercury (Hg) in fish muscle (*1*). The MLs are 0.050 $\mu$g $g^{-1}$ of fresh weight for Cd (raised to 0.10–0.30 for some selected species), 0.30 $\mu$g $g^{-1}$ of fresh weight for Pb, and 0.50 $\mu$g $g^{-1}$ of fresh weight for Hg (raised to 1.0 for some predatory fish). The higher MLs for mercury reflect the tendency of this element to build up in fish muscle, largely as methylmercury ($CH_3Hg^+$, hereafter MeHg), the chemical form of most concern from the toxicological point of view.

The uptake of trace elements in fish occurs through food ingestion and water via the gills. For a given trace element, accumulation sites within fish may vary with route of uptake (i.e., food vs water) and also with the intensity and duration of exposure (*2*). In the case of farmed fish, if water is under control (i.e., noncontaminated or previously treated water is used), the diet is the only significant source of trace elements. In the safety assessment of farmed fish, knowledge about the possible bioaccumulation of foodborne toxic trace elements, such as Cd, Pb, Hg, and arsenic (As) in its inorganic forms, is of importance.

Fish farming is the main activity in global aquaculture, a sector that has witnessed a steady increase in the past 15 years (*3*). The Food and Agricultural Organization reported that of 132.2 million tons of fish that represented the worldwide

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production in 2003, 32.9 million tons resulted from aquaculture (*4*). In Italy, the contribution of the aquaculture industry is estimated at 43.5% of the national fish and seafood production, and rainbow trout (*Oncorhyncus mykiss* Walbaum, 1792) is the major farmed species, constituting 58% of the total aquaculture production in 2004 (*5*).

The main aim of the present study was to characterize the distribution of dietary As, Cd, Pb, and Hg in organs and tissues of rainbow trout and assess whether these trace elements bioaccumulate during fish growth, with special emphasis on muscle tissue. The other tissues and organs included in the study, namely, gills, kidney, liver, and skin, are important sites for element uptake and storage, and a dynamic equilibrium implying element exchange exists among them and muscle tissue.

Because Hg (as MeHg) is of greatest concern in edible fish, the study focused particularly on this element. A mass balance model was applied to get insight into the process of Hg uptake and accumulation in rainbow trout by comparing measured concentrations with theoretical ones.

Arsenic speciation in muscle tissue of rainbow trout was another objective of the study. In fact, for the assessment of the health risks related to human consumption of fish, the toxic species must be differentiated from the nontoxic one. Thus, the identification of the As compounds present in muscle tissue was undertaken to distinguish the nontoxic organic species such as arsenobetaine [$(CH_3)_3As^+CH_2COO_2^-$, hereafter AB] from the toxic ones (primarily inorganic As).

To have a more complete picture of trace element accumulation and distribution in rainbow trout, selenium, that is, an essential trace element for which a physiological requirement exists in fish, was included in the study as well. Because fish is an important source of selenium in the human diet, this element was included in the study also for the purpose of a risk/benefit assessment.

The uptake and distribution of trace elements in rainbow trout, including As (*6–9*), Cd (*9–29*), Hg (*10, 21, 30–37*), Pb (*9, 21, 38–40*), and Se (*37, 41–43*) have already been extensively studied.

However, in the vast majority of the studies performed so far, acute exposures have been addressed, trace elements having been administered at levels close to or above those eliciting adverse responses with consequent alteration of normal distribution patterns and absorption/elimination pathways. Furthermore, trace elements have often been added in their inorganic form to either food or water. If exposure to waterborne inorganic element species can be a relatively realistic exposure scenario, this is not the case as far as the diet is concerned, at least for elements such as As, Hg, and Se, the organic forms of which predominate in food and feed matrixes. The current awareness of the crucial role played by speciation in the absorption and metabolic fate of trace elements dictates that if a sound knowledge about their transfer from food to fish is to be gained, the naturally occurring element species have to be addressed. Therefore, in this study fish were exposed to foodborne trace elements at the levels and in the chemical forms found in normal commercial feed. Fish were reared in clean water without exogenous addition of trace element, which made the uptake of waterborne trace elements negligible.

## MATERIALS AND METHODS

**Fish.** Rainbow trouts were grown at the Ichthyologic Institute in Rome, Italy. Fish were reared in a 1000 L round flow-through tank under a natural light regimen. Tap water dechlorinated by activated charcoal filtration was used. The tank water was aerated to 86% oxygen saturation ($O_2$ concentration of 9.5 mg $L^{-1}$). The pH and water hardness (as $CaCO_3$) were 7.5 and 250 mg $L^{-1}$, respectively. The water flow into the tank was set at 20 L $min^{-1}$, and the temperature was monitored continuously by means of two digital thermometer probes placed close to the water inflow and outflow. The temperature range was found to be 11–12 °C.

Fish were fed a standard fish food prepared with commercial fish meal and containing fish oil as a source of lipids. Fish were fed a daily ration of 0.7–0.9% of fresh body weight, depending on growth stage.

Procedures for trout care and management complied with those required by Italian law and adhered to the standards for treatment of experimental animals established by the ethical committee of the Istituto Superiore di Sanità.

**Sample Treatment.** Groups of five trouts were randomly collected at four different growth stages over 3.3 years, that is, from juveniles to adults of commercial size. The four groups were aged 10, 14, 16, and 40 months, respectively. The fish were killed by a blow to the head followed by decapitation, and their length and weight were measured. The average weight ranged from 102 g of the first group to 828 g of the fourth group.

All sample manipulations in the laboratory were carried out in clean room conditions under a laminar flow box (Spetec GmbH, Erding, Germany). Gills, kidney, liver, muscle, and skin were dissected out with titanium and tungsten carbide instruments (Fine Science Tools, Heidelberg, Germany). After dissection, gills were rinsed with a 0.6% solution of ultrapure NaCl to eliminate any fine particulate matter.

Both inflow and outflow water samples were collected from the fish farming tank every 6 months over the duration of the study. Water samples were filtered through a 0.45 $\mu$m membrane filter, acidified (1% $HNO_3$), and stored at 4 °C for the analysis of total dissolved elements. Fish food pellets were sampled every 6 months over the duration of the study, sealed in decontaminated polyethylene bags, and stored at −20 °C pending analysis of total elements. Samples were thoroughly pulverized before subsampling for analytical determinations.

**Total Element Analysis.** For the determination of total trace elements, aliquots of previously homogenized tissue samples were placed in high-pressure Teflon containers, with the addition of 4 mL of $HNO_3$ and 1.5 mL of $H_2O_2$, and then mineralized by microwave-assisted digestion (Milestone Ethos Pro microwave labstation, FKV, Bergamo, Italy). In the case of muscle tissue, a predigestion step was performed before microwave irradiation. At the end of the irradiation process, samples were transferred to decontaminated disposable tubes and diluted to a volume of 25 mL with deionized water. The same procedure was applied to pulverized fish food pellets.

All of the chemicals used during the analytical procedure were of ultrapure grade. Deionized water employed for solution preparation and cleaning was obtained by a Milli-Q Element System (Millipore, Molsheim, France). Calibrants and the internal standard solution (rhodium) used for total element measurements were obtained from standard certified solutions with a content of 1 mg $mL^{-1}$ (Spex, Metuchen, NJ), followed by dilution with acidified ($HNO_3$) deionized water as necessary.

Quantification of total As, Cd, Pb, and Se in fish tissues and feed pellets as well as water was performed by ICP-MS using an Elan DRC II spectrometer (Perkin-Elmer, Norwalk, CT). The sample introduction systems consisted of a peristaltic pump, a Meinhard quartz concentric nebulizer and a cyclonic quartz spray chamber. Measurements were carried out in the standard mode after assessment of the absence of spectroscopic interferences by use of $NH_3$ as the reactive gas in the DRC. The analytical masses and other details can be found in a previous work (*44*). Hg determination was carried out by flow injection cold vapor atomic absorption spectrometry (FI-CV-AAS) using a FIMS-400 flow injection mercury system (Perkin-Elmer). $NaBH_4$ (0.2% w/v in NaOH 0.05% w/v) and ultrapure HCl (3% v/v), both from Merck (Darmstadt, Germany), were used as the reductant and carrier, respectively. Measurements were made at a wavelength of 253.7 nm.

The certified reference material BCR CRM 422 (cod muscle) from IRMM (Geel, Belgium) was used for analytical quality control. The accuracy of determinations as assessed through CRM analysis is shown in **Table 1**.

**Table 1.** Accuracy As Assessed through BCR CRM 422 (Cod Muscle)

| element | certified concn ($\mu$g g$^{-1}$ of dry wt) | found concn[a] ($\mu$g g$^{-1}$ of dry wt) |
|---|---|---|
| As | 21.1 ± 0.5 | 20.0 ± 1.8 |
| Cd | 0.017 ± 0.002 | 0.016 ± 0.003 |
| Hg | 0.559 ± 0.017 | 0.560 ± 0.010 |
| Se | 1.63 ± 0.07 | 1.63 ± 0.15 |
| Pb | 0.085 ± 0.015 | 0.081 ± 0.007 |

[a] Data expressed as mean value ± SD ($n$ = 9).

**Table 2.** Chromatographic Conditions for Arsenic Speciation Analysis

| | Anion Exchange Chromatography | |
|---|---|---|
| column | ION-120 | |
| column temp | 23 °C | |
| injection vol | 50 $\mu$L | |
| mobile phase | 20 mM $(NH_4)_2CO_3$ in 3% (v/v) MeOH, adjusted to pH 10.3 with $NH_3$ | |
| elution mode | isocratic | |
| flow rate | 0.8 mL min$^{-1}$ | |

| | Cation Exchange Chromatography | |
|---|---|---|
| column | Chrompack IonoSpher-5C | |
| column temp | 35 °C | |
| injection vol | 50 $\mu$L | |
| mobile phase | (A) 3% (v/v) MeOH<br>(B) 50 mM pyridinium formate in 3% (v/v) MeOH, pH 2.7 | |
| flow rate | 1.0 mL min$^{-1}$ (unless otherwise stated) | |
| gradient program | 0–3 min | 99% A |
| | 3–4 min | 90% A−10% B |
| | 4–15 min | 90% A−10% B |
| | 15–16 min | 99% A (1.5 mL min$^{-1}$) |
| | 16–20 min | 99% A (1.5 mL min$^{-1}$) |

**Arsenic Speciation Analysis.** For the quantification of water-soluble arsenic species in muscle tissue, samples were extracted using a 1:1 (v/v) methanol/water mixture. After a first extraction with mechanical agitation overnight, another two consecutive extractions were carried out. Concentration of sample extracts was achieved by a Zymark TurboVap II Concentrator Workstation (FKV, Bergamo, Italy).

Arsenic compounds were determined by high-performance liquid chromatography with online inductively coupled plasma mass spectrometry (HPLC-ICP-MS). A Perkin-Elmer 200 series quaternary pump equipped with an autosampler and a column thermostat was used as the chromatographic system. The outlet of the HPLC column was directly connected via PEEK capillary tubing to the nebulizer of the ICP-MS instrument, which served as the As specific detector. A PC$^3$ Peltier-cooled quartz cyclonic spray chamber (Elemental Scientific Inc., Omaha, NE) set at 2 °C was used for sample introduction.

Separations were carried out by a cation exchange column (Chrompack IonoSpher-5C, 100 × 3.0 mm i.d., 5 $\mu$m particles; Varian, Middelburg, The Netherlands) using aqueous pyridine formate at pH 2.7 with gradient elution and by an anion exchange column (ICSep ION-120, 120 × 4.6 mm i.d., 10 $\mu$m particles; Transgenomics, San Jose, CA) with 20 mM ammonium carbonate at pH 10.3 as mobile phase. Arsenic species in extracts of the samples were identified by matching retention times with those of standard compounds. The selected chromatographic conditions (**Table 2**) enabled the separation of 15 different As species for which standards were available, including the most relevant from the toxicological point of view, that is, arsenous acid or As(III), arsenic acid or As(V), monomethylarsonic acid, and dimethylarsinic acid. However, only As(V) (standard from Merck) and AB (standard from IRMM, CRM 626 arsenobetaine solution) were detected in sample extracts.

**Data Treatment.** To study accumulation patterns in different tissues in successive growth stages, the analytical results were expressed both as tissue concentrations and as tissue contents. The content of each element in different tissues was derived from concentration data expressed in micrograms per gram of fresh weight multiplied by whole tissue fresh weights expressed in grams. Because the majority but not the whole of muscle and skin tissues were sampled from each fish in the laboratory, the weight of both tissues was estimated using the percent contributions of different tissues with respect to total fish weight as reported by Giblin and Massaro (*30*). According to these authors, in rainbow trout muscle and skin account for 54.50 and 8.30% of total fish weight, respectively.

Statistical analysis was performed by means of Statistica (version 7.0, StatSoft, Inc.). The distribution of data was evaluated by the Kolmorogov−Smirnov test with Lilliefors probabilities and appeared to deviate from normality even after all of the common transformations had been applied. Thus, the element content in each tissue as a function of age-related variations in tissue weight was investigated through nonparametric regression. The abbreviated Theil−Kendall method was manually computed into a spreadsheet to calculate the regression equations of tissue weights on element contents ($n$ = 20) (*45*). A significance level of $p < 0.01$ corresponding to a critical value of $\tau = 0.421$ in the $\tau$ Kendall correlation test was obtained in all cases. The differences among element contents/concentrations in various organs and tissues were tested by Kruskal–Wallis ANOVA and related multiple comparisons. The same method was applied to check differences in element concentrations of a single organ or tissue as a function of age. A significance level of $p = 0.05$ was used in all cases.

**Mercury Mass Balance Model.** The organs and tissues included in this study made up a sizable portion of the fish body. Thus, it was reasonable to calculate a Hg "whole body" concentration by summing up the contributions of these organs and tissues by using the formula

$$C_{wb} = \frac{\sum_{i=1}^{n} C_i W_i}{W_s} \quad (1)$$

where $C_{wb}$ is the whole body Hg concentration ($\mu$g g$^{-1}$ of fresh weight), $C_i$ is the Hg concentration in tissue $i$ ($\mu$g g$^{-1}$ of fresh weight), $W_i$ is the weight of tissue $i$ (g), and $W_s$ is the sum of all tissue weights (g).

The Hg whole body concentration found experimentally by eq 1 was compared with that calculated by means of a first-order single-compartment kinetic model for MeHg accumulation in fish. MeHg was considered because it is the predominant form of mercury in fish, especially at higher trophic levels (≥95% of total Hg). Under the assumption of a negligible MeHg uptake from water, the accumulation of MeHg in fish can be expressed as

$$\frac{dC}{dt} = (AE \cdot IR \cdot C_f) - (K_e + g)C \quad (2)$$

where $C$ is the concentration of MeHg in fish ($\mu$g g$^{-1}$), AE is the assimilation efficiency of MeHg (decimal fraction, unitless), IR is the weight-specific daily ingestion rate (day$^{-1}$), $C_f$ is the MeHg concentration in food ($\mu$g g$^{-1}$), $K_e$ is the elimination rate (day$^{-1}$), and $g$ is the specific growth rate (day$^{-1}$).

The MeHg concentration in fish was calculated using the following equation, obtained by integrating equ 2:

$$C_{t+\Delta t} = C_t e^{-(K_e+g)\Delta t} + \frac{AE \cdot IR \cdot C_f}{K_e + g}(1 - e^{-(K_e+g)\Delta t}) \quad (3)$$

Experiments with diets containing $^{203}$Hg-labeled MeHg showed that rainbow trout assimilates 70–80% of the MeHg they are fed (*33*), and thus an AE = 0.75 was used in eq 3. IR was determined as ca. 90% of daily ration. For the calculation of $K_e$, the model of MeHg elimination in fish developed by Trudel and Rasmussen for chronic exposures (*46*)

$$\ln K_e = 0.066T - 0.20 \ln \ BW + 5.83 \quad (4)$$

was used,where $T$ is the water temperature (°C) and BW is the fresh body weight of fish (g).

Finally, $g$ was determined by fitting weight data into an exponential growth model (*47*)

$$g = \frac{\ln \frac{BW_{t+\Delta t}}{BW_t}}{\Delta t} \quad (5)$$

where $BW_t$ and $BW_{t+\Delta t}$ are the fresh body weights of fish (g) at time $t$ and $t + \Delta t$ (days).

The mass balance model was applied on a daily basis by interpolating fish size between age classes (*47*). MeHg concentration predicted at the end of a day (i.e., $C_{t+\Delta t}$) was used as the initial concentration in fish (i.e., $C_t$) for the following day. The Hg concentration in fish of a given age class calculated by eq 1 was used as the input for the initial concentration in fish of the following age class, assuming that 95% of Hg was in the form of MeHg (*48*).

## RESULTS

**Concentrations of Trace Elements in Water and Food.** Trace element levels in both inflow and outflow water samples as well as in fish food are reported in **Table 3**.

The element concentrations measured in water were in the range of typical background levels. They ranged from 0.1% (Cd) to 2% (Se) of the MLs established at the EU level for water intended for human consumption (*49*). Arsenic concentration amounted to 5% of the ML, which is however within the normal background range for this element in water. Due to the levels found, the contribution of water to the uptake of trace elements by fish was considered to be negligible compared to that of the diet (see below).

The element concentrations in fish food pellets were 4, 26, 37, and 39% of the MLs established at the EU level for Pb, As, Cd, and Hg in complete feedingstuffs for fish, respectively (*50*). Although complying with the existing legislation, it is clear that the selected fish food did represent a sizable source of foodborne elements for reared fish. The Se content was also relatively high, if the range separating requirement from adverse exposures in rainbow trout, that is, approximately 0.1–10 $\mu$g g$^{-1}$ of feed (as selenite), is considered (*41, 43*).

**Concentrations of Trace Elements in Tissues and Organs.** Element concentrations in gills, kidney, liver, muscle, and skin of fish collected in four different growth stages are summarized in **Table 4**. With a few exceptions, for all elements there was no concentration increase with age.

Arsenic showed the highest concentration in muscle tissue ($p < 0.001$). This element peaked at 14 months and then markedly decreased on consecutive age stages in muscle. The same pattern was observed in all tissues, levels at 14 months being always higher than those in adult specimens ($p < 0.01$, except $p = 0.017$ in kidney).

The highest Cd concentrations were found in kidney, followed by liver. In the other tissues Cd concentrations were significantly lower than those in kidney and liver ($p < 0.01$) and did not exceed 5 ng g$^{-1}$ of fresh weight at any growth stage. In all tissues, except skin, the Cd level decreased from specimens aged 10 months to those aged 14–16 months and then increased to a maximum in adult specimens (the difference between the fourth and second age groups was always significant, $p < 0.005$).

Hg reached the highest levels in kidney, followed by gills, muscle, liver, and skin. Hg concentration in muscle was independent of fish growth and significantly higher than that in skin ($p = 0.005$). A similar pattern was observed for gills. Hg concentration in kidney had a minimum at 14 months and then increased to a maximum at 40 months. The curve for liver was similar, but the minimum was reached later (i.e., at 16 months) than in the case of the kidney. The adult specimens exhibited the highest Hg concentration in kidney and liver and the lowest Hg concentration in skin.

**Table 3.** Element Concentrations in Pelletized Fish Food and in Tank Water[a]

| element | pellet ($\mu$g g$^{-1}$ of fresh wt) | tank water | |
|---|---|---|---|
| | | inflow water ($\mu$g L$^{-1}$) | outflow water ($\mu$g L$^{-1}$) |
| As | 1.53 ± 0.08 | 0.52 ± 0.15 | 0.47 ± 0.16 |
| Cd | 0.373 ± 0.008 | 0.0048 ± 0.0018 | 0.0088 ± 0.0032 |
| Hg | 0.039 ± 0.020 | <QL[b] | <QL |
| Pb | 0.188 ± 0.054 | 0.078 ± 0.021 | 0.057 ± 0.034 |
| Se | 1.22 ± 0.09 | 0.177 ± 0.035 | 0.268 ± 0.059 |

[a] Data expressed as mean value ± SD ($n = 5$). [b] Quantification limit (0.006 $\mu$g L$^{-1}$).

Skin showed the highest concentrations of Pb, followed by kidney ≈ gills, liver, and muscle. Pb concentrations in muscle tissue remained at a constant value of 5 ng g$^{-1}$ of fresh weight at any growth stage, a significantly lower level than that found in skin, kidney, and gills ($p < 0.001$).

With regard to Se, kidney and liver showed a significantly higher concentration compared to the other tissues ($p < 0.001$). Se levels in kidney and in muscle did not change significantly with age, whereas a decrease in adult specimens was observed in gills, liver, and skin ($p < 0.05$).

For adult specimens, when element concentrations in muscle were compared to those in food, it became evident that no biomagnification occurred in the edible tissue of fish. Hg concentration in muscle tissue was equal to that in food, whereas for As, Se, Pb, and Cd the concentrations in muscle tissue were 26, 15, 3, and 1% of that in food, respectively. As a result, the levels of Cd and Hg in muscle tissue of adult specimens were only 8% of the relevant EU MLs, whereas for Pb the figure amounted to 3%.

**Element Burden of Tissues and Organs.** The relative element burden of the different tissues and organs gives a picture of their respective importance as storage sites. On average, muscle tissue accounted for 94, 86, and 72% of the total measured burden of As, Hg, and Se, respectively. However, it contained only 57 and 51% of the total Cd and Pb. Kidney accounted for 25% of the total Cd burden, followed by liver (9%) and skin (8%). On the other hand, skin was the major Pb sink after muscle (41%).

The element burden of tissues and organs as a function of increasing tissue weight with fish aging was also investigated. A nonparametric linear model was used to study the relationship between tissue weight and element content. The regression equations thus obtained and their significance values are shown in **Table 5**. As expected, a positive relationship between element content and tissue weight was obtained in all cases with a high level of significance ($p < 0.01$).

However, the rate at which the burden of a given element increases varies widely among the different organs and tissues. For As, the slope of the regression equation was clearly steeper for muscle tissue with respect to all other tissues. With regard to Cd and Se, the steepest slope, that is, the fastest buildup, was obtained in the case of kidney. The same was found for Hg, but in this case after kidney, gills, muscle, and liver showed relatively similar rates of increase in their element burden. In the case of Pb, the slopes of the regression equations obtained for kidney and gills were similar.

**Hg Whole Body Accumulation.** As shown above, Hg is the element with the highest retention in rainbow trout, including the edible portion of the fish. This is not surprising because it is known that Hg is efficiently assimilated from food, where it is largely present as easily absorbable MeHg, and the ac-

**Table 4.** Element Concentrations in Organs and Tissues of Rainbow Trout Collected at Four Different Growth Stages ($\mu$g g$^{-1}$ of Fresh Weight)[a]

| tissue | growth stage | As | Cd | Hg | Pb | Se |
|---|---|---|---|---|---|---|
| gills | 1 | 0.200 ± 0.028 | 0.003 ± 0.001 | 0.051 ± 0.006 | 0.023 ± 0.008 | 0.320 ± 0.021 |
| | 2 | 0.320 ± 0.027 | 0.002 ± 0.000 | 0.070 ± 0.012 | 0.024 ± 0.011 | 0.361 ± 0.025 |
| | 3 | 0.234 ± 0.095 | 0.003 ± 0.000 | 0.055 ± 0.017 | 0.013 ± 0.004 | 0.269 ± 0.043 |
| | 4 | 0.156 ± 0.027 | 0.005 ± 0.001 | 0.063 ± 0.007 | 0.021 ± 0.004 | 0.280 ± 0.034 |
| kidney | 1 | 0.233 ± 0.053 | 0.072 ± 0.015 | 0.135 ± 0.033 | 0.013 ± 0.004 | 1.464 ± 0.362 |
| | 2 | 0.425 ± 0.102 | 0.039 ± 0.003 | 0.104 ± 0.012 | 0.031 ± 0.007 | 1.179 ± 0.180 |
| | 3 | 0.218 ± 0.030 | 0.080 ± 0.012 | 0.130 ± 0.029 | 0.019 ± 0.007 | 1.351 ± 0.273 |
| | 4 | 0.215 ± 0.048 | 0.114 ± 0.024 | 0.256 ± 0.066 | 0.018 ± 0.001 | 1.635 ± 0.333 |
| liver | 1 | 0.198 ± 0.033 | 0.014 ± 0.004 | 0.026 ± 0.004 | 0.004 ± 0.001 | 1.661 ± 0.386 |
| | 2 | 0.377 ± 0.042 | 0.009 ± 0.001 | 0.024 ± 0.003 | 0.019 ± 0.008 | 1.280 ± 0.295 |
| | 3 | 0.242 ± 0.029 | 0.014 ± 0.002 | 0.021 ± 0.003 | 0.007 ± 0.001 | 1.150 ± 0.264 |
| | 4 | 0.167 ± 0.021 | 0.023 ± 0.006 | 0.036 ± 0.006 | 0.012 ± 0.004 | 0.750 ± 0.086 |
| muscle | 1 | 0.848 ± 0.131 | 0.003 ± 0.001 | 0.040 ± 0.004 | 0.005 ± 0.002 | 0.193 ± 0.012 |
| | 2 | 0.948 ± 0.148 | 0.001 ± 0.000 | 0.042 ± 0.010 | 0.005 ± 0.002 | 0.206 ± 0.012 |
| | 3 | 0.667 ± 0.104 | 0.001 ± 0.000 | 0.035 ± 0.005 | 0.005 ± 0.002 | 0.257 ± 0.102 |
| | 4 | 0.399 ± 0.100 | 0.004 ± 0.001 | 0.039 ± 0.007 | 0.005 ± 0.001 | 0.181 ± 0.015 |
| skin | 1 | 0.224 ± 0.055 | 0.003 ± 0.001 | 0.017 ± 0.003 | 0.024 ± 0.008 | 0.311 ± 0.020 |
| | 2 | 0.469 ± 0.089 | 0.003 ± 0.001 | 0.021 ± 0.002 | 0.049 ± 0.018 | 0.297 ± 0.056 |
| | 3 | 0.180 ± 0.016 | 0.003 ± 0.001 | 0.030 ± 0.013 | 0.037 ± 0.005 | 0.293 ± 0.031 |
| | 4 | 0.089 ± 0.018 | 0.003 ± 0.001 | 0.006 ± 0.001 | 0.022 ± 0.006 | 0.115 ± 0.009 |

[a] Each value represents the mean ± SD ($n$ = 5).

**Table 5.** Relationship between Element Content in Tissues and Tissue Weight for Rainbow Trout Aged 10–40 Months, Regression Equations, and Corresponding $\tau$ Kendall Values[a]

| element | tissue | regression eq | $\tau$ Kendall |
|---|---|---|---|
| As | gills | $y = 0.147x + 0.220$ | 0.565 |
| | kidney | $y = 0.232x + 0.110$ | 0.695 |
| | liver | $y = 0.190x + 0.290$ | 0.695 |
| | muscle | $y = 0.798x + 1.670$ | 0.660 |
| | skin | $y = 0.229x + 0.390$ | 0.477 |
| Cd | gills | $y = 0.004x - 0.002$ | 0.491 |
| | kidney | $y = 0.100x - 0.02$ | 0.599 |
| | liver | $y = 0.018x - 0.010$ | 0.579 |
| | muscle | $y = 0.003x - 0.090$ | 0.474 |
| | skin | $y = 0.003x - 0.020$ | 0.848 |
| Hg | gills | $y = 0.062x + 0.008$ | 0.607 |
| | kidney | $y = 0.214x - 0.080$ | 0.589 |
| | liver | $y = 0.028x - 0.010$ | 0.797 |
| | muscle | $y = 0.037x + 0.050$ | 0.800 |
| | skin | $y = 0.005x + 0.180$ | 0.589 |
| Pb | gills | $y = 0.018x + 0.003$ | 0.446 |
| | kidney | $y = 0.021x + 0.005$ | 0.674 |
| | liver | $y = 0.014x - 0.007$ | 0.620 |
| | muscle | $y = 0.006x - 0.040$ | 0.621 |
| | skin | $y = 0.015x + 0.340$ | 0.779 |
| Se | gills | $y = 0.261x + 0.078$ | 0.765 |
| | kidney | $y = 1.364x + 0.010$ | 0.599 |
| | liver | $y = 0.705x + 1.170$ | 0.642 |
| | muscle | $y = 0.204x + 1.640$ | 0.768 |
| | skin | $y = 0.103x + 2.190$ | 0.821 |

[a] Each equation was calculated on the basis of the data obtained from 20 fish collected at four subsequent growth stages (age 10–40 months).

cumulated MeHg is slowly excreted, due to the low MeHg elimination rates in fish.

To get insight into the dynamics of Hg in rainbow trout, we compared experimentally derived concentrations with that obtained through eq 3. The whole body Hg concentration calculated on the basis of experimental data through eq 1 for fishes aged 40 months (average weight = 828 g) was 0.038 $\mu$g g$^{-1}$. It can be observed that this concentration is almost the same as that in muscle tissue (0.039 $\mu$g g$^{-1}$). As reported by other authors, the concentrations of Hg in muscle tissue and in the whole body are generally equal in fish (*47*).

The "theoretical" whole body MeHg concentration was calculated through eq 3 from the Hg concentration measured in fish food, assuming that about 85% of Hg in fish food was in the monomethylated form (*51*). The assimilation efficiency of inorganic Hg from food by fish is 5–10-fold lower than that of MeHg, whereas the clearance is about 3-fold faster (*46*); therefore, the contribution of the inorganic Hg present in fish food to the whole body accumulation of Hg was ignored in this study.

The physiological parameters calculated by eqs 4 and 5 were $K_e$ = 0.0016 day$^{-1}$ and $g$ = 0.0023 day$^{-1}$, respectively. A MeHg whole body concentration of 0.036 $\mu$g g$^{-1}$ was obtained by applying the kinetic model. This value matches the whole body Hg concentration of 0.038 $\mu$g g$^{-1}$ calculated on the basis of experimental data, taking into account that almost the entirety of Hg (≥95%) in rainbow trout tissues is in the form of MeHg (*48*). It is worth noting that because individuals aged 40 months are sexually mature, Hg loss to the gonads should be considered in eq 2 (*47*). Gonads may be considered as a separate compartment that receives a continuous input of Hg from the body. Assuming that a constant fraction of Hg is lost from the body to the gonads each day without any exchange back to the fish, an additive term ($K_g$, per day) should be included together with $K_e$ and $g$ in eq 3 as follows:

$$C_{t+\Delta t} = C_t e^{-(K_e+g+K_g)\Delta t} + \frac{AE \cdot IR \cdot C_f}{K_e + g + K_g}(1 - e^{-(K_e+g+K_g)\Delta t}) \quad (6)$$

$K_g$ was calculated as $Q \times GSI \times (1/365)$, where $Q$ is the ratio of Hg concentration in the gonads and the whole fish (i.e., $C_g/C_f$), and GSI is the gonadosomatic index; a 1:1 female/male

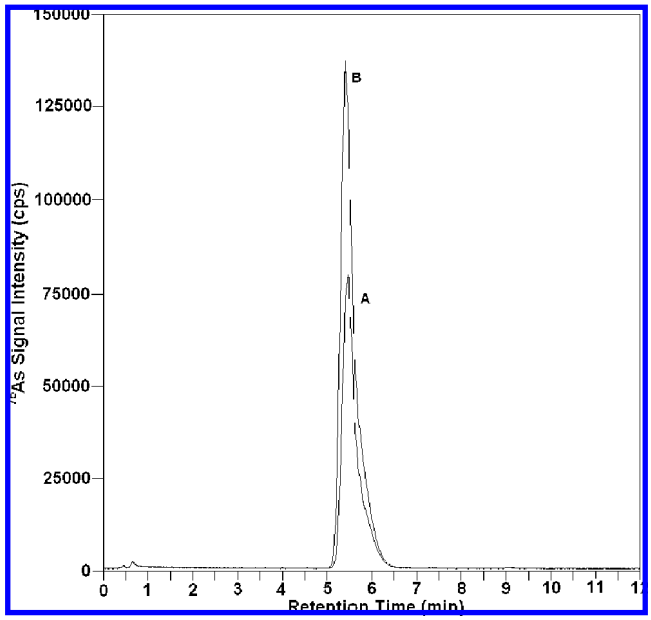


**Figure 1.** Cation exchange HPLC-ICP-MS chromatogram of muscle extract of rainbow trout: (**A**) chromatogram of muscle extract of rainbow trout; (**B**) chromatogram of the same sample with an AB spike.

ratio was assumed (*47*). The $Q$ values reported by Trudel and Rasmussen (*47*) (0.12 and 0.59 for females and males, respectively) were used, and a GSI values of 0.10 and 0.02 were estimated from the literature for females and males, respectively (*52, 53*). However, correction for loss to the gonads turned out to be very small (about 1%) and had a negligible influence on the final result.

The same approach described above for adult specimens was used to predict the concentration of individuals aged 14 and 16 months ($K_g = 0$), and a good agreement with the concentrations calculated on the basis of experimental data was obtained as well. Overall, these findings show that a dynamic equilibrium of MeHg input and output existed and that uptake was balanced by elimination and growth to give an almost constant concentration in fish.

**As Speciation.** On average, 99% of the arsenic present was extracted from muscle tissue by the methanol/water procedure employed. The identification and quantification of the arsenic species by means of cation and anion exchange chromatography coupled online with ICP-MS highlighted that AB was by far the major compound in muscle tissue.

**Figure 1** shows a typical cation exchange chromatogram with very minor peaks eluting with and right after the void volume, corresponding to poorly retained As species, and a major AB peak. The As species poorly retained on the cation exchange column at pH 2.7 are anionic and neutral molecules. The most toxic inorganic arsenic species, that is, As(III) and As(V), show this chromatographic behavior. Anion exchange chromatography confirmed that inorganic arsenic was virtually absent in muscle tissue, with only traces of As(V) appearing in some samples.

Depending on the samples, up to 18% of the total extracted As did not elute from the analytical column and thus remained unidentified. Šlejkovec et al. (*54*) found satisfactory postcolumn recoveries for aqueous methanol extracts of rainbow trout muscle tissue but succeeded in extracting only 75–80% of the total arsenic present in the samples, thus obtaining global recoveries slightly lower than in this study.

## DISCUSSION

In general terms, the concentration of a given trace element in fish is the result of the chemical fluxes into the fish, that is, uptake from food and water, the chemical fluxes out of the fish, that is, elimination due to respiration and excretion, and chemical concentration reduction by growth dilution. A mass balance can be drawn by summing the relevant chemical fluxes into and out of the fish and expressed in the form of eq 2, where the uptake from water does not appear as being negligible in the conditions of this study. Speciation is important because it influences uptake and elimination of a trace element; for example, MeHg is better absorbed and more slowly eliminated than inorganic Hg.

Inside the fish, as is clearly obvious from the results of this study, the fate of each trace element (species) is different, in terms of distributions into different organs and tissues as well as possible biotransformation and, eventually, elimination. Some tissues can act as accumulation sites; others are primarily involved in the possible biotransformation of foreign compounds (e.g., liver) or their elimination (e.g., kidney). In general terms, it can be speculated that trace elements accumulate in multiple compartments in fish, each with a different elimination kinetic (*2*). However, in many practical cases, single-compartment models can be used to predict trace element accumulation, for example, when different compartments have similar turnover times or when the exchangeable pool is large relative to the total (*2*).

As shown under Results, As was relatively well absorbed from food and retained in rainbow trout. Within fish, muscle acted as the most efficient storage site, even though concentrations in muscle tissue decreased in fish older than 14 months due to biodilution with growth. AB represented the only significant As species identified in fish muscle extracts. Fish was exposed to arsenic almost exclusively through food, and AB is the major water-soluble arsenical in fish feed products (*55–57*). Intestinal uptake and efficient transfer of AB from blood to muscle have been reported in yelloweye mullet (*Aldrichetta forsteri*), a marine fish (*58*), and in freshwater and seawater-adapted Atlantic salmon (*Salmo salar*) (*59*). A 40–50% retention of orally administered arsenobetaine in muscle tissue was found in these earlier studies (*58, 59*). Absorption of AB from feed and disposition of the unchanged compound into muscle tissue is a common pattern in teleosts; however, at least at high exposure levels, different species may handle the compound differently (*57*). Atlantic salmon exposed to AB at 25 $\mu$g As g$^{-1}$ accumulated AB in muscle and, at a lower extent, in liver and kidney (i.e., the organs of elimination), whereas in Atlantic cod (*Gadus morhua*) accumulation was observed only in muscle (*57*). Atlantic cod exhibited a higher absorption efficiency and a longer elimination rate of AB in muscle compared to the Atlantic salmon, which was speculated to be at least partially due to the differences in the volume of muscle tissue available to receive distribution of AB (*57*). In fact, muscle tissue of Atlantic cod contains $<1\%$ fat, whereas in Atlantic salmon it contains $>10\%$ fat. Muscle tissue of farmed rainbow trout has a lipid content of 4.1–5.4% (*60, 61*). In this study, the As present in muscle as AB appeared to be exchanged with other tissues because the same concentration pattern was observed in all of the tissues as fish grew, that is, a maximum at 14 months and then a decline. This suggests that muscle represents the storage site of AB, which is then redistributed to other tissues, similarly to what was observed for another salmonid, that is, Atlantic salmon, by Amlund et al. (*57*).

Our result of AB as the major As compound in rainbow trout muscle matches previous findings of Shiomi et al. (*62*) and Šlejkovec et al. (*54*). However, it should be noted that AB is not the only significant arsenical occurring in fish feed. Fish oil, which is an important ingredient of salmonid diets, contains relatively high levels of As (*55*). Arsenic in fish oil is present in the form of arsenolipids (*63*), a class of compounds for which the metabolic fate is still poorly understood. It seems that these arsenicals can be at least partially absorbed and biotransformed to various compounds, including water-soluble As species (*64*). Arsenolipid compounds have also been found in the muscle of marine fish (*65*). However, in rainbow trout, water-soluble arsenicals amounted to about 99% of the total As found in muscle tissue, and AB was the only As species identified in fish muscle extracts. In terms of food safety, this means that As in farmed rainbow trout does not represent any health risk to consumers.

MeHg is the predominant form of mercury in fish feed (>80%) (*51*) and was efficiently absorbed in rainbow trout in the conditions of this study. Muscle was the major storage site of MeHg and exhibited a Hg concentration equal to that of the administered fish food. The kidney was clearly the target organ showing the highest Hg concentrations, possibly due to accumulation of inorganic Hg resulting from demethylation of MeHg (*32, 36*). These findings match those of previous studies, which reported that after exposure to MeHg only spleen showed a higher concentration compared to kidney, as spleen directly reflected the high Hg level in blood (*30, 36*). However, these earlier studies found somewhat high Hg levels in liver (*30, 36*), which may be ascribed to the different mode of MeHg exposure (i.e., acute vs chronic in this study). Next to kidney, the gills showed higher concentrations compared to the other tissues (statistically significant difference with liver and skin, $p < 0.0001$). Penetration of the lipophilic MeHg molecule across the branchial membranes may underlie the Hg tissue levels observed in the gills.

The mass balance model employed in this study accurately predicted Hg concentrations in fish at different growth stages. The model assumed that the elimination rate of Hg (as MeHg) was a first-order process expressed by an allometric relationship (eq 4). Earlier studies showed that the elimination of Hg by acutely exposed fish is generally biphasic, with fast and slow components having half-lives of days to weeks and of months to years, respectively (*30, 46*). However, in this study fish were chronically exposed to foodborne MeHg at low levels and chronically exposed fish excrete MeHg almost exclusively from the slow component (*46*). Due to its satisfactory prediction power, the model was also used to estimate the MeHg concentration of rainbow trout of different weights, for example, 500 and 250 g. The experimental age−weight relationship obtained by exponential regression was used to estimate the average age from the corresponding weights for inclusion in eq 5. Interestingly enough, the MeHg concentration for 500 g-fish was close to the (almost constant) value obtained for the other age classes, whereas for fish weighing 250 g a lower MeHg concentration ($\sim$0.032 $\mu$g g$^{-1}$) was found. More importantly, the mass balance model provided a theoretical basis for explaining the evidence of almost equal Hg concentrations in feed and in rainbow trout in the conditions of this study over a period of >3 years as a result of age (weight)-specific growth and elimination rates. The application of the model enabled the assessment that even when reared with feed at the Hg maximum permitted levels (0.1 $\mu$g g$^{-1}$), farmed rainbow trout have Hg concentrations in muscle tissue of about 0.1 $\mu$g g$^{-1}$, that is, well below the ML established for human consumption (0.5 $\mu$g g$^{-1}$). It is worth noting that the low water temperature in this study (11–12 °C) caused the elimination of MeHg in fish to be particularly slow (eq 4). Therefore, a relatively low bioaccumulation potential of MeHg in farmed rainbow trout is apparent independent of the experimental conditions we used. The Hg concentrations in the muscle tissue measured in this study are strikingly similar to those reported for rainbow trout either farm-raised, that is, 0.035 $\mu$g g$^{-1}$ (*66*), 0.032–0.039 $\mu$g g$^{-1}$ (*67*), or 0.029–0.031 $\mu$g g$^{-1}$ (*68*), or grown in natural environments with background Hg levels, that is, 0.036 $\mu$g g$^{-1}$ (*48*). Commonly found concentrations of Hg in farmed rainbow trout are in the range of 0.01–0.08 $\mu$g g$^{-1}$ (*66–69*). The $\sim$1:1 ratio for Hg in feed and rainbow trout muscle also finds confirmation in data from earlier studies (*66*).

Cd is poorly absorbed in the gastrointestinal tract, and the concentrations measured in organs and tissues were consistently low. The absorption efficiency of foodborne Cd by rainbow trout has been previously estimated to be around 3% (*17*). The highest concentrations of Cd were found in kidney and liver, with levels approximately 1 order of magnitude above those of the other tissues. Earlier studies showed that selective sequestration of Cd in kidney and liver of orally exposed rainbow trout via binding with metallothioneins takes place (*28*). As a result of the progressive accumulation during fish growth, the percentage of the Cd body burden in kidney and liver increased from 6 to 13% and from 11 to 20%, respectively, in the 3.4 year duration of the study.

Gills, which play a primary role in Cd uptake from contaminated water and may reach relatively high concentrations when Cd enters the fish body through this route of exposure (*17, 18, 28*), turned out to contain very low levels of the element, without significant differences with muscle and skin. Cd concentrations almost identical to those found in this study have been previously reported in kidney, muscle, and gills of hatchery-reared rainbow trout (*21*). However, in this study changes in Cd concentrations of all tissues, skin being the only exception, were clearly detected with fish aging. Cd levels decreased from specimens aged 10 months to those aged 14–16 months and then increased to a maximum in adult specimens, which could reflect age-related variations in physiological parameters such as the Cd elimination rate.

Similarly to Cd, also foodborne Pb is poorly absorbed. Alves and Wood (*40*) observed that 10 of 13 tissues of rainbow trout were within a factor of 3 of background concentrations when fish was exposed to an exceedingly high concentration of 50 $\mu$g of Pb g$^{-1}$ of diet and put forward some sort of homeostatic regulation of this element. Evidence in favor of this hypothesis arises from the constant concentration of Pb in muscle tissue found in the present study at any growth stage. Moreover, we observed that the overall variation in Pb whole body concentration with fish aging was limited, similar to that of Se and second only to Hg. However, we did notice changes in the concentration of Pb in gills and, especially, skin, kidney, and liver at different growth stages, with a maximum at 14 months, notwithstanding the constant Pb dietary exposure. On the other hand, it must be noted that the major storage site of Pb is the bone, which has not been examined in this study, and this necessarily leaves the picture of Pb disposition inside the fish body incomplete. Perhaps of more importance is the fact that, despite some similarities with Cd (divalent cations, chemistry dominated by inorganic forms, no biomagnification, etc.), in this study the Pb body burden was on average nearly 2-fold that of Cd on a molar basis in fish at early growth stages. Considering the lower

concentration of Pb in the fish food (of a factor ~3.5 on a molar basis), this highlights a better retention of Pb from the diet compared to Cd in rainbow trout of ≤16 months. However, a somewhat different trend was apparent in adult specimens because the body burden of Cd at 40 months turned out to be slightly higher on a molar basis, which could be due to a slower elimination of the element compared with Pb.

Pb concentrations in internal organs and tissues followed the order kidney ≈ gills > liver ≥ muscle, which matches earlier studies (*38*). Surprisingly, the skin showed the highest concentration of Pb. Apparently this would not be connected to either direct uptake or passive binding from waterborne Pb due to the very low levels present in the tank water (<100 ng $L^{-1}$). At a closer examination, however, Pb is the only element for which a clear drop in the levels of the outflow water was detected compared to the inflow water (**Table 3**). Therefore, the question remains open whether the higher concentration in the skin was the result of dietary Pb disposition inside the body or skin interaction with waterborne Pb.

Se is an essential element for animals and is generally readily absorbed from the gastrointestinal tract and the surrounding water by fish (*70*). Se route into the diets of higher animals is unique among the minerals in that it follows sulfur amino acid pathways. In fact, in most cases the major organoselenium compounds in living organisms are protein-bound selenomethionine and (at higher trophic levels) selenocysteine. The Se requirement of fish varies with the form of Se ingested, polyunsaturated fatty acid, and vitamin E content of the diet and concentration of waterborne selenium (*71*). The Se requirement determined on the basis of optimum growth and maximum plasma glutathione peroxidase activity was estimated to be 0.15−0.38 mg of Se $kg^{-1}$ of diet (as selenite) for rainbow trout (*41*). At high concentrations Se exerts toxic effects, and rainbow trout reared on a 10–13 mg of Se $kg^{-1}$ of diet as selenite showed renal calcinosis, reduced growth, poor feed efficiency, and higher mortality (*41, 43*).

The bioavailability of Se to fish differs depending on Se speciation and fishmeal digestibility (*71*). The Se present in the fish food used in this study was relatively well retained in rainbow trout. The highest concentrations of Se were found in kidney and liver, which are known target organs of Se metabolism, even though Se levels in liver decreased with fish age. Earlier studies highlighted that the liver/kidney ratio provides a useful measure of Se loading and that rainbow trouts exposed to exceedingly high dietary levels show an increase of Se concentration in liver with respect to kidney (*42*).

Se concentration in muscle tissue was almost constant at a level of 0.2 $\mu g\ g^{-1}$. Soluble selenocompounds in muscle tissue of rainbow trout have been shown to belong mainly (87%) to the high molecular weight range (>10 kDa), with only 10% of low molecular weight compounds (*72*). Even though little is known about the exact nature of these selenocompounds, a recent study highlighted that trout is a highly bioavailable source of dietary Se in humans and that cooking does not affect Se apparent absorption or retention from fish (*73*). Therefore, due to the sizable concentrations in muscle tissue as well as high bioavailability rainbow trout appears to be a good source of Se in the human diet.

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