

PHARMACOKINETICS OF PYRENE AND OXYTETRACYCLINE IN SALMONIDS

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**THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
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Pharmacokinetics of Pyrene and Oxytetracycline in Salmonids

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ABSTRACT

The presence of chemical residues in fish has received considerable attention recently due to an increased awareness of potential human health effects related to the consumption of contaminated fish. Contamination of fish by chemicals could be the result of industrial and/or aquaculture activities.

Two different fish contaminants were selected for the present study. Pyrene, a polycyclic aromatic hydrocarbon (PAH), is a ubiquitous environmental pollutant released from the incomplete combustion of organic matter and from petrochemical pollution. Oxytetracycline (OTC), an antibiotic, is widely used in the aquaculture industry.

The purposes of this study were: (1) to investigate the toxicokinetics of pyrene in rainbow trout (*Oncorhynchus mykiss*) following the branchial and dermal routes of exposure, (2) to compare the disposition kinetics of OTC in chinook salmon (*Oncorhynchus tshawytscha*) and coho salmon (*Oncorhynchus kisutch*), and (3) to test a previously reported physiologically-based pharmacokinetic model (PBPM) of OTC with data obtained from farm treated salmon.

Trout were exposed to water-borne pyrene in a flow-through aquarium. Pyrene was absorbed rapidly but eliminated slowly by trout. Pyrene was absorbed by the branchial route at a much faster rate than the dermal route. The elimination kinetics of pyrene in trout following branchial and dermal exposure could be described by a two-compartment and a three-compartment open toxicokinetic model, respectively. Tissue concentrations of pyrene following whole-fish exposure decreased in the order of: liver > carcass > gill > kidney > blood > gut > muscle. Pyrene tissue concentrations following dermal exposure decreased in the order of: kidney = blood > muscle > liver > gill > gut.

Farm chinook salmon and laboratory kept coho salmon were fed OTC-medicated feed. A similar OTC tissue distribution pattern was observed in both fish species. However, OTC elimination rates from coho salmon tissues were about 2 - 3 times faster than those of chinook salmon. Empirical OTC tissue concentration data of farm salmon were compared with the PBPM-predicted values. Model-predicted OTC tissue concentrations agreed with the experimental data. These results indicate that the PBPM can be used as a supplement or a replacement for current methods of OTC residue determination in farm fish.

Dedication

This thesis is dedicated to the holy spirit of
my cousin, brother and friend
Daryoosh Namdari.

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List of Abbreviations

AIC	Akaike's information criterion
AUC	area under the curve
C	centigrade
EDTA	ethylenediamine-tetraacetic acid
g	gram
hr	hour
HPLC	high-performance liquid chromatography
i.a.	intraarterial
i.d.	inside diameter
I.U.	international Unit
l	liter
K _{ow}	octanol/water partition coefficient

M	molar
min	minute
m	meter
MS 222	3-aminobenzoic acid ethyl ester
N	normality
ND	not detectable
NS	not sampled
o.d.	outside diameter
OTC	oxytetracycline
PAH	polycyclic aromatic hydrocarbon
PBPM	physiologically-based pharmacokinetic model
PMT	photomultiplier tube
sd	standard deviation

vs.	versus
w/v	weight to volume
w/w	weight to weight
WSSR	weighted sum of squares residuals
<i>g</i>	acceleration of gravity

prefixes for units of measurement:

n	nano
μ	micro
m	milli
k	kilo

Part I Toxicokinetics of Pyrene in Trout

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants which are released into the environment as a result of incomplete combustion of organic matter and from petrochemical pollution (CBEAP, 1972; Hase and Hites, 1978; NRC, 1985). Natural processes such as marine seeps, forest fires and volcanic activities (Wilson *et al.*, 1977; Youngblood and Blumer, 1975) also contribute significantly to PAHs pollution in the environment. Therefore, PAHs have been found in ambient air, waters, soils, marine sediments, and foods (Santadonato *et al.*, 1981; NRC, 1983). However, the major environmental source of PAHs are from anthropogenic activities (NRC, 1983; Hites *et al.*, 1980).

PAHs are organic compounds which are comprised of carbon and hydrogen. They consist of two or more fused benzene rings in linear, angular or cluster arrangements. Heteroaromatic compounds containing nitrogen, sulfur or oxygen in place of carbon are also referred to as PAHs. Therefore, there are hundreds of PAHs ranging in molecular weight from 128.16 (naphthalene) to 300.36 (coronene), each recognized by the number of fused rings and the number of

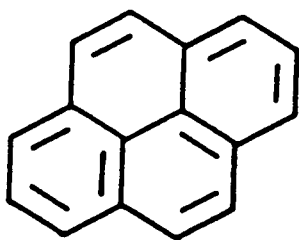
substitutions on aromatic rings.

Pyrene (Figure 1.1) was selected as a model PAH in the present study since high concentrations of pyrene are found in waters contaminated by PAHs (EPA, 1975; Marrich and Lenkevich, 1973; Diehl *et al.*, 1967). A significant level of pyrene has also been found in sediments (Youngblood and Blumer, 1975) and fish taken from a polluted aquatic environment (Krahn *et al.*, 1978). Moreover, a high concentration of pyrene also has been detected in certain type of soils, raw vegetables and fruits, cooking oils, various types of smoked/cooked fish and meats (Lijinsky and Subik, 1965; Howard *et al.*, 1966; Lijinsky and Ross, 1967; Masuda and Kuratsune, 1971 Kolar *et al.*, 1975).

Although pyrene is not a potent carcinogen, it has been shown to cause skin papillomas (Dipole *et al.*, 1984). It is probably a cocarcinogen (Weinstein and Troll, 1977). Pyrene also has been shown to be mutagenic (Kinae *et al.*, 1981).

Previous studies have shown that the toxicokinetics of pyrene in trout following intraarterial administration could be described by a three-compartment open toxicokinetic model (Kennedy and Law, 1990). Results of this study also indicated that pyrene is rapidly absorbed by trout since it

Figure 1.1. The chemical structure of pyrene.



is detected in the blood of trout within 5 min after exposure to water-borne pyrene.

The biological fate of PAHs in aquatic organisms has been thoroughly reviewed by Neff (1979), NRCC (1983) and Eisler (1987). Previous toxicokinetic studies have shown that the uptakes of PAHs by fish vary with the physico-chemical properties of the PAHs, exposure routes, physiological status of the fish and the hydrophobicity of the surrounding materials. In general, water-borne PAHs and those associated with sediments are rapidly taken up by different aquatic organisms (Lee et al., 1972; Stein et al., 1984). For example, PAHs have been shown to reach an equilibrium concentration in fish tissues within 24 hr after exposure to water-borne hydrocarbons (Lee et al., 1972). In contrast, uptake of PAHs by fish from food and sediments are much slower. Whittle et al., (1977) have shown that in most fish species, more than half of the orally administered PAHs are unabsorbed but associated with the digestive tract or its contents. Although the major route of chemical uptake by the fish is the gills (Hunn and Allen, 1974; Hamelink and Spacie, 1977; Neff, 1979; Hayton and Barron, 1990), Saarikoski et al., (1986) have suggested that dermal uptake may also be an important route of chemical absorption in fish. However, definitive empirical evidence to verify that xenobiotics are also taken up directly from water by the

skin is limited.

Fish were used in these studies for several reasons. Fish are an important natural resource as well as being a food supply for humans and other wildlives. In the aquatic environment fish may be exposed to harmful xenobiotics such as PAHs. The Environmental Protection Agency has published guidelines for the formulation of water quality criteria for the protection of aquatic species including fish (U.S. EPA, 1983). There is also an increased interest in the use of fish as alternative model systems for toxicological research and biological monitoring (Hoover 1984; Payne *et al.*, 1987; Power 1989). Rainbow trout were selected for this study since they are readily available, easy to maintain, have local commercial value and they attained world-wide use as a representative of the cool-water salmonid.

The objectives of this study were to examine the toxicokinetics and tissue distribution of pyrene in rainbow trout (*Oncorhyncus mykiss*) following different routes of exposure and also to determine the bioavailability of pyrene following dermal exposure.

MATERIALS AND METHODS

I) Fish

Rainbow trout (*Oncorhynchus mykiss*) weighing 60 - 450 g were obtained from Spring Valley Trout Farm (Langley, B.C.). They were kept in fiberglass tanks with dechlorinated, flowing water. The tanks were located in an indoor aquatic facility at Simon Fraser University. The fish were acclimated to the desired temperature ($12\pm 1^\circ\text{C}$) and photoperiod (12-hr dark, 12-hr light) for at least three weeks before an experiment. The fish were fed daily with New Age Feed from Moore Clarke Co. (Vancouver, B.C.), but were not fed for three days prior to an experiment.

II) Chemicals

Unlabeled pyrene and 1,2-benzanthracene were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). The PAHs were purified by repeated recrystallization from methanol. Chemical purities of these chemicals were determined by high-pressure liquid chromatography (HPLC) and were found to exceed 99%. Ethyl-N-aminobenzate methane sulfonic acid (MS 222), heparin and polyoxyethylene sorbitan monooleate (Tween 80) were purchased from Sigma Chemical Co. (St. Louis, MO.).

Organic solvents were of HPLC grade. All other chemicals were of analytical grade or better.

III) Surgical Preparation

Rainbow trout weighing 250 - 450 g were used in these studies. Trout were anesthetized in a water solution of MS 222 (0.1 g/l) and NaHCO_3 (0.1 g/l). The dorsal aorta of trout was cannulated by a modified procedure of Smith and Bell (1964) as follows: The fish was placed dorsally on a soft sponge holder and kept anesthetized by flowing water containing MS 222 (50 mg/l) and NaHCO_3 (50 mg/l) over its gills. A sharpened stainless steel wire (0.5 mm i.d.) inside a piece of PE50 tubing (0.58 mm i.d., 0.965 mm o.d., Clay Adams, Parsippany, NJ.) was used to puncture the dorsal aorta. The PE50 tubing was inserted with a 35 to 45 degrees angle at the midline of the first gill arch. Entry of the cannula into the dorsal aorta was indicated by a sudden rush of blood into the cannula after withdrawal of the stainless steel wire. The cannula was flushed with heparinized saline (5 I.U./ml), heat sealed and sutured securely on the roof of the fish mouth with silk surgical sutures (size 3.0, Ethicon, Inc., Somerville, NJ.) at two different locations. The free end of the cannula was passed through a hole made previously on the snout of the trout, and secured with

suture on the dorsal fin. The entire operation took approximately 20 min. The fish was allowed to recover from surgery for 24 hr in a darkened Plexiglass chamber or in an aquarium supplied with aerated, flowing water. The aquarium was covered by a piece of black plastic to minimize stress to the fish. The cannula was washed twice daily with heparinized saline to prevent clotting.

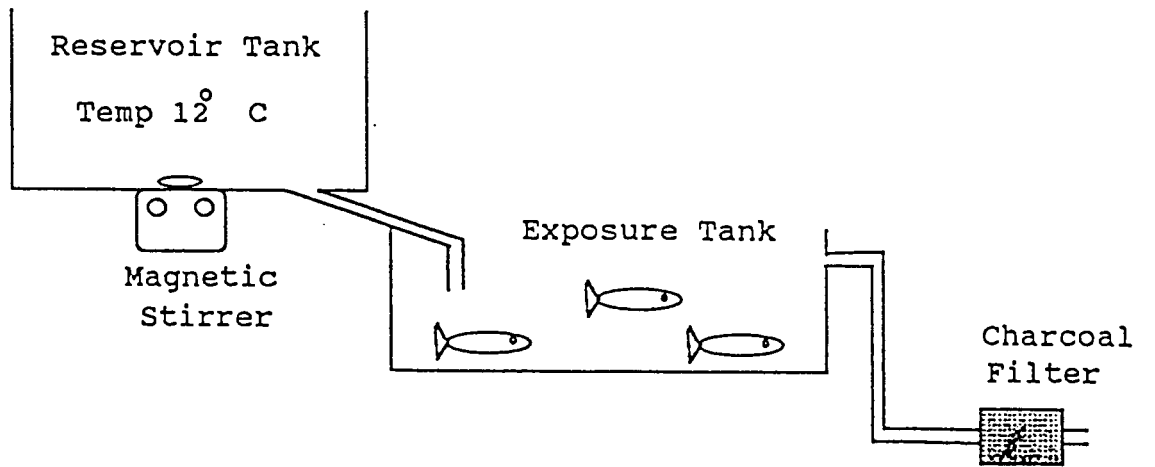
IV) Exposure Systems

Trout were exposed to an aqueous solution of pyrene in either a flow-through aquarium or a holding chamber as follows:

i) Flow-Through Aquarium

Figure 1.2 shows the flow-through system which was used to maintain a constant pyrene concentration in the water of the exposure aquarium. Glass aquaria were used to minimize pyrene absorption. The reservoir and the exposure aquarium were connected by a polyethylene tubing. A magnetic stirrer was used to mix the solution in the reservoir. Water flow rate in the exposure aquarium was adjusted to about 425 ml/min. Water temperature was maintained at 12°C.

Figure 1.2. The flow-through system used for trout exposure.



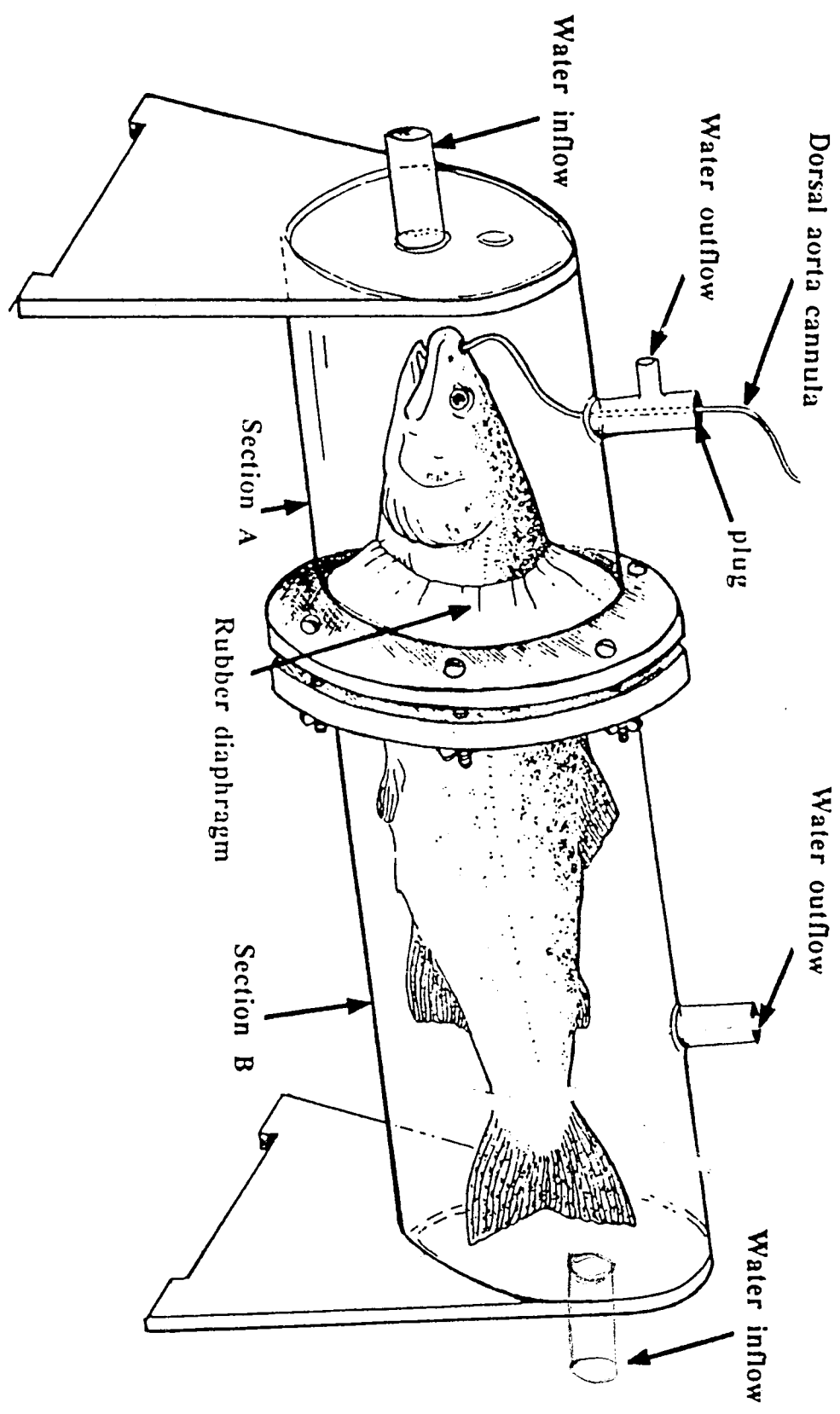
ii) Holding Chamber

A compartmentalized holding chamber modified from the metabolic chamber of Maren *et al.*, (1968) was used to study pyrene uptake by trout following exposure by different routes. Figure 1.3 shows a schematic diagram of the holding chamber (Kennedy, 1990). The chamber was divided by a rubber diaphragm into two different sections. A polyethylene tubing delivered water containing pyrene into the front chamber at 425 ml/min. Since the fish was placed inside a holding chamber, it was termed a "chambered fish".

V) Toxicokinetic Studies of pyrene in the blood of trout after different routes of exposure

These studies were carried out with different exposure scenarios (see below). For each exposure scenario, a control blood sample was taken from the fish before the experiment. At different time points during and after a trout was exposed to pyrene, a 0.2 ml blood sample was withdrawn through the cannula. An equal volume of heparinized saline solution was injected into the cannula after a blood sample was taken to replace the blood removed and to prevent blood clot formation inside the cannula.

Figure 1.3. Schematic diagram of the chamber used to examine the different uptake routes of pyrene by trout. (Kennedy, 1990).



i) Whole-Fish Chambered Exposure

A stock solution of pyrene was prepared by dissolving pyrene in Tween 80 (0.1% w/v) and 10 ml of distilled water. The stock solution was diluted to a final concentration of 8 mg/l with water. In all experiments, pyrene concentrations in water were nominal concentrations. The aqueous solution was poured into a reservoir which was connected to the front section of a holding chamber by a polyethylene tubing. Water flow rate to the chamber was adjusted to approximately 425 ml/min. A cannulated trout was placed inside the chamber (without a partitioning diaphragm) and exposed to a constant concentration of pyrene (8 mg/l) for 4-hr. At the conclusion of a 4-hr exposure, the trout was exposed to uncontaminated, flowing water for an additional 96 hr for depuration. Blood samples were taken as described above.

ii) Whole-Fish Free-Swimming Exposure

To investigate whether confinement of fish in a holding chamber has any effect on the uptake kinetics of pyrene, a free-swimming, cannulated trout was also kept in an aquarium containing 8 mg/l of pyrene. After a 4-hr exposure, the fish were transferred to a depuration tank containing uncontaminated, flowing water. Blood samples were taken as explained in section V.

iii) Head-Only Exposure

A cannulated trout was placed inside a holding chamber equipped with a partitioning diaphragm to separate water in the front (head) chamber from those of the back (body) chamber. The front chamber enclosed the head and the opercula of the trout. It received flowing water containing 8 mg/l of pyrene at a flow rate of 425 ml/min. After a 4-hr exposure period, uncontaminated water was delivered to the front chamber. The back chamber received clean, flowing water during the experiment. Blood samples were taken as mentioned in section V. Water samples (0.2 ml) were taken from the back chamber. The samples were analysed for pyrene by HPLC to ensure that there was no mixing of water between the front and rear chamber during exposure.

iv) Body-Only Flow-Through Exposure

Body-only exposure is defined as the exposure of the skin of trout posterior to the gills. To carry out such a study, a cannulated trout was placed inside the compartmentalized holding chamber. The front chamber was supplied with uncontaminated, flowing water while the rear chamber received water containing 8 mg/l of pyrene at a flow rate of 425 ml/min. At the conclusion of a 4-hr exposure, uncontaminated water was delivered to the rear chamber.

Blood samples (0.3 ml) were taken as described in section V.

v) Body-Only Static Exposure

A cannulated trout was put into a holding chamber. Clean water was supplied to the front chamber as described in section IV. The rear section of the chamber was filled with a stagnant solution of pyrene (24 mg/l). At the conclusion of a 4-hr exposure, contaminated water in the rear section was replaced with clean, flowing water. Blood samples (0.3 ml) were taken as mentioned previously.

VI) Tissue Distribution Studies of pyrene

Trout weighing 60 - 70 g were exposed to 8 or 0.8 mg/l of pyrene in a flow-through aquarium for 4-hr before being transferred to clean water. At different times after exposure: 0, 0.5, 1, 4, 10, 18, 24, 48, 72, 96 and 144 hr (0-hr indicated the end of the 4-hr exposure) three fish were sacrificed and blood samples taken via the caudal vein. Major organs and tissues were removed from trout, weighed and stored at -4°C until analysis.

In a separate experiment, three trout 400 - 500 g were sacrificed after a 4-hr static, body-only exposure to water

containing 24 mg/l of pyrene. Blood and tissue samples were taken as described above.

VII) Extraction of Pyrene from trout tissues

Blood samples (0.2 ml) were pipetted separately into 15 ml glass centrifuge tubes containing 0.5 ml sulfuric acid (1 N) to deproteinize the blood. After the addition of distilled water (3 ml), the tube was vortexed for 3 min. The mixture was extracted with 6 ml hexane containing benzantracene (0.04 $\mu\text{g}/\text{ml}$) as an internal standard. For the static exposure study, 0.3 ml of blood was extracted with 6 ml hexane to increase the amount of pyrene extractable from the samples. The centrifuge tube was shaken for 30 min on a mechanical shaker and centrifuged at 2000 g for 15 min to separate the organic and aqueous layers.

Tissues (0.5 g) were homogenized separately in distilled water (2 ml) and 0.5 ml of 1 N sulfuric acid with a Polytron homogenizer (Brinkman Co., Rexdale, Ont.) and transferred to 15 ml centrifuge tubes. The polytron probe was rinsed with 2 ml distilled water. The rinse was added to the tissue homogenates which were extracted with 6 ml hexane containing 0.04 $\mu\text{g}/\text{ml}$ of benzantracene, the internal standard. Recoveries of pyrene from tissues were examined by spiking the tissues with known amounts of pyrene and

extracted with hexane as described above.

XIII) Analysis of Pyrene

An aliquot of the hexane extract was injected into a Hewlett-Packard Liquid Chromatograph (Model 1050) equipped with an ODS-Hypersil column (Phenomenex 5 μ m, 250 X 4.6 mm i.d.) and a Hewlett-Packard programmable fluorescence detector (Model 1046 A). The settings of the fluorescence detector were as follows: EX = 239 nm, EM = 400 nm, PMT Gain = 12 and lamp = -1. The column was eluted isocratically with methanol:water (78%:22%) at a flow rate of 1.8 ml/min.

IX) Toxicokinetic Analysis

The blood concentration-time profile of pyrene was initially fitted by RSTRIP (MicroMath Scientific Software, 1989) to determine the appropriate number of exponential components used to analyse the data. These initial estimates were then used to fit the data with NONLIN (Metzler *et al.*, 1984). Akaike's information criterion (AIC) (Yamaoka *et al.*, 1987), weighed sum of squares residuals (WSSR) (Balant and Garrett, 1983) and the scatter of observed values about the predicted values were applied to select the most appropriate model. The data points of individual fish were weighted according to the following equation proposed by Albert *et*

al., 1974:

$$\ln \sigma^2 = \ln a + n \ln C \quad (1)$$

Where σ is the variance corresponding to the mean concentration (C) for a group of subjects at each sampling time, and a and n are constants. A plot of $\ln \sigma$ versus $\ln C$ yields a straight line with the intercept $\ln a$ and a slope n. If $n = 1$, then an appropriate weighing factor is the reciprocal of blood chemical concentration; if $n = 2$, the squared reciprocal of the blood chemical concentration should be used.

The time course of pyrene depuration from the blood of trout after pyrene exposure was best described by the equation:

$$C_b = \sum A_i e^{-X_i t} \quad (2)$$

Where C_b is the blood concentration of pyrene at time t, and A_i and X_i are the coefficients and constants of the exponential components, respectively. The overall goodness of fit was determined by comparing the sum of the squared deviations and by the scatter of the data points around the fitted function. Area under blood concentration-time curves

(AUC) were calculated using the following equations (Gibaldi and Perrier, 1985):

$$AUC_{0 \rightarrow \infty} = AUC_{0 \rightarrow t} + AUC_{t \rightarrow \infty} \quad (3)$$

where t represents the time when the last blood sample was taken. $AUC_{0 \rightarrow t}$ was determined using the trapezoidal rule and the estimate of $AUC_{t \rightarrow \infty}$ was calculated using the following equation (Gibaldi and Perrier, 1985):

$$AUC_{t \rightarrow \infty} = C_t / \beta \quad (4)$$

where β is the terminal elimination rate constant and C_t is the concentration of pyrene in the last blood sample.

The apparent bioavailability (A) of pyrene administered dermally to trout was calculated by the following equation:

$$A(\%) = \frac{(AUC_d) (dose_{i.a.})}{(AUC_{i.a.}) (dose_d)} \times 100 \quad (5)$$

where $AUC_{i.a.}$ and $dose_{i.a.}$ are the area under the blood concentration-time curve and dose, respectively, after intraarterial administration. AUC_d and $dose_d$ are the corresponding parameters of dermal exposure (Gibaldi and

Perrier, 1985). The apparent bioavailability is a measurement of the relative amount of the dermally administered dose, as compared to the same dose given by the intraarterial route, which actually reaches the general circulation. The intraarterial dose is assumed to be 100% bioavailable. The values for intraarterial administration were adapted from a previous study by Kennedy and Law (1990).

The *t* test was used for statistical comparisons, the level of significance was chosen at 0.05.

RESULTS

I) Chromatographic Analysis of Pyrene

Extraction recoveries of tissues spiked with a known amount of pyrene were: 98, 78, 84, 86, 79, 84, and 82% for liver, kidney, muscle, gills, gut, blood, and carcass, respectively. The detection limit of pyrene by the HPLC system ranged from 10 to 20 ng/ml. Figure 1.4 shows a typical HPLC elution profile of pyrene. The retention time of pyrene and benzoanthracene (the internal standard) were 5.5 min and 6.3 min, respectively.

II) Toxicokinetics of Pyrene in the Blood of Trout Following Different Routes of Exposure

i) Whole-Fish and Head-only Exposures

Figures 1.5 depict the concentration vs. time curves of pyrene in the blood of trout following chambered, free-swimming and head-only exposure to 8 mg/l of pyrene. Maximum blood concentration of pyrene at the conclusion of a 4-hr exposure period for the chambered, free-swimming and head-only exposed fish were 14.25 ± 4.11 , 9.09 ± 1.52 and 12.10 ± 3.79 $\mu\text{g/ml}$, respectively. These values are not significantly different ($P > 0.05$) from each other.

Figure 1.4. A typical HPLC elution profile of pyrene and benzantracene (internal standard). Peaks I and II indicate pyrene and benzantracene, respectively.

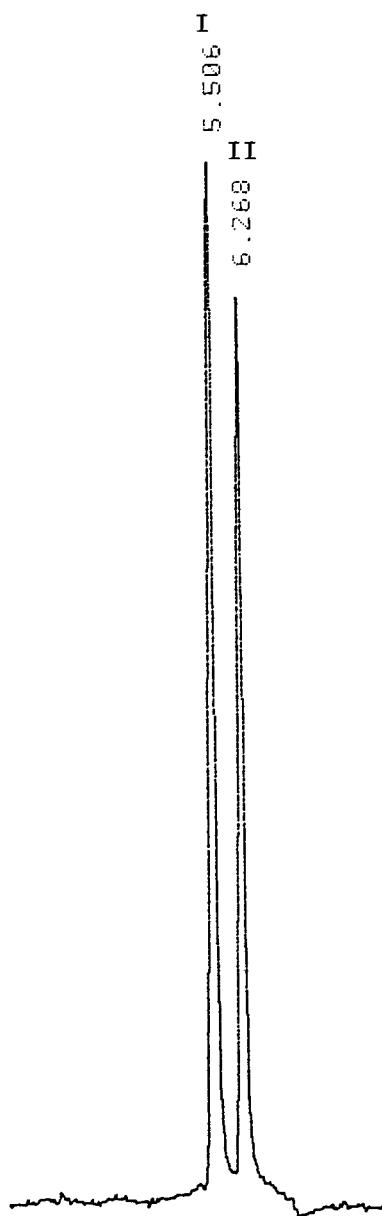
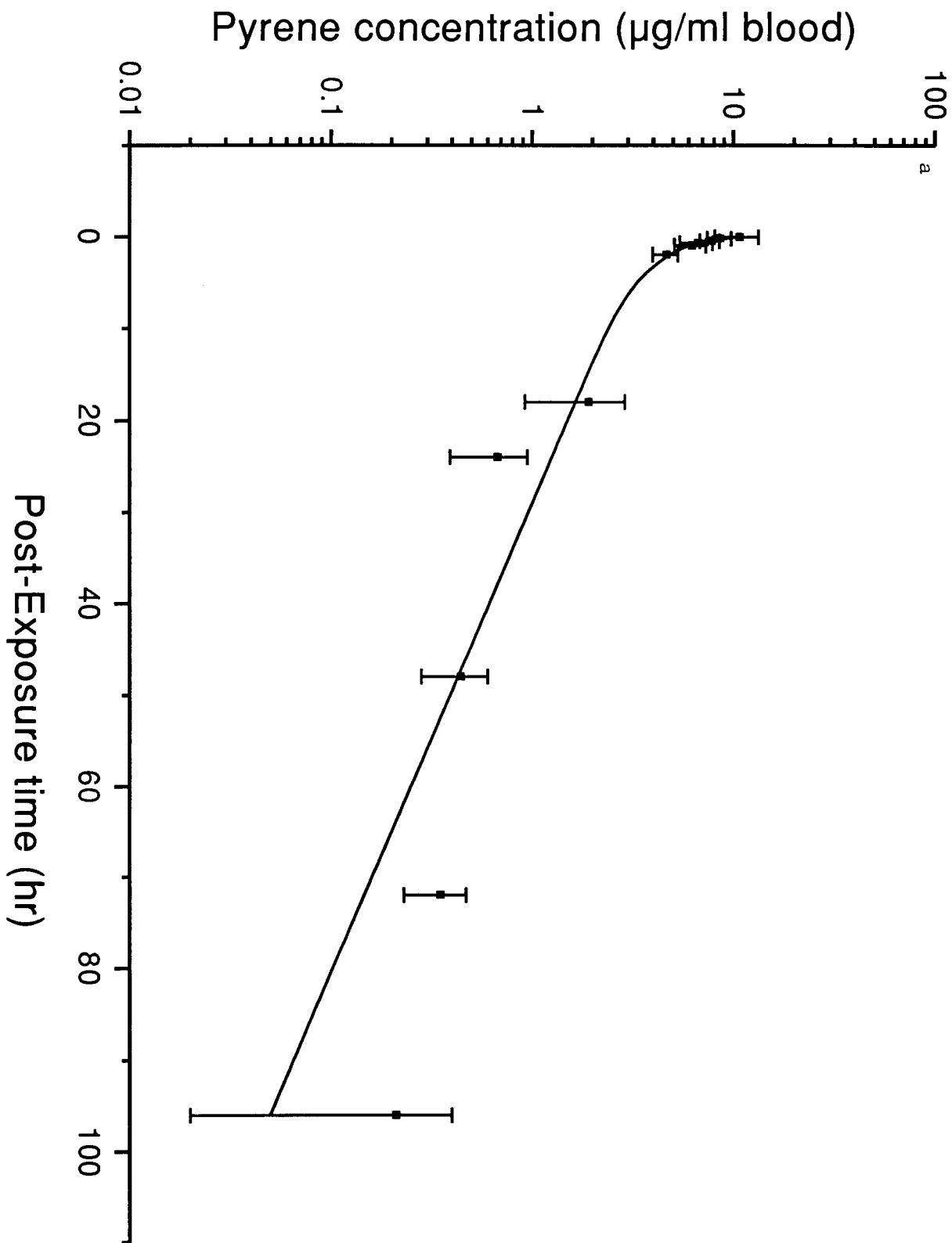
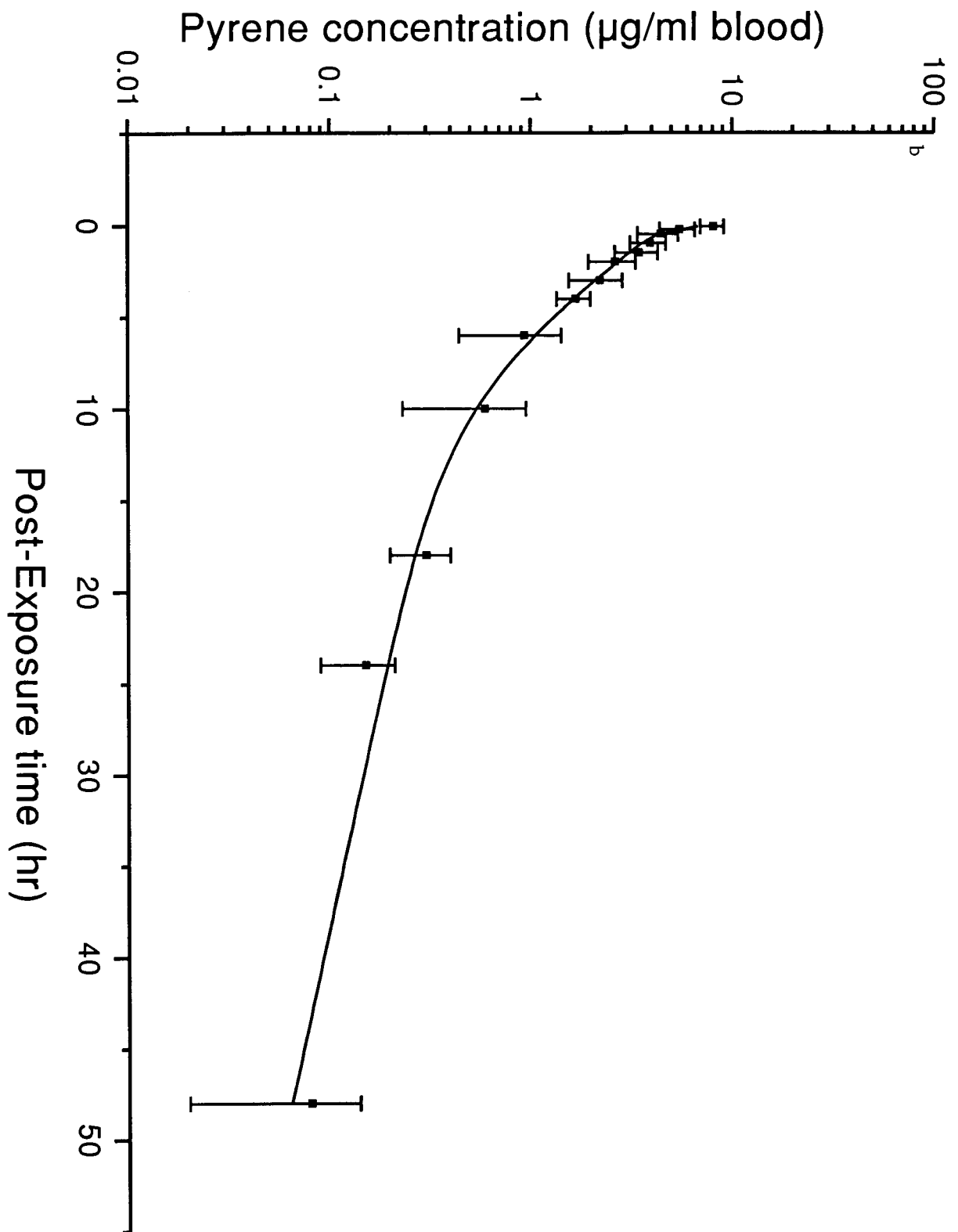
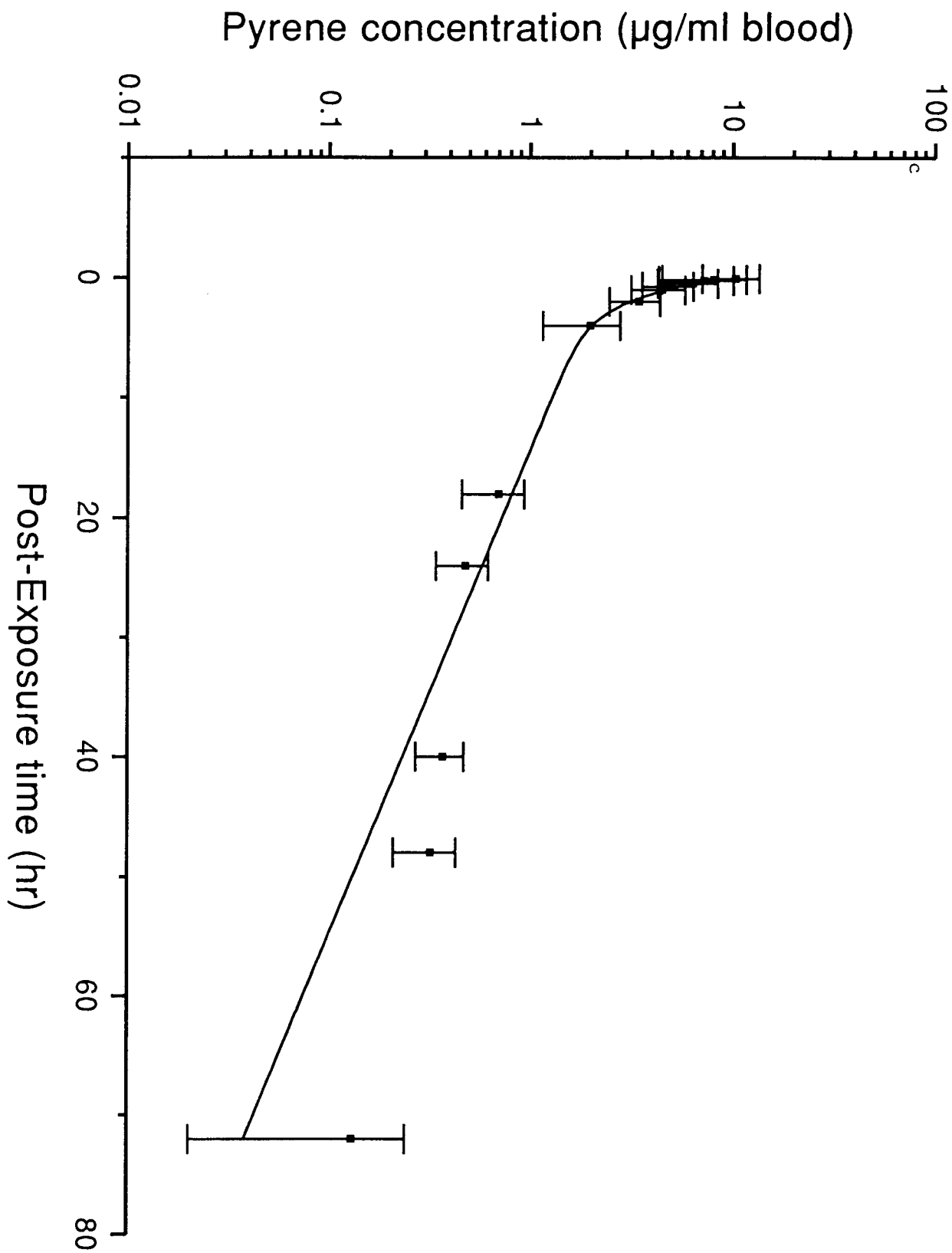


Figure 1.5. Time course of pyrene concentration in the blood of trout following different routes of exposure. Each point represents the mean \pm sd of three fish. The curve represents the three-compartment model prediction of the data. a) chambered, b) free-swimming and c) head-only exposure.







Since the slope of the $\ln \sigma$ versus $\ln C$ plot (see equation 1) were 0.68 and 0.80 for chambered and free-swimming fish, respectively, a weighing factor of $1/C$ was chosen to fit these data. In contrast, a weighing factor of $1/C^2$ was chosen for the data of head-only exposed fish since the slope of the $\ln \sigma$ versus $\ln C$ plot was 1.56. Each blood concentration-time curve was fitted to a two-compartment and a three-compartment toxicokinetic models with NONLIN. Since a three-compartment fit gives a lower WSSR and AIC values (data not shown) and the scattering of observed values about the predicted values was randomly distributed (data not shown), the three-compartment model was chosen over the two-compartment model to analyze these data. The parameter estimates of the fitted equation, along with the derived toxicokinetic parameters are shown in Table 1.1. The terminal elimination half-life of pyrene in blood was 13.27, 14.46 and 12.19 hr for the chambered, free-swimming and head-only exposed fish, respectively. Pyrene blood concentration-time data are shown in the Appendix (Table A.1).

ii) Body-Only Exposure

After a 4-hr, flow-through, body-only exposure of trout to 8 mg/l of pyrene, the chemical was not detected in the blood of trout in the initial 3 hr. In the static exposure

Table 1.1. Model parameters describing blood concentration of pyrene in trout following different routes of exposure^a.

Parameter	Exposure Route		
	Whole-Fish ^b	Whole-Fish ^c	Head-Only
A ₁ (μg/ml)	12.36	6.17	8.72
A ₂ (μg/ml)	5.52	4.58	5.81
A ₃ (μg/ml)	4.42	0.63	2.28
X ₁ (hr ⁻¹)	28.15	9.22	14.39
X ₂ (hr ⁻¹)	1.04	0.35	0.83
X ₃ (hr ⁻¹)	0.052	0.048	0.057
X _{1HL} (hr)	0.025	0.075	0.048
X _{2HL} (hr)	0.67	1.97	0.84
X _{3HL} (hr)	13.27	14.46	12.19
K ₁₂ (hr ⁻¹)	14.48	4.67	6.51
K ₂₁ (hr ⁻¹)	12.88	4.17	7.26
K ₁₃ (hr ⁻¹)	1.14	0.28	0.92
K ₃₁ (hr ⁻¹)	0.48	0.09	0.27
K ₁₀ (hr ⁻¹)	0.25	0.42	0.35
AUC _{4→t} (μg.hr/ml)	90.23	26.83	47.75
AUC _{0→∞} (μg.hr/ml) ^d	132.81	51.13	85.20

a) Trout were exposed to 8 mg/l of pyrene for 4 hr.

b) Chambered-Fish

c) Free-Swimming

d) $AUC_{0 \rightarrow \infty} = AUC_{0 \rightarrow 4} + AUC_{4 \rightarrow t} + AUC_{t \rightarrow \infty}$

of trout to 24 mg/l of pyrene, pyrene was detected in the blood in the first 1 hr. At the conclusion of a 4-hr flow-through and static body-only exposure, maximum blood concentration of pyrene were 0.08 and 0.37 $\mu\text{g/ml}$, respectively. Since pyrene blood concentration dropped below the HPLC detection limit at 5 hr post-exposure only a limited number of data points were available for toxicokinetic analysis (Table 1.2).

Figure 1.6 depicts the pyrene blood concentration vs. time curve of trout following static, body-only exposure to 24 mg/l of pyrene. Pyrene concentration in the blood appeared to decline biphasically with time. Therefore, a two-compartment open toxicokinetic model was used to fit the data. The parameter estimate of the fitted equation, along with the derived toxicokinetic parameters are shown in Table 1.3. The terminal elimination half-life of pyrene was 16.04 hr. Numerical data of the blood concentration vs. time curves are presented in the Appendix (Table A.2).

III) Tissue Distribution of Pyrene

Tables 1.4 and 1.5 summarize the results of pyrene tissue distribution in free-swimming trout after whole-fish exposure in a flow-through aquarium containing 8 or 0.8 mg/l of pyrene. At the cessation of the 4-hr exposure period, the

Table 1.2. Concentration of pyrene ($\mu\text{g/ml}$) in the blood of trout^a during and after body-only exposure.

Time (hr)	Concentration
1.00	ND ^b
2.00	ND
3.00	0.04 \pm 0.03
4.00	0.08 \pm 0.03
4.50	0.07 \pm 0.02
9.00	0.05 \pm 0.02
12.0	ND
20.0	ND
28.0	ND

a) Trout exposed to 8 mg/l of pyrene in a flow-through compartmentalized chamber for 4 hr. Each value represents the mean \pm sd of three fish.

b) Not detected

Figure 1.6. Time course of pyrene concentration in the blood of trout following static body-only exposure in a chamber containing 24 mg/l of pyrene. Each point represents the mean \pm sd of three fish. The curve represents the two-compartment model prediction of the data.

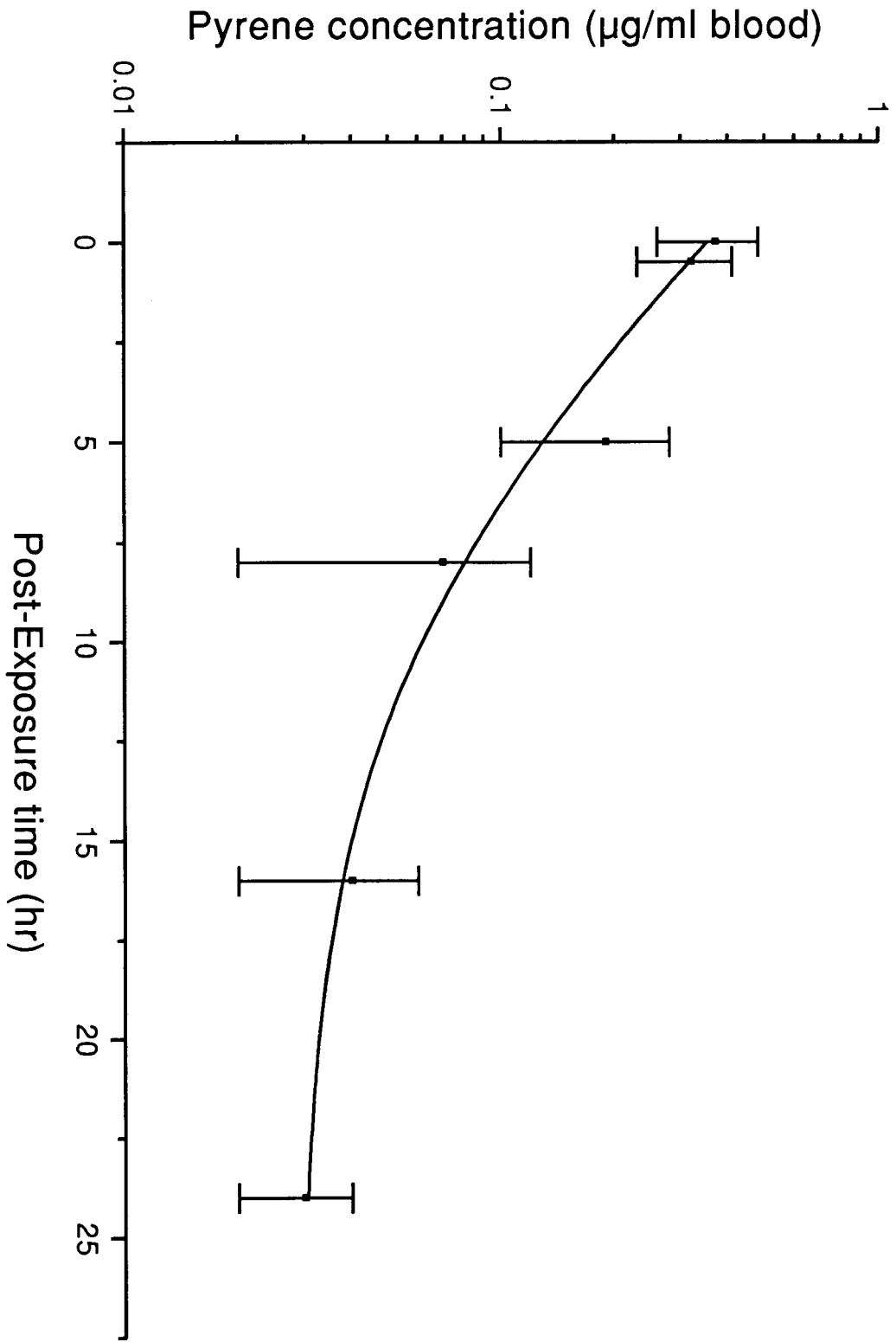


Table 1.3. Model parameters describing blood concentrations of pyrene in trout^a following static body-only exposure.

Parameters	Value
A ₁ (μg/ml)	0.52
A ₂ (μg/ml)	0.08
X ₁ (hr ⁻¹)	1.53
X ₂ (hr ⁻¹)	0.043
X _{1HL} (hr)	0.45
X _{2HL} (hr)	16.04
K ₁₂ (hr ⁻¹)	1.33
K ₂₁ (hr ⁻¹)	0.19
K ₂₀ (hr ⁻¹)	0.05
AUC _{4→t} (μg.hr/ml)	2.23
AUC _{0→∞} (μg.hr/ml) ^b	3.66

a) Trout exposed in a compartmentalized chamber containing 24 mg/l of pyrene for 4-hr.

$$b) \text{AUC}_{0 \rightarrow \infty} = \text{AUC}_{0 \rightarrow 4} + \text{AUC}_{4 \rightarrow t} + \text{AUC}_{t \rightarrow \infty}$$

Table 1.4. Concentration of pyrene ($\mu\text{g/g}$) in tissues of trout^a following free-swimming fish exposure to water containing 8 mg/l of pyrene.

Time	Tissue						
	Liver	Kidney	Muscle	Gill	Gut	Blood	Carcass
0 ^b	26.35±1.57	15.76±2.44	6.52±0.48	17.82±0.96	7.99±1.85	11.55±2.30	22.12±6.51
0.5	20.51±3.45	14.27±0.59	6.26±0.98	13.45±1.21	7.80±2.16	7.13±2.25	17.43±0.95
1	15.36±2.81	10.51±3.58	5.44±1.38	12.84±10.0	6.64±1.42	6.65±2.46	13.55±4.74
4	14.64±6.10	9.66±3.77	4.59±0.87	9.22±2.08	5.71±2.28	3.27±2.23	12.07±0.68
10	11.79±3.89	6.43±2.55	4.40±0.80	3.38±1.29	2.81±1.73	2.72±1.37	9.59±0.65
18	6.79±0.51	4.85±0.56	3.83±1.65	2.57±0.78	1.32±0.86	2.60±0.21	8.93±0.84
24	4.01±1.96	3.02±1.86	3.93±0.36	1.20±0.06	0.72±1.01	1.51±1.11	7.76±1.19
48	1.77±1.34	0.30±0.41	0.43±0.28	0.15±0.26	0.39±0.46	0.24±0.10	7.25±0.51
72	0.10±0.13	ND	0.18±0.16	ND	ND	ND	2.74±0.30
96	ND ^c	ND	ND	ND	ND	ND	1.04±0.34
120	NS ^d	NS	NS	NS	NS	NS	0.68±0.62

a) Trout were exposed in a flow-through aquarium for 4-hr. Each value represents the mean±sd of three fish.

b) 0 hr indicates the conclusion of 4-hr exposure period.

c) Not detected

d) Not sampled

Table 1.5. Concentration of pyrene ($\mu\text{g/g}$) in tissues of trout^a following free-swimming fish exposure to water containing 0.8 mg/l of pyrene.

Time	Tissue						
	Liver	Kidney	Muscle	Gill	Gut	Blood	Carcass
0 ^b	4.28±1.49	3.55±0.91	1.39±0.07	3.09±0.44	0.95±0.21	1.49±0.15	1.76±0.10
0.5	1.84±0.30	1.69±0.39	1.84±0.69	1.72±0.43	0.69±0.08	0.70±0.20	1.18±0.45
1	1.75±0.16	1.11±0.29	1.34±0.12	1.43±0.51	0.63±0.05	0.68±0.11	1.15±0.46
4	0.96±0.38	0.74±0.10	0.67±0.10	0.94±0.33	0.55±0.11	0.61±0.08	1.07±0.48
10	0.77±0.16	0.41±0.04	0.48±0.05	0.55±0.39	0.40±0.08	0.33±0.06	0.85±0.16
18	0.34±0.03	0.35±0.09	0.30±0.04	0.40±0.08	0.30±0.18	0.32±0.06	0.75±0.24
24	0.33±0.12	0.28±0.08	0.27±0.18	0.20±0.03	0.20±0.08	0.15±0.01	0.64±0.34
48	0.12±0.06	0.06±0.01	0.08±0.01	ND ^c	ND	ND	0.34±0.09

a) Trout were exposed in a flow-through aquarium for 4-hr. Each value represents the mean±sd of three fish.

b) 0 hr indicates the conclusion of 4-hr exposure period.

c) Not detected

highest concentration of pyrene was found in the liver and the lowest level in the muscle or gut. The following orders summarize the tissue distribution pattern of pyrene in trout after whole-fish exposure to different concentrations of pyrene: a) 8 mg/l, b) 0.8 mg/l.

a) Liver > Carcass > Gill > Kidney > Blood > Gut > Muscle

b) Liver > Kidney > Gill > Carcass > Blood > Muscle > Gut

The pattern was quite similar for both pyrene concentrations except the order of carcass. The carcass represents the remaining tissues of the fish after the removal of organs needed for analysis.

The elimination of pyrene from the whole-fish and major tissue depots conformed to simple first-order kinetics. Table 1.6 shows the terminal elimination rate constants and half-lives of pyrene in different tissues of trout following whole-fish exposure. Tissue depuration of pyrene shows a heterogeneous pattern among the different organs and tissues examined, but the trend was similar for both low and high pyrene concentrations. The longest terminal elimination half-life was found in the carcass and the shortest in the gills. The following patterns summarize the ranking order of

Table 1.6. Terminal elimination rate constant (hr^{-1}) of pyrene in selected tissues of trout^a following free-swimming fish exposure to different concentration of pyrene.

Tissue	Pyrene Concentration			
	8 mg/l		0.8 mg/l	
Liver	0.072	(9.63) ^b	0.049	(14.14)
Kidney	0.064	(10.83)	0.049	(14.14)
Muscle	0.058	(14.14)	0.044	(15.75)
Gills	0.085	(8.15)	0.071	(9.76)
Gut	0.048	(14.14)	0.049	(14.14)
Carcass	0.026	(26.65)	0.025	(27.72)

a) Trout exposed in a flow-through aquarium containing 8 or 0.8 mg/l of pyrene for 4 hr.

b) Corresponding half-life of the terminal elimination rate rate constant.

these data for different exposure concentrations a) 8 mg/l,
b) 0.8 mg/l:

a) Carcass > Gut > Muscle > Kidney > Blood > Liver > Gill

b) Carcass > Muscle > Gut = Kidney = Liver > Blood > Gill

Tissue concentrations of pyrene after a 4-hr static body-only exposure to water containing 24 mg/l of pyrene are shown in Table 1.7. Pyrene distribution showed a different pattern from that of the whole free-swimming fish exposure in a flow-through system, with the following order:

Kidney = Blood > Muscle > Liver > Gill > Gut

Table 1.7. Concentration of pyrene ($\mu\text{g/g}$) in the tissues of trout^a following static body-only exposure.

Tissue	Concentration
Liver	0.17 \pm 0.15
Kidney	0.22 \pm 0.13
Muscle	0.18 \pm 0.14
Gill	0.09 \pm 0.05
Gut	0.03 \pm 0.03
Blood	0.22 \pm 0.13

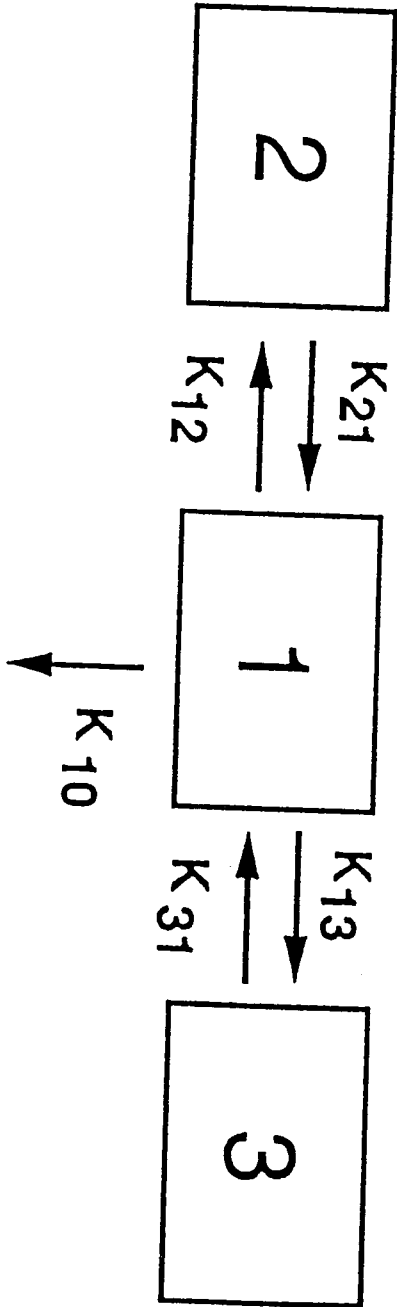
a) Trout exposed in a holding chamber containing 24 mg/l of pyrene for 4 hr. Each value represents the mean \pm sd of three fish.

DISCUSSION

Results of the present studies indicate that the elimination of pyrene from the blood of trout following branchial and dermal routes of exposure could be described adequately by a three-compartment and a two-compartment open toxicokinetic model, respectively.

Figure 1.7 shows a schematic diagram of a three-compartment toxicokinetic open model which is used to describe the kinetics of pyrene in the blood of trout following the whole-fish or head-only exposure. The model consists of a central compartment, a shallow peripheral compartment and a deep peripheral compartment. The central compartment represents the vascular system, the shallow peripheral compartment represents highly perfused tissues (such as liver and kidney) and the deep peripheral compartment represents low perfused tissues of the trout (such as muscle and fat). The triexponential decline of the blood concentration-time curve may be associated with the following processes: the first slope represents the rapid distribution of pyrene to both the shallow and deep compartments. The second slope represents elimination and metabolism of pyrene. The third slope represents a slower elimination of pyrene from the deep compartment.

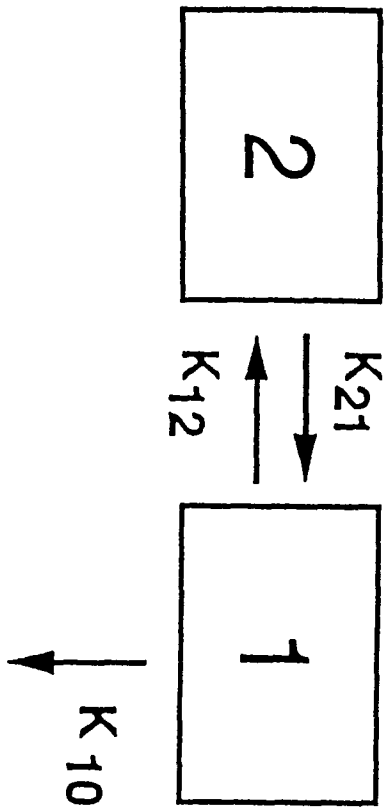
Figure 1.7. Schematic representation of a three-compartment model describes the disposition of pyrene in trout following whole-fish or head-only exposure. Where compartments 1, 2 and 3 represent central, shallow and deep peripheral compartments, respectively. k_{12} , k_{21} , k_{13} , k_{31} are intercompartmental transfer rate constants and k_{10} is the elimination rate constant from the central compartment.



The terminal elimination half-life (X_{3HL}) of pyrene in trout is very large (Tables 1.1 and 1.3). This is consistent with the lipophilic and bioaccumulative characteristics of pyrene. This also supports the rapid removal of pyrene from the blood by other tissues and the sequestration of pyrene by the fatty tissues. Kennedy and Law (1990) have reported that the terminal elimination half-life of pyrene in the blood of trout following intraarterial administration is 12.80 hr. This is in agreement with an estimated 13.99 hr (range from 12.20 - 16.04 hr) mean terminal elimination half-life of pyrene in the present study.

In contrast, the toxicokinetics of pyrene in the blood of trout following a static, body-only exposure to 24 mg/l pyrene could be described by a two-compartment open toxicokinetic model. Figure 1.8 shows a schematic diagram of the two-compartment open model which consists of a central compartment and a deep compartment. In the body-only mode of exposure, skin is the barrier of systemic pyrene absorption. In the whole-fish or head-only mode of exposure, both the skin and gills are the barriers of absorption. A comparison of the maximal pyrene blood concentration at the conclusion of a 4-hr head-only (12.10 $\mu\text{g/ml}$) and body-only (0.08 $\mu\text{g/ml}$) exposures indicate that pyrene uptake by the gills of trout is much faster than that of the skin. Since dermal

Figure 1.8. Schematic representation of a two-compartment model describes the disposition of pyrene in trout following body-only exposure to 24 mg/l of pyrene. Where compartments 1 and 2 represent central and deep compartments. K_{12} and k_{21} are inter-compartmental transfer rate constants and k_{10} is the elimination rate constant from the central compartment.



absorption of pyrene is slow, the rapidly equilibrating compartment (shallow compartment) observed after whole-fish or head-only exposure was obscured following body-only exposure. In other words, the shallow and central compartments in body-only exposed fish filled up simultaneously due to the slow dermal absorption of pyrene and therefore, they can be represented as a single compartment in the body-only exposed fish. A similar approach was used by Schultz and Hayton (1993) to explain the toxicokinetics of trifluralin in rainbow trout following different routes of exposure. Barron *et al.*, (1990) also suggested that slow absorption may obscure the early exponential phases of a particular chemical elimination.

A steady-state concentration of pyrene apparently was attained in the blood of trout after a 4-hr, head-only exposure, but it was not achieved by a 4-hr body-only exposure. Although these studies differed by a 3 fold difference in exposure concentration (8 mg/l compare to 24 mg/l of pyrene), the area under the blood concentration-time curve ($AUC_{0 \rightarrow \infty}$) of the head-only exposed trout (85.20 $\mu\text{g}\cdot\text{hr}/\text{ml}$) was about 23 times higher than that of the body-only exposed fish (3.66 $\mu\text{g}\cdot\text{hr}/\text{ml}$). These results indicate that pyrene uptake following branchial exposure is more extensive than that of dermal exposure. It should be pointed

out that, head-only exposure probably includes uptake of pyrene by the nasal and eye epithelia, lining of the oral cavity and the skin around the head area. However, the amounts of pyrene absorbed by these tissues are negligible due to their small surface area. Since trout in freshwater do not drink water, it is unlikely that pyrene is absorbed via the gastrointestinal tract of trout during the head-only exposure study. Supporting this assertion are data from Kennedy and Law (1990) who have reported that little or no pyrene is absorbed into the blood of trout following the intragastric route of administration.

Fish gills are comprised of thin, lipid bilayer membranes (2-4 μm) which permits rapid and ready diffusion of lipophilic organic molecules. In contrast, fish skin consists of dense, nonliving layers of tissue which is covered by mucus. The countercurrent blood flow pattern in the gills of fish also helps to remove a chemical from the site of diffusion and maintains a large concentration gradient across the membrane, resulting in more efficient absorption. The gills have a large surface area (typically 2-10 times more than the body surface area in fish) which also maximizes chemical absorption (Rand and Petrocelli, 1985). Pyrene is a lipophilic chemical with a logarithm of octanol-water partition coefficient ($\log K_{ow}$) of 4.9. This

also enhances the uptake of pyrene by the fish gills. Faster and more extensive pyrene uptake by the gills of trout compare to that of skin is consistent with the gill structure, its anatomical arrangement, and the K_{OW} of pyrene. Kennedy and Law (1990) have demonstrated that pyrene is found in the blood of trout almost immediately after exposing fish to water-borne pyrene. McKim and Heath (1983) have shown that about 76% of a polychlorinated biphenyl (PCB) congener in inspired water is absorbed, in one pass, by the gills of two species of trout.

As discussed above, the present studies show clear evidence of pyrene absorption by the skin of trout. The maximal pyrene concentration of 0.08 $\mu\text{g/ml}$ and 0.37 $\mu\text{g/ml}$ was detected in the blood of trout following body-only exposure to 8 mg/l and 24 mg/l pyrene, respectively. Saarikoski *et al.*, (1986) showed that the skin might be an important route of chemical uptake in fish. Balk *et al.* (1984) suggested that benzo(a)pyrene could be absorbed directly by the skin of English Sole from water. In the present study, the apparent bioavailability of pyrene in trout following dermal (body-only) exposure was estimated to be 1.02%. This is consistent with finding of McKim and Nichols, (1991) who demonstrated that 2-5% of the total dose of chloroethanes absorbed by rainbow trout was due to dermal absorption. Lien and McKim (1993) have suggested that fish

size may be a limiting factor in the absorption of chemicals by the skin. A comparison of the log K_{OW} of several chemicals by Ng et al., (1992) indicated that a log K_{OW} of around 5 was the maximum limit for a significant transfer of chemical from the skin into the blood by a passive diffusion process. Since the log K_{OW} of pyrene (4.9) is very close to the maximal limit of 5 suggested by Ng et al. (1992) it may enhance its absorption by the skin.

The results of present studies also show that a chambered trout absorbs more pyrene than a free-swimming trout since the $AUC_{0 \rightarrow \infty}$ of a chambered fish (132.81 $\mu\text{g/hr/ml}$) is about three times higher than that of a free-swimming trout (51.13 $\mu\text{g/hr/ml}$). This is probably due to the level of stress to the fish and the K_{OW} of pyrene. Since a chambered trout was restrained inside a plexiglass box and pyrene-contaminated water "forced" toward its head, it experienced more stress than the free-swimming fish. Previous studies showed that stress can increase gill ventilation resulting in a higher chemical uptake (Hoar and Randall, 1978). For a highly lipophilic chemical such as pyrene (log K_{OW} = 4.9), the capacity of blood to transport chemical is high enough so that delivery of chemical by water to the gill surface (ventilation volume) and not blood flow limits chemical uptake. Erickson and Mckim (1990a) have suggested that chemical delivery to the gills by water is a limiting

factor in the uptake of chemicals with a log K_{OW} of more than 3. Similarly, Hayton and Barron (1990) have predicted that uptake clearance (the volume of water totally cleared of chemical per unit time) is dependent on the water flow of a compound with high log K_{OW} . Schmieder and Weber (1992) also have shown that the uptake of decanol (log K_{OW} of 4.51 which is very close to the log K_{OW} for pyrene) by trout gills is water flow limited.

Tissue distribution results are consistent with the blood flows and lipid contents of different trout tissues. Thus, the highest pyrene concentration is found in the liver, a highly vascularized, well perfused organ which also contains a high level of fat. In contrast, the muscles are low in lipid content and blood flow; they accumulate only a low level of pyrene. Previous studies have shown that among the various fish tissues, the liver of fish contains the highest level of PAHs (Kennedy *et al.*, 1989; Melcanon and Lech, 1978; Roubal *et al.*, 1977). However, pyrene concentration in the liver declines faster than that of the muscle. This is probably due to the high mixed-function oxidase activities of the liver (Law, 1981).

Toxicokinetic studies in fish allows for a better understanding of species differences in the disposition and

metabolism of xenobiotics. This will facilitate the evaluation of aquatic animals as surrogates of human health as well as ecological risk assessment of chemicals. Results of present experiments also show that fish can be used for PAHs monitoring in polluted aquatic environments, since fish can rapidly absorb, accumulate and slowly eliminate these chemicals.

PART II: Pharmacokinetics of OTC in Salmon

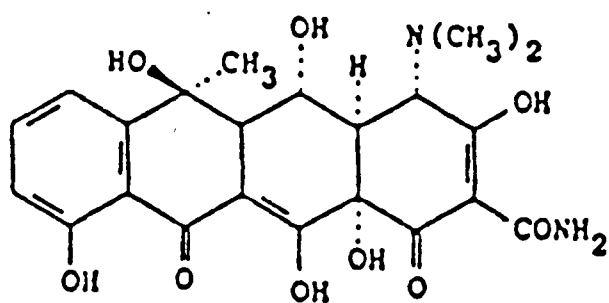
INTRODUCTION

As a result of widespread use of drugs in modern aquaculture to treat and prevent fish diseases, concern has arisen over the presence of drug residues in aquatic species reared for human consumption.

Oxytetracycline (OTC) is a commonly used antibiotic on freshwater and marine fish farms (Grondel *et al.*, 1987). Figure 2.1 shows the chemical structure of OTC. OTC is a bacteriostatic compound with a broad antibacterial activity against both aerobic and anaerobic, Gram-positive and Gram-negative species (Neu, 1978). It is recommended for the treatment and prevention of several fish diseases including bacterial kidney disease (BKD), vibriosis, furunculosis, edwardsiellosis and enteric red mouth disease. OTC is usually mixed in feed and administered to fish orally at a rate of 50-100 mg OTC/kg fish per day for 3 - 14 days depending upon the infection.

A number of reports have been published on the health and environmental impacts of OTC. OTC has an adverse effect on both fetuses and infants; it may cause major and minor malformations (Heinonen *et al.*, 1977). Musculo-skeletal

Figure 2.1. The chemical structure of oxytetracycline.



abnormalities were found among infants receiving OTC. A yellow, grayish-brown or brown discoloration of the teeth has frequently been observed in children who received OTC preparations during mineralization of deciduous or permanent teeth. Liver injury including hepatic dysfunction was reported in association with use of OTC in human (Lepper *et al.*, 1951; Schultz *et al.*, 1963; Combes *et al.*, 1984; Weinstein, 1970). Nephro-toxicity was also observed in patients receiving OTC.

Tetracyclines are also known to induce resistance to antibiotics in the aquatic microflora and in bacteria which are pathogenic to fish (Toranzo *et al.*, 1984; Austin, 1985; Schlotfeldt *et al.*, 1985; Jones *et al.*, 1986). Jacobsen and Berglind (1988) found that OTC persisted in fish farm sediments. The drug was found in concentrations capable of causing antimicrobial effects up to 12 weeks after administration. OTC-resistant bacteria have been isolated from wild fish (Björklund *et al.*, 1990). The resistance factors can be transferred to potential human pathogens like *Escherichia coli* (Toranzo *et al.*, 1984). It is also possible that humans who consume OTC-treated fish have a greater chance of developing antibiotic resistant strains of bacteria.

Many previous studies have examined the residues and withdrawal time of OTC in fish (Silven *et al.*, 1968; Herman *et al.*, 1969; Liungberg *et al.*, 1969; McCracken *et al.*, 1976; Salte, 1982; Salte & Liestol, 1983; Keck *et al.*, 1984; Jacobsen, 1989). Withdrawal time is defined as a time period required after the cessation of treatment for OTC levels to drop to a "acceptable" level for human consumption. This level in Canada is 0.05 μg OTC/g fish muscle. It is difficult, if not impossible, to determine a withdrawal time from previous studies since they have been carried out with different dosage regimens, treatment durations, fish sizes, fish species and water temperatures. Although these factors can significantly affect the pharmacokinetics of OTC in treated fish, current legislation on the withdrawal times of OTC-treated fish does not take all of these into consideration. Therefore, the withdrawal periods of OTC-treated fish vary from one jurisdiction to another. For example, the withdrawal periods of OTC at water temperature of 9°C and above is 40 days in Norway and Canada, 30 days in Sweden, 21 days in the United States and no specific time point in Denmark since no detectable drugs should be present in fish destined for consumption according to Danish general practice. When the water temperature is below 9°C, the withdrawal period is 80 days in Norway and Canada, 60 days in Sweden and in the United States OTC is not allowed to be used for fish farming. To ensure that levels of antibiotics

in fish tissues are within acceptable limits, monitoring of drug residues is an essential aspect of public health. It is also important to establish a specific, rapid and sensitive method of predicting OTC residues in farmed fish under different treatment and environmental conditions.

The objectives of the present study were to investigate the absorption, distribution and elimination of OTC in farm chinook salmon (*Oncorhynchus tshawytscha*) at different water temperatures, to test a physiologically-based pharmacokinetic model (PBPM) of OTC in fish by comparing model-predicted OTC tissue concentrations with data from the farm and to examine the disposition kinetics of OTC in coho salmon (*Oncorhynchus kisutch*) treated in the laboratory.

MATERIALS AND METHODS

I) Fish

A field study was carried out with chinook salmon (*Oncorhynchus tshawytscha*) at two different fish farms (A and B) in British Columbia. Fish were fed by hand twice daily with pelleted dry feed at 2% body weight. Pellets were purchased from Moore Clark Co. Inc. (Vancouver, Canada).

The laboratory study was performed at the Simon Fraser University seawater facilities. Coho salmon (*Oncorhynchus kisutch*) weighing 15 - 20 g were obtained from Rosewall Creek Hatchery (Canada Department of Fisheries and Oceans). Fish were kept in a large glass aquarium (100 l) and 75% of the water was changed daily. Coho were fed by hand twice daily with small pellets (3.5 mm) at 2% body weight. The feed used was 3/32 semi-moist Biodiet (Bioproducts).

II) Chemicals

OTC and tetracycline were purchased from Sigma Chemical Co. (St. Louis MO). Solvents were of analytical or HPLC grade. All other chemicals were of analytical grade or better. OTC medicated feed pellets were obtained from Moore Clark Co. Inc. (Vancouver, Canada).

III) Absorption and depletion Studies

i) Field Studies

a) Farm A Treatment and Sampling

The Farm A study was carried out in two different seapens (Pen # 10 and # 11) containing Chinook salmon weighing 250 - 400 g at different times of the year: chinook salmon in Pen # 10 were treated in October, 1991 for 10 days and fish samples were collected from October to the first week of December. Salmon in Pen # 11 received medicated feed in February, 1992 for 10 days and fish samples were removed from February to the end of March. Water temperatures on the surface and at a depth of 5 and 10 meters were monitored throughout the experiment.

Medicated feed containing 1% OTC (w/w) was given to the salmon at the rate of 100 mg OTC/kg fish/day for 10 days. This was followed by daily feeding with non-medicated feed. At specific time points during and after OTC medication, 6 salmon were randomly selected from the seapens. Pen # 10 sampling time points were: 0, 2, 6, 9, 15, 17, 22, 28, 35 and 42 days (day 0 was the day prior to medication). Pen #11 sampling time points were: 0, 2, 4, 6, 9, 13, 15, 17, 22, 28, 36 and 43 days. The fish were sacrificed by a blow to

the head, labeled and transferred on dry ice to Simon Fraser University. The fish were kept frozen at -20°C until analysis.

b) Farm B Treatment and Sampling

The Farm B study was carried out concurrently for 70 days in two separate seapens (Pen # 108 and Pen # 104) with Chinook salmon weighing approximately 350 g. Water temperatures of the seapens were periodically monitored and recorded at a depth of three meters.

Salmon in Pen # 108 were treated with medicated feed containing 1% OTC (w/w) at a rate of 100 mg OTC/kg fish/day for 14 days (treatment I). At the conclusion of the 14-day treatment period, the fish were treated with non-medicated feed. Salmon in Pen # 104 received medicated feed in the same manner as Pen # 108 during the initial 2 days. The fish then received OTC medicated feed at the rate of 50 mg/kg fish/day for the remaining 55 days, (treatment II). At specific time points: 0, 14, 28, 70 (day 0 was the day before medication) 3 fish were randomly collected from the seapens. They were sacrificed by a blow to the head, labeled, frozen, transferred to Simon Fraser University, and stored at -20°C until analysis.

ii) Laboratory Study

Coho salmon weighing 15 - 20 g were treated with medicated feed at 100 mg OTC/kg fish per day for six weeks. This was followed by daily feeding with non-medicated feed for an additional three weeks. Five randomly selected fish were collected each week during and after medication. Control fish were sampled before the initiation of medication. The fish were sacrificed, labeled, weighed and stored at -20°C until analysis. Water temperature in the tank was $10\pm 1^{\circ}\text{C}$ throughout the experiment.

IV) Extraction of OTC from Fish Tissues and Medicated Feed

The fish were thawed at room temperature and weighed individually before dissection. Major organs and tissues were removed from the fish. The organs were blotted, weighed and homogenized separately. OTC extracted by a modified procedure of Björklund (1988). All the reagents and tissues were kept on ice.

i) Reagent Preparation**a) McIlvaine Buffer**

McIlvaine buffer was prepared as follows: Citric acid monohydrate (21.01 g) was dissolved in 1 l of distilled water in a 2 l flask. Dibasic sodium phosphate (17.76 g) was dissolved in 625 ml of distilled water. The solutions were mixed and the pH adjusted to 4.0 ± 0.1 .

b) McIlvaine Buffer/EDTA Buffer

McIlvaine buffer/0.1 M disodium ethylenediamine tetraacetate (EDTA) was prepared by dissolving the appropriate amount of disodium EDTA (60.49 g) in 1.625 l of McIlvaine buffer.

ii) Extraction of OTC from Soft Tissues

After placing 0.5 - 1 g of tissue into a 20 ml disposable test tube, five ml of McIlvaine buffer/EDTA solution containing $4 \mu\text{g}$ tetracycline ($2 \mu\text{g}/\text{ml}$) as an internal standard was added to the test tube. The tissue was homogenized with a Polytron homogenizer (Brinkman Co., Rexdale, Ont.) for 30 sec. The Polytron probe was rinsed twice with 2.5 ml plain buffer (containing no internal standard). The rinses were combined and added to the homogenate which was subsequently transferred to a 15 ml centrifuge tube. The tube was capped before being shaken on a mechanical shaker for 10 min.

The homogenate was centrifuged at 2500 g for 10 min. The supernatant was transferred to a 50 ml centrifuge tube. The tissue pellet was resuspended with 10 ml McIlvaine buffer/EDTA solution and mixed by vortexing. The supernatant was separated by centrifugation, removed and added to the first extraction. The procedure was repeated by 5 ml of fresh buffer.

The combined supernatant was filtered with a Whatman filter paper (GF/B) fitted into a 50 ml conical flask. The centrifuge tube was rinsed twice with 2 ml buffer which was also filtered. A set of Bond elute C-18 columns (Varian, Harbor City, CA) with 15 ml reservoirs was set up with the Baker-10 SPE system (J.T.Baker Inc., Phillipsburg, N.J.) connected to a vacuum source. The bond elute C-18 columns were pre-washed with 10 ml methanol and 10 ml distilled water before the tissue extract was allowed to pass through the column. The conical flask was rinsed with water (15 ml) which was also extracted by the Bond elute C-18 column. The column was washed with water and allowed to run dry for at least 2 min.

OTC was eluted from the column with 2 ml methanol. An aliquot of the methanolic eluate was analyzed for OTC by HPLC.

iii) Extraction of OTC from Bone

HCl (1 N) was added to 0.5 g fish bone (1:1/w:v) and incubated with pepsin (200 mg/ml) for 2 hr at 37°C. McIlvaine buffer/EDTA solution (5 ml) containing tetracycline as an internal standard was added. The solution was adjusted to pH 4 by adding 7 drops of saturated NaOH. The solution was extracted by Bond elute C-18 column as described above.

iv) Extraction of OTC from Medicated Feed

OTC medicated feed (0.5 g) was weighed into a 250 ml volumetric flask. After the addition of 10 ml 0.1 N HCl, the medicated feed was dissolved with ultrasonication. The mixture was diluted to 250 ml with McIlvaine buffer/EDTA. Extraction was carried out with Bond elute C-18 column as described above. OTC was eluted from the column with 2 ml methanol.

V) Analysis of OTC

An aliquot of the methanol extract was analyzed on a Hewlett Packard Liquid Chromatograph (Model 1050, Vandal, PA) equipped with an ODS-Hipers column (Phenomenex, 5 μ m, 100 X 4.6 mm i.d.) and a diode-array UV detector set at 356

nm. The mobile phase was prepared by dissolving 4 g of diamoniumhydrophosphate, 5 ml diethanolamine and 60 ml dimethylformamide in a solution of distilled water and acetonitrile (80%:20%). The pH of the mobile phase was adjusted to 2.5 by orthophosphoric acid. The mobile phase was filtered and degassed with Supelco HPLC solvent filtration system. OTC and tetracycline were eluted from the HPLC column isocratically at a flow rate of 1 ml/min (Nordlander *et al.*, 1987).

VII) Validation of OTC PBPM with Fish Farm Data

Tissue OTC concentration data obtained from the field studies were used to test a PBPM of OTC developed previously in our laboratory (Law, 1992). The model had predicted successfully OTC tissue concentrations in fish treated under laboratory conditions (Law, 1992). The present study was performed to investigate whether the model could also be used to describe OTC tissue concentrations in salmon treated in farms. Computer modelling of PBPM were carried out by Dr. Francis Law.

VIII) Classical Pharmacokinetic Analysis

OTC depletion data were analysed by plotting the natural logarithm of drug concentrations against time. Least

squares linear regression analysis was used to determine the slopes of the regression lines. The apparent terminal elimination rate constant was obtained from the slope of the linear terminal part of the depletion curve. The last few data points of the post-treatment period were used to calculate the slope. The terminal elimination half-life of OTC in different tissues of salmon was calculated from $t_{1/2} = 0.693/\beta$, where β is terminal elimination rate constant.

Student's t test was used for statistical comparisons. The level of significance was chosen at 0.05.

RESULTS

I) Chromatographic Analysis of OTC

Extraction recoveries of liver, kidney, muscle, skin, bone and non-medicated feed pellets spiked with a known amount of OTC were 82, 71, 79, 78, 72 and 69%, respectively. HPLC analysis showed that medicated feed contained $0.96 \pm 0.29\%$ OTC (w/w). The detection limit of OTC for tissues ranged from 0.05 to 0.1 $\mu\text{g/g}$. Figure 2.2 shows a typical HPLC elution profile of OTC. The retention time of OTC and tetracycline (the internal standard) were 6.4 and 7.8 min, respectively.

II) Absorption and depletion of OTC

i) Field Studies

a) Farm A

Figure 2.3 shows a record of water temperature in Farm A. The average water temperature from mid-October to the end of November was $12 \pm 0.5^\circ\text{C}$ (Pen # 10). This will be referred to as the 12°C water temperature group. The average water temperature from February to the end of March (pen # 11) was $9.1 \pm 0.5^\circ\text{C}$. This will be referred as the 9°C water

Figure 2.2. A typical HPLC elution profile of OTC and tetracycline (internal standard). Peaks I and II indicate OTC and tetracycline, respectively.

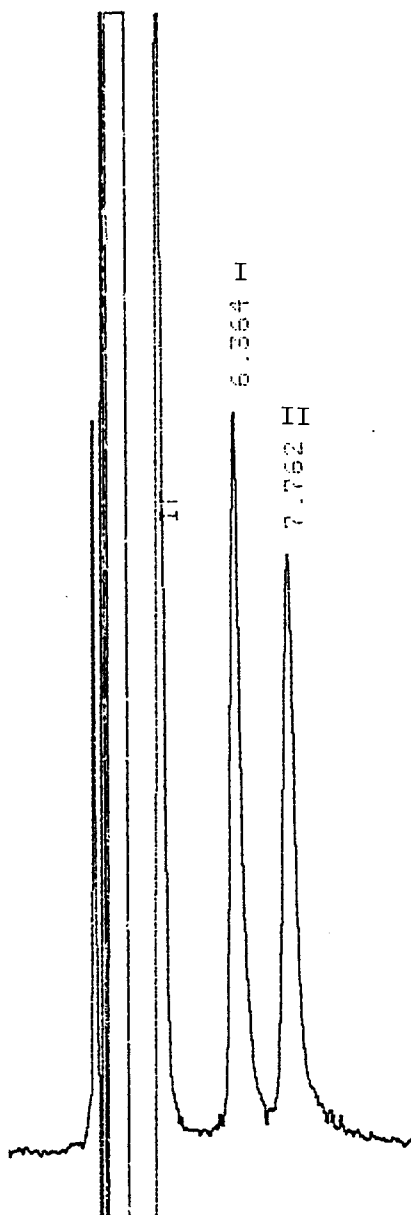
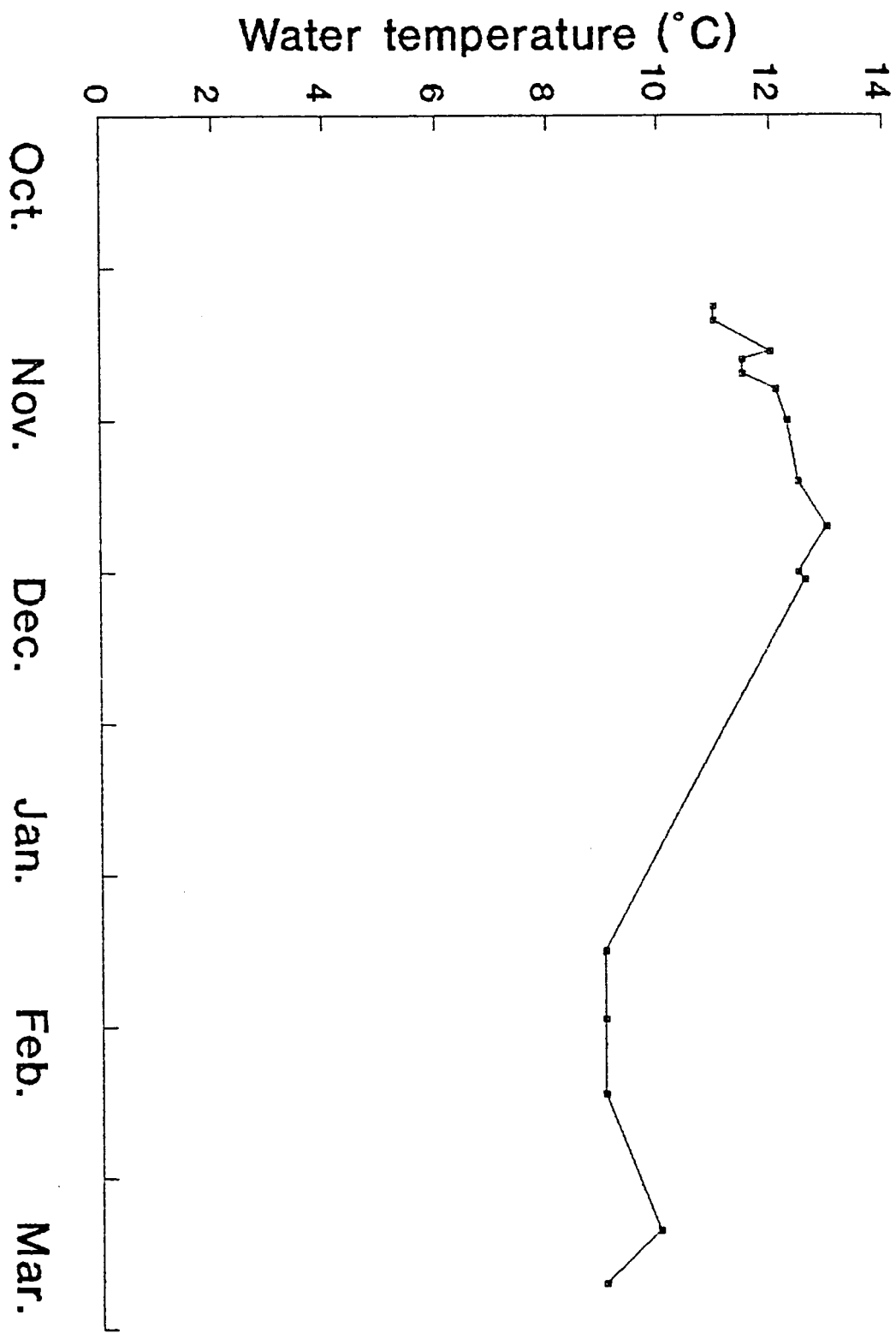


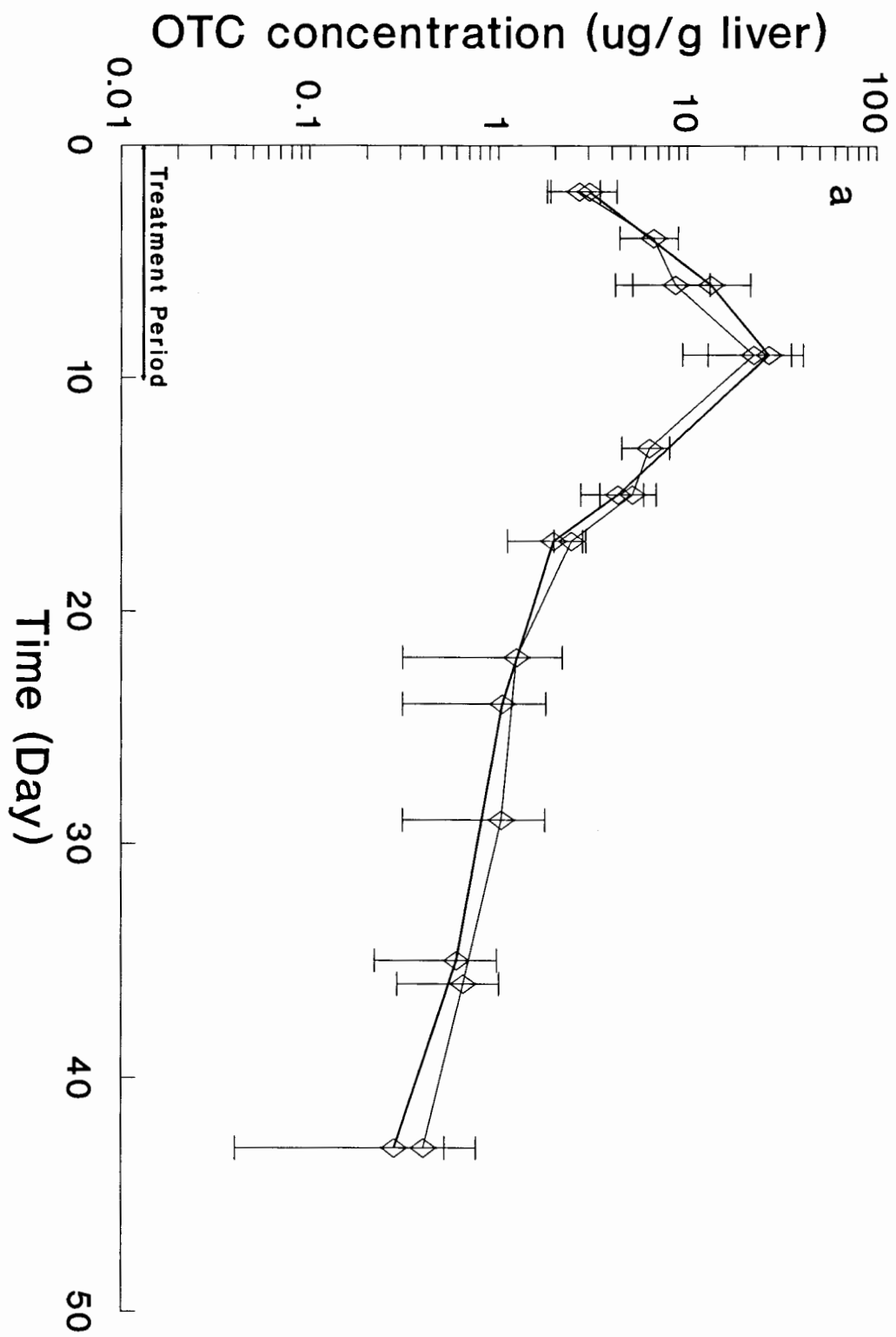
Figure 2.3. Water temperature profile of Farm A from October, 1991 to March, 1992. Each point represents the mean of water temperature at the surface, depth of 5 and 10 m.

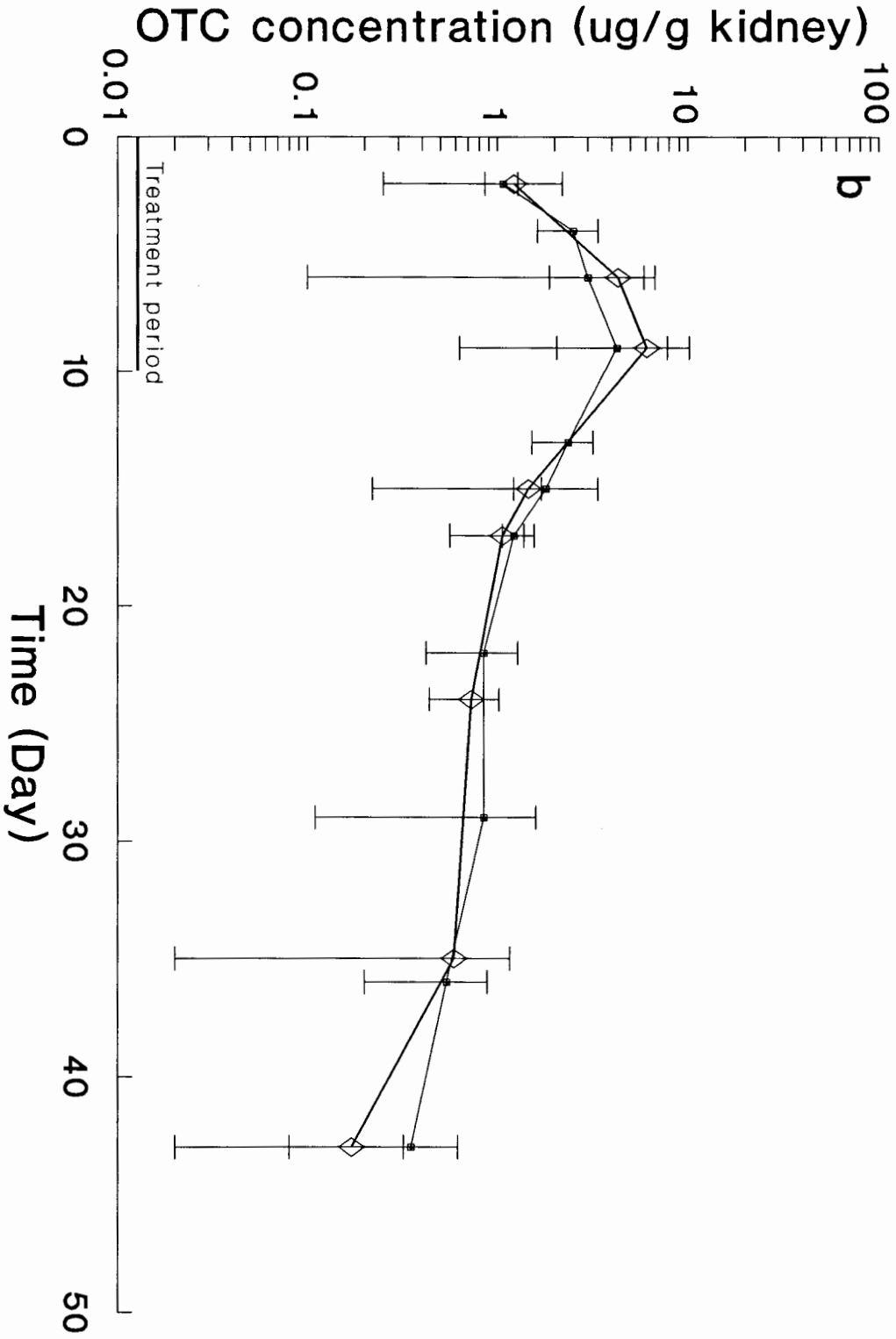


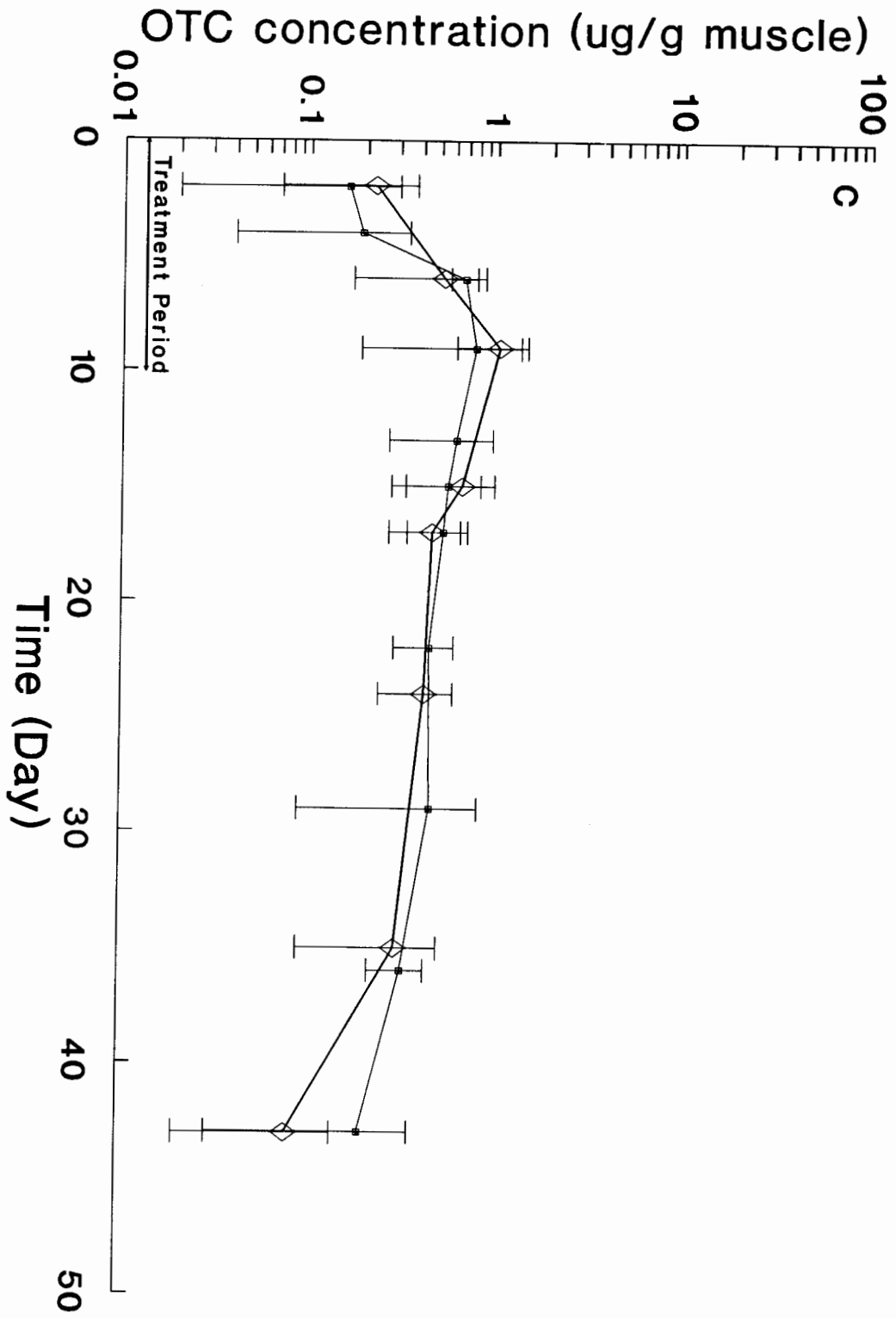
temperature group. The average fish weight for 12°C and 9°C water temperature groups were 262±63 g and 404±119 g, respectively.

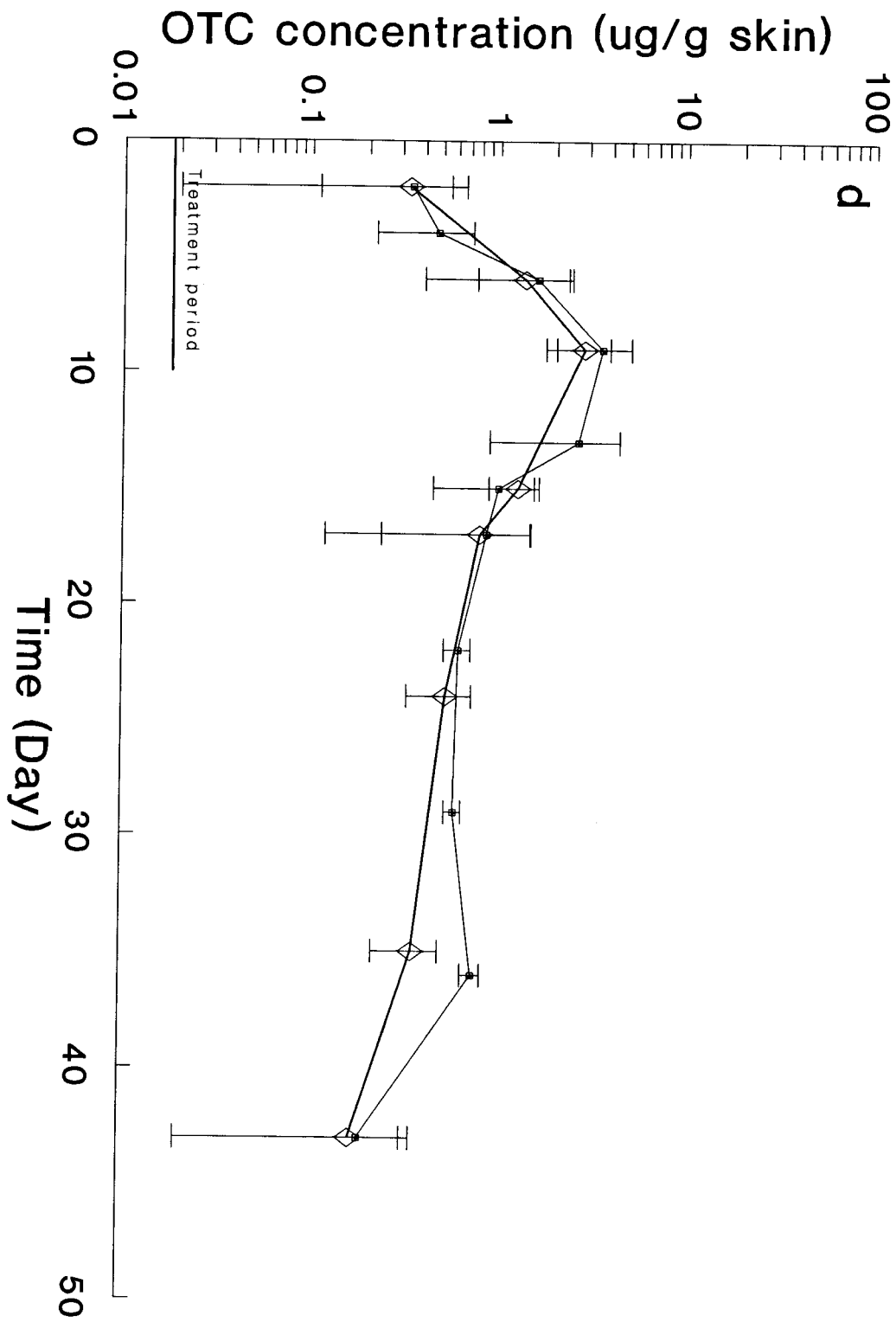
HPLC analysis showed that OTC was not detectable in the tissues of control fish. Figures 2.4 show the absorption and elimination of OTC in the tissues of Farm A salmon at different water temperatures. There was considerable variation in the residue levels of each time point. However, peak tissue concentrations of OTC were reached at the cessation of treatment for both water temperatures. An exception was the bone which peaked at 5 days post-dosing. In general, maximal OTC tissue concentrations were higher for the 12°C fish than the 9°C fish with the exception of the skin and bone. However, the maximal OTC tissue concentration of the two water temperatures were not significantly different ($P>0.05$). The highest OTC concentration was found in the liver whereas the lowest OTC concentration was in the muscle. Within two days after the initiation of the experiment, OTC concentration in the liver was 16 to 17 times higher than those in the muscle. At 33 days post-dosing, liver OTC concentration had declined significantly and reached the levels close to those found in the muscle. The following order of OTC tissue concentrations were observed in salmon one day before (day 9) and 33 days

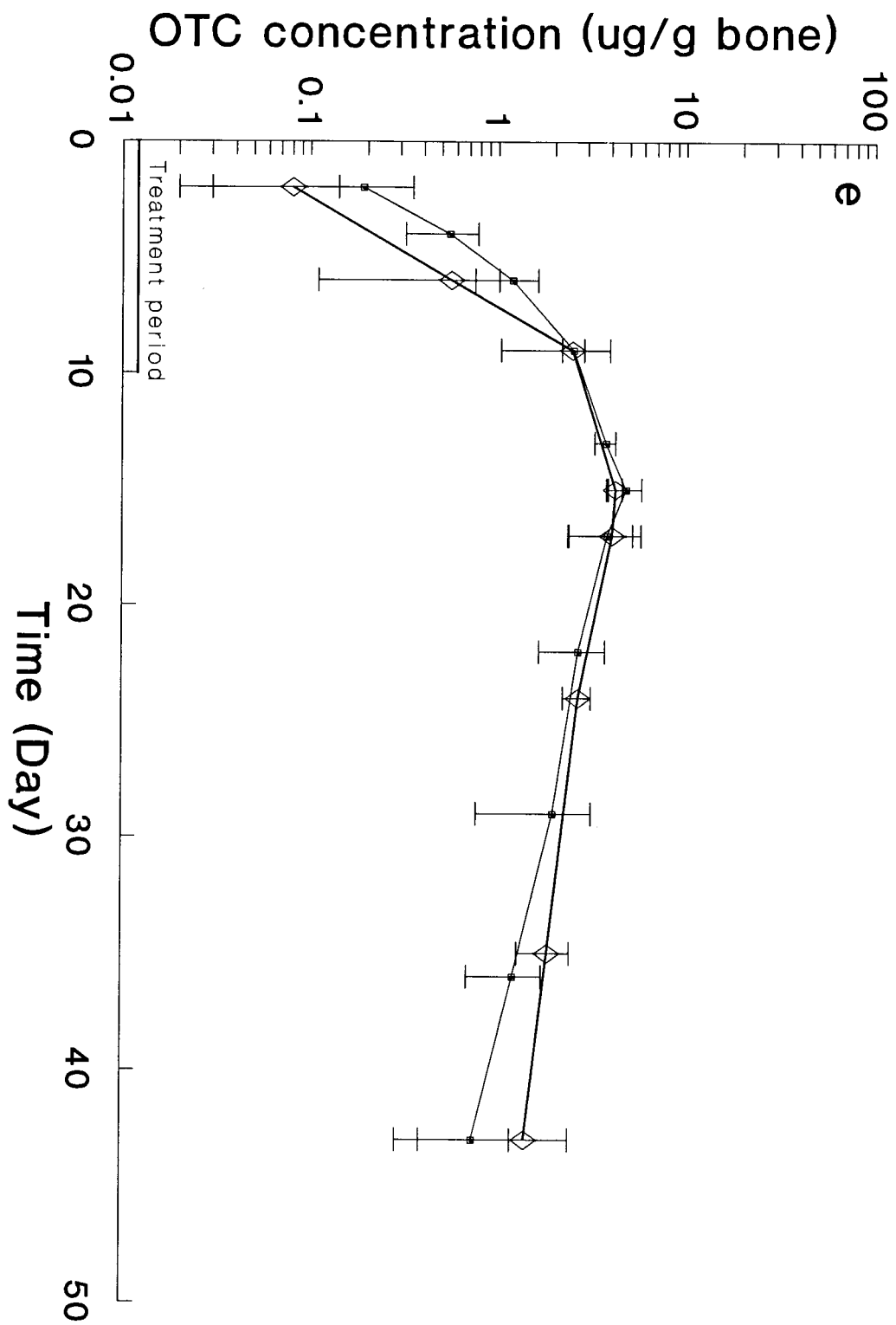
Figure 2.4. Time course of OTC concentration in tissues of salmon maintained at different water temperatures in Farm A. Each point represents the mean \pm SD of six fish. The thin line represents the data from 9°C water temperature and the thicker line for 12°C. a, liver; b, kidney; c, muscle; d, skin; e, bone.











after (day 43) the cessation of medication:

(Day 9) Liver > Kidney > Skin > Bone > Muscle

(Day 43) Bone > Liver > Kidney > Skin > Muscle

Table 2.1 shows the estimated terminal elimination rate constant (β) and corresponding half-life ($t_{1/2}$) of OTC in salmon tissues. The β value of each tissue at 12°C water temperature was not significantly different ($P>0.05$) from that of 9°C. Neither were the β values significantly different among the different tissues at a particular water temperature.

Figure 2.5 shows the empirical and PBPM-predicted tissue concentrations of OTC in salmon treated at 9°C water temperature. Model predicted results closely simulated most of the experimental data. Experimental data are shown in the Appendix (Tables A.3).

b) Farm B

Figure 2.6 shows the water temperature profile of Farm B. The average water temperature from October to January was $11\pm 2^\circ\text{C}$. The average fish weight was 325 ± 50 g. OTC was not

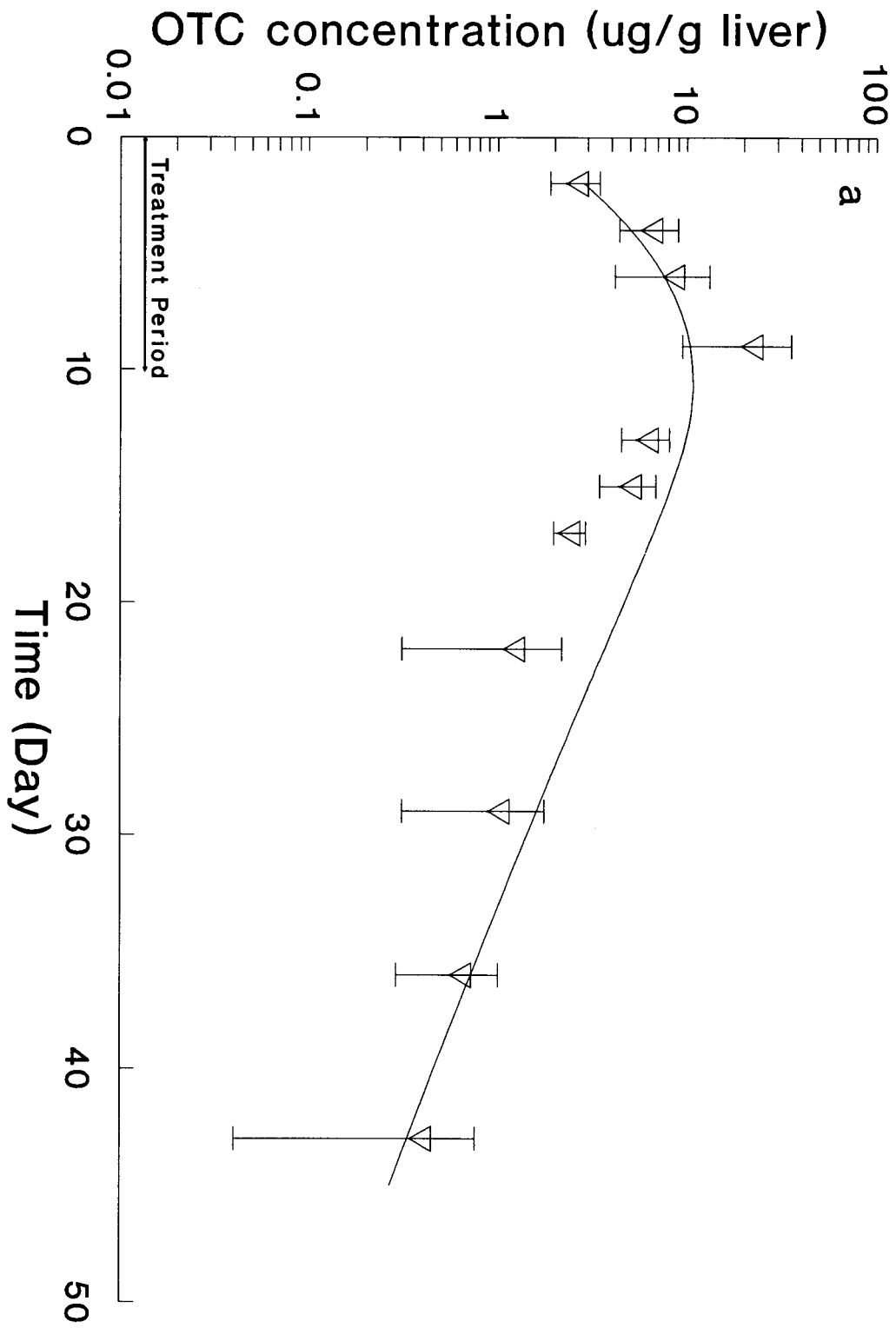
Table 2.1 Terminal elimination rate constant (day^{-1}) of OTC in tissues chinook salmon^a.

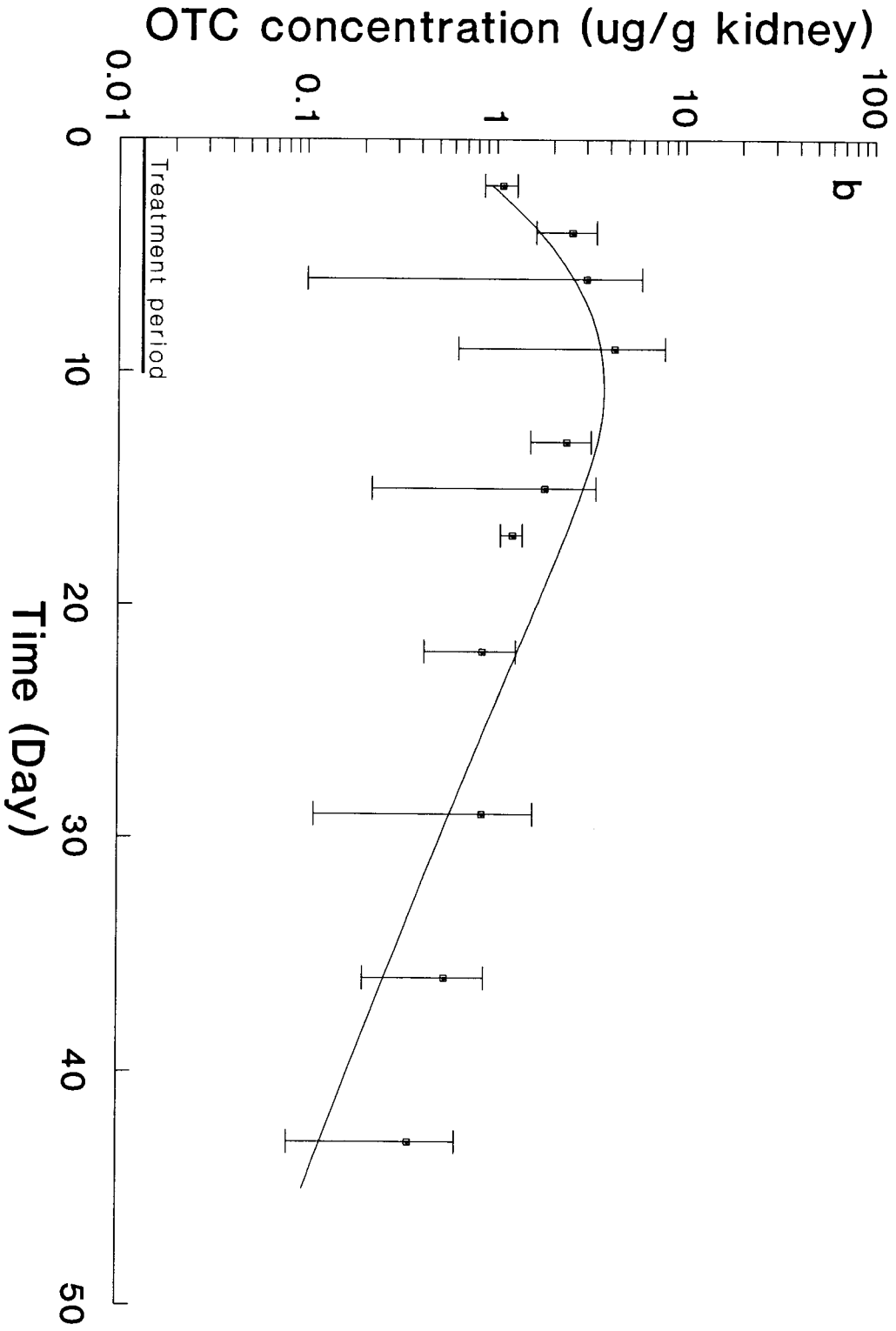
Tissue	Water Temperature	
	12°C	9°C
Liver	0.085 (8.15) ^b	0.088 (7.88)
Kidney	0.064 (10.83)	0.057 (12.16)
Muscle	0.067 (10.34)	0.054 (12.83)
Skin	0.063 (11.18)	0.059 (11.75)
Bone	0.046 (15.07)	0.057 (12.16)

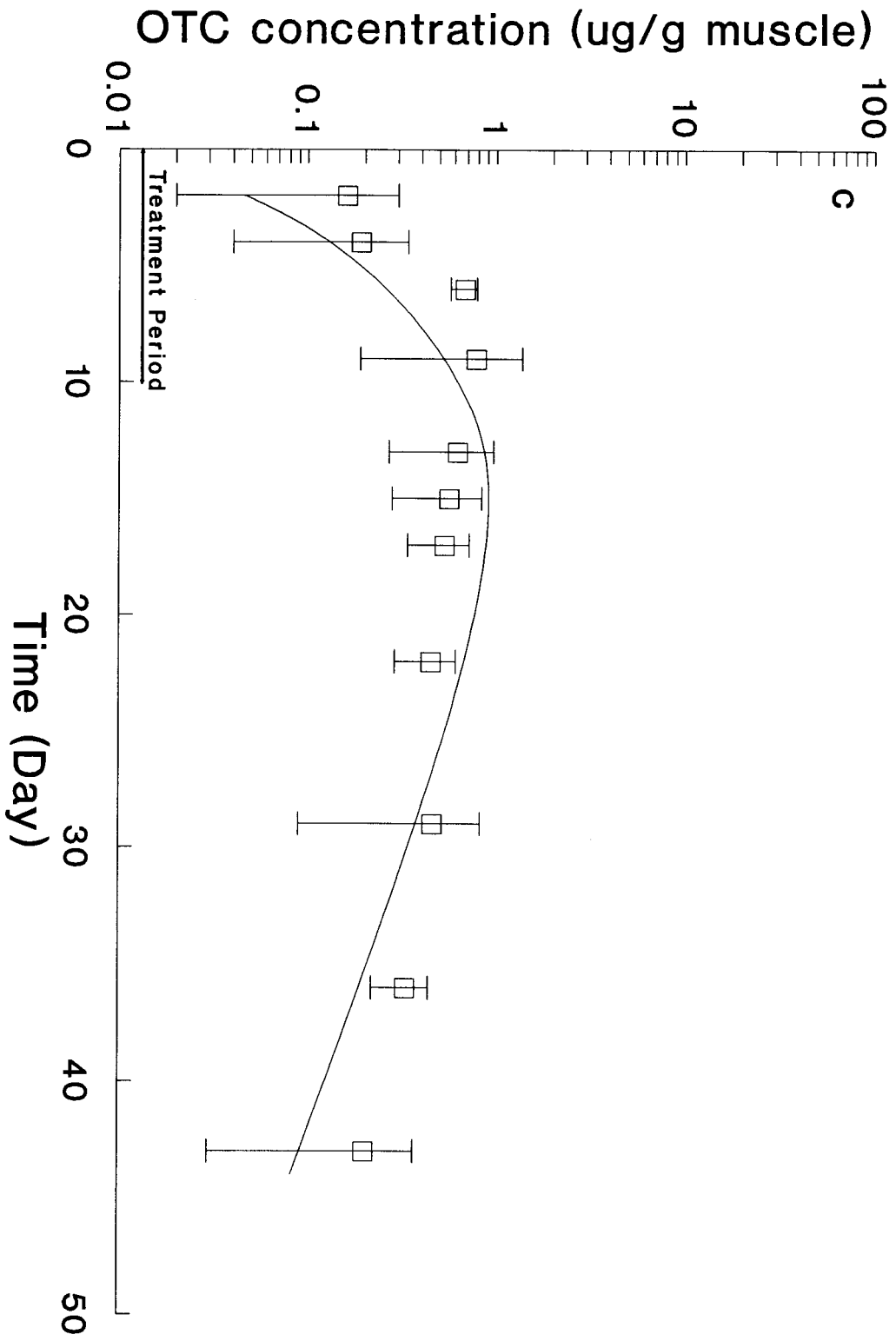
a) Salmon received OTC-medicated feed at the rate of 100 mg OTC/kg fish/day for 10 days in Farm A.

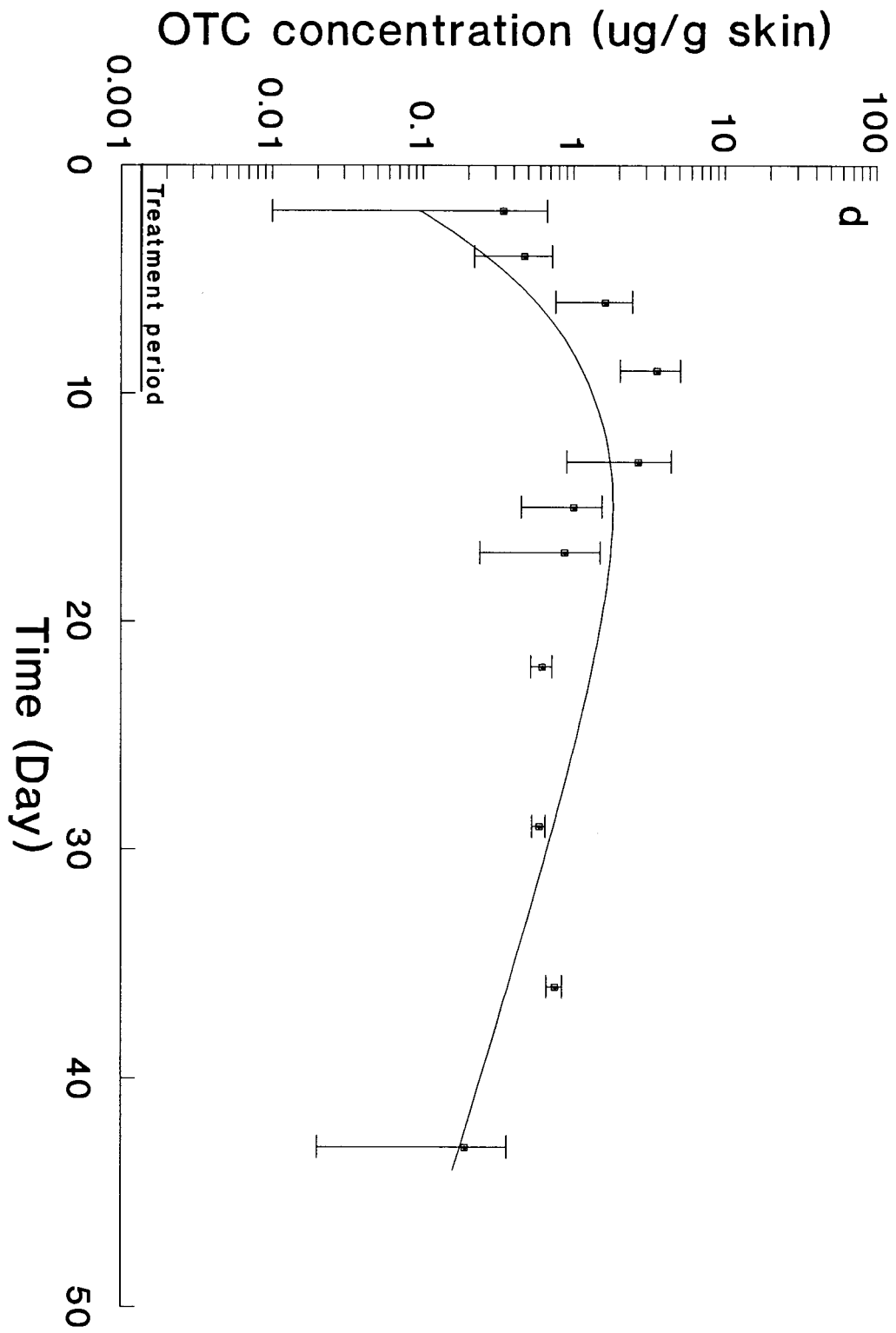
b) Corresponding half-life of the terminal elimination rate constant.

Figure 2.5. Measured vs. predicted concentrations of OTC in salmon tissues from Farm A during and after multiple dosing at 9°C water temperature. Each point represents the mean \pm sd of six fish. Solid curve represents simulation using the model. a, liver; b, kidney; c, muscle; d, skin; e, bone.









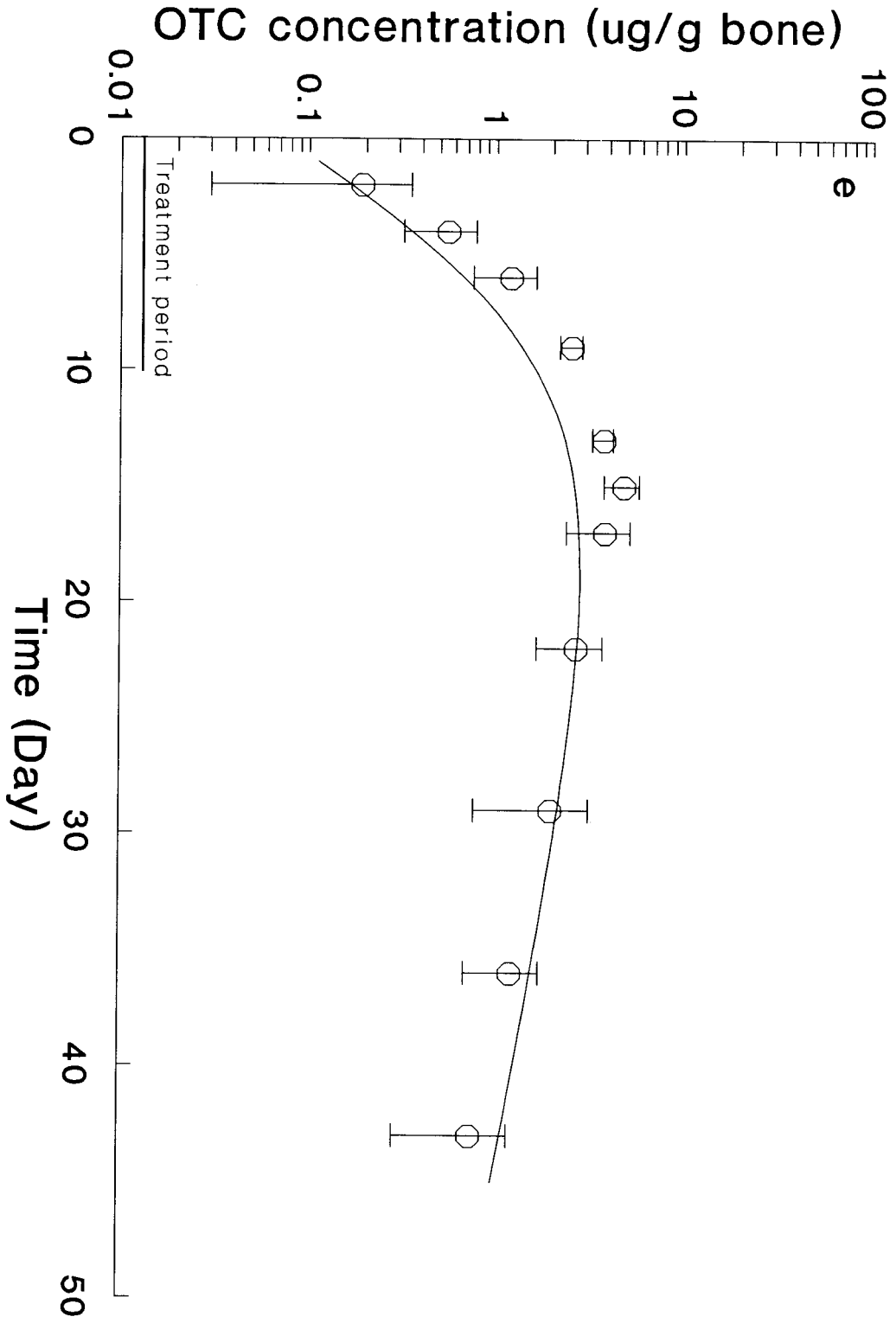


Figure 2.6. Water temperature profile of Farm B at 3 m from the surface from October, 1992 to January, 1993.

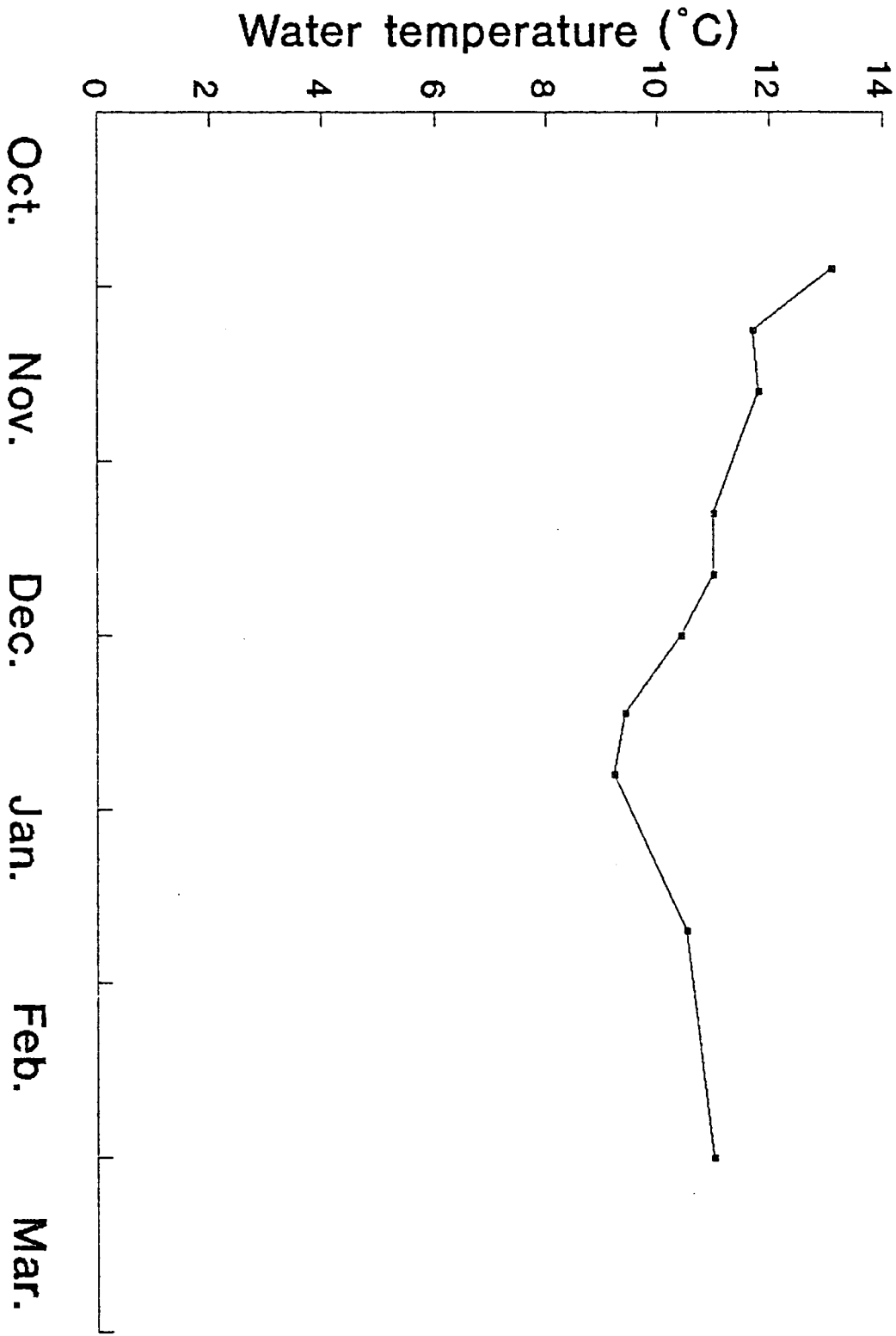


Table 2.2 Concentration of OTC ($\mu\text{g/g}$) in the muscle of chinook salmon treated in Farm B.

Time (day)	Pen #	
	108 ^a	104 ^b
14	1.44 \pm 0.87 (1.45) ^c	1.21 \pm 0.63 (0.83)
28	0.88 \pm 0.23 (0.83)	1.84 \pm 1.08 (1.26)
70	ND (0.01)	0.65 \pm 0.16 (0.81)

- a) Salmon in Pen # 108 treated with OTC medicated feed at the rate of 100 mg OTC/kg fish per day for 14 days (treatment I).
- b) Salmon in Pen # 104 treated with OTC-medicated feed at the rate of 100 mg OTC/kg fish per day for 2 days and then received 50 mg OTC/kg fish per day for additional 55 days (treatment II).
- c) PBPM model-predicted result

detected in the muscle of control fish. Table 2.2 shows the experimental and PBPM-predicted OTC muscle concentrations of salmon treated in Farm B. A high OTC concentration ($0.65 \pm 0.16 \mu\text{g/g}$) was found in the muscle of salmon at the conclusion of the experiment (day 70) in Pen # 104. OTC was not detected in the muscle of salmon 56 days post-dosing in Pen # 108. Data from Table 2.2 also shows model-predicted results closely simulate the corresponding experimental values.

ii) Laboratory Study

The fish weight was significantly increased ($P > 0.05$) from the first week of treatment ($14.9 \pm 1.56 \text{ g}$) to the last week of sampling ($29.7 \pm 6.65 \text{ g}$). The average fish weight during the experiment was $21.8 \pm 6.9 \text{ g}$. OTC was not detected in the tissues of control fish. Table A.5 summarizes the results of the OTC tissue distribution in coho salmon. Tissue OTC concentrations peaked at the cessation of medication, except for bone which peaked at 7 days post-dosing. The highest OTC concentrations were found in the gut and the lowest in the muscle. A slight increase was observed in the OTC tissue concentrations from day 21 to day 28 and from day 21 to day 42. However, the increase were not statistically significant ($P > 0.05$). Therefore, an apparent steady-state concentration of OTC was reached in all tissues

at about 21 days after treatment, except for the bone. The rank order of OTC tissue concentrations at the last day of medication (day 42) and 21 days post-dosing (day 63) are shown below:

(day 42) Liver > Kidney > Skin > Bone > Muscle

(day 63) Bone > Skin > Liver > Kidney > Muscle

Table 2.3 shows the β and corresponding $t_{1/2}$ of OTC in different tissues of coho salmon. The β values for liver, kidney and muscle were significantly higher than that of the bone ($P > 0.05$).

Table 2.3 Terminal elimination rate constant (day^{-1}) of OTC in the tissues of coho salmon^a.

Tissue	Water Temperature	
	10°C	
Liver	0.159	(4.36) ^b
Kidney	0.140	(4.95)
Muscle	0.157	(4.41)
Skin	0.092	(7.53)
Bone	0.044	(15.75)

a) Salmon were kept in laboratory and treated with medicated feed at the rate of 100 mg/kg fish/day for 42 days.

b) Corresponding half-life of the terminal elimination rate constant.

DISCUSSION

Although peak tissue concentrations of OTC in salmon maintained at 9°C and 12°C water temperatures (Tables A.3 and A.4) were not significantly different, an increase of 3°C water temperature produced about 20% - 45% increase in OTC accumulation in the liver, kidney and muscle. These data suggest that an increase in water temperature more than 3°C may significantly increase the OTC tissue concentrations. Previous studies showed that OTC was absorbed at a faster rate by trout maintained at a warmer water temperature (Jacobsen, 1989; Björklund and Bylund, 1990).

The tissue concentration results show that highly vascularized and well-perfused organs such as the liver, kidney and gut had a high level of OTC. In contrast, the slowly perfused tissue like the muscle has a very low OTC level. These data suggest that tissue distribution of OTC is determined by the blood flow to different organs or tissues of fish. It should be pointed out that OTC concentration of a particular tissue varied considerably. This may be due to the variable intake of medicated feed by fish. Previous research has shown that food uptake by fish can be very uneven (Rae, G. H., 1992).

Two different salmon species were used in the present study. The pattern of OTC tissue distribution was very similar in both species. OTC concentrations in the liver, kidney, skin and bone were significantly higher ($P>0.05$) than that of the muscle. For example, the liver OTC concentrations were 3 - 9 fold higher than that of the muscle six days after medication and concentrations in the skin were 2.5 - 4.5 fold higher than that of the muscle (Tables A.3, A.4, A.5). Several other investigators also have reported a higher concentration of OTC, oxolinic acid and trimethoprim in the liver, kidney, skin and gall-bladder of fish treated with these drugs. (Herman *et al.*, 1969; McCarthy *et al.*, 1974; Cartmell *et al.*, 1976; Keck *et al.*, 1984; Fujihara *et al.*, 1984; Kasuga *et al.*, 1984). For example, Nordlander *et al.* (1987) have reported 2 - 3 fold higher concentrations of OTC in the liver compare to the muscle of rainbow trout (*Salmo gairdneri*) six days after drug administration. Jacobsen (1989) showed that OTC concentration in the skin was about 3.5 times higher than that of the muscle in rainbow trout. These results are consistent with those of the present studies.

Several studies have demonstrated the accumulation of OTC or other tetracyclines in the bone of fish (Herman 1969; Grondel *et al.*, 1980; Ingebrigtsen *et al.*, 1985). Results of the present studies confirm these findings. The binding of

tetracyclines to di- and trivalent cations such as Ca^{+2} , Mg^{+2} , Fe^{+2} , Fe^{+3} , Al^{+3} has been well characterized (Albert, 1953; Albert and Rees, 1956; Clive, 1968). The binding is especially pronounced for hydrophilic tetracyclines such as OTC and tetracycline.

Although a 3°C increase in water temperature did not significantly increase the OTC elimination rate from salmon soft tissues (Figure 2.4), water temperature could still affect OTC elimination by fish. For example, although the OTC tissue concentrations of the 12°C group were higher than those of 9°C at the conclusion of medication, they dropped below the levels of 9°C group at 43 days post-dosing. Salte and Liestøl (1983) have suggested that OTC elimination by fish was temperature-dependent; about 10% increase/decrease per 1°C change in water temperature. They have reported a higher OTC terminal elimination rate constant (β) for trout muscle at a higher water temperature (0.069 day⁻¹ at 9.6°C) than that of the lower temperature (0.056 day⁻¹ at 7.5°C). In the present study, the estimated β value of muscle at 12°C water temperature is 0.067 day⁻¹. This is 24% higher than the β value of 9°C water temperature, 0.054 day⁻¹.

It is interesting to note that with the exception of the bone the terminal elimination rate constants of coho salmon tissues were about two to three times higher than

those of the corresponding chinook salmon tissues. This could be due to the difference in fish size, growth, experimental conditions and/or fish species. The mean fish weight in field studies were 12 to 18 times higher than the mean fish weight of the laboratory study. It is possible that small fish have a faster urinary and/or biliary excretion than that of the larger fish. Gobas and McCorquodale (1992) suggested that fish growth results in an increase of the fish body mass and a decrease in drug concentration. Therefore, fish growth could lead to an overestimation of the elimination rate constant in the present study. Sijm *et al.*, (1992) also suggested that growth dilution may increase the overall elimination rate of a chemical. In addition, growth may also change the physiological parameters of fish such as percentage of cardiac output to different organs and tissues, result in altering the disposition kinetics of OTC in these fish. Similarly interspecies physiological differences may change the drug kinetics as well. Laboratory conditions do not exactly mimic the field situation. Since experimental condition such as level of stress may have a profound effect on the pharmacokinetics of fish, the terminal elimination rate constant of salmon kept in farm could be significantly different from those treated in laboratory. Björklund and Bylund (1990) found that the β value of trout muscle in farm treated fish was higher than that of laboratory dosed fish.

Results of the farm fish studies indicate that OTC has a very long elimination half-life in salmon. Probably this is explainable by the absence of OTC metabolism in fish. Björklund and Bylund (1990) stated that OTC metabolites were not found in rainbow trout or salmon exposed to the drug. Since OTC is absorbed by the gastrointestinal tract at a slow rate, this may contribute to the persistence of OTC in fish tissues (Horsberg and Berge, 1986). Enterohepatic circulation and slow release of OTC from the bone depot may also contribute to the persistence of OTC in the fish. Enterohepatic circulation of tetracyclines in fish was reported by Fanelli and Nigrelli, 1963; and Cravedi *et al.*, 1987. In addition, Huber (1986) suggested that the long $t_{1/2}$ of tetracyclines was due to enterohepatic circulation.

Current method of OTC withdrawal time determination is based on classical pharmacokinetic principles. However, estimates of OTC withdrawal periods for different doses, treatment scenarios (duration, frequency) and/or water temperatures cannot be done by this method due to the limited prediction capability of classical pharmacokinetic models (Salte and Liestøl, 1983). In contrast, PBPMs have a greater extrapolation capability since they are developed from physiological, biochemical and physico-chemical parameters. PBPM provides a means of estimating the chemical tissue concentrations over different dose regimens,

treatment scenarios, exposure conditions (such as water temperature), various animal species and different routes of administration (Harvey *et al.*, 1987; Leung, 1991).

Moreover, the currently recommended method is based only on data of OTC residues in muscle. However, due to cultural eating preferences, some individual consume fish liver, skin and head (containing bone and cartilage). Since OTC concentrations in liver, skin and bone are significantly higher and more persistent than that of the muscle these tissues/organs may still have a high OTC concentration even though the residues may be undetectable in the muscle. Therefore, withdrawal time of OTC should be based on the drug residue in whole gutted fish rather than the muscle alone. Jacobsen (1989) also recommended that the residual quantities of OTC in both muscle and skin should be considered for OTC withdrawal time determination. Therefore, knowing the withdrawal period from different tissues of OTC-treated fish would be useful for public safety and regulatory decisions.

The PBPM provides an adequate prediction of OTC concentrations in the muscle of salmon treated with different dosage regimens at different water temperatures. In addition, our data demonstrate that the model can reasonably predict the time course of OTC concentration in

different tissues of farm salmon. These results suggest that PBPM of OTC can be used as a supplement and eventually replacement for current methods of OTC withdrawal time determination in farm fish.

APPENDIX

Table A.1. Concentration of pyrene ($\mu\text{g/ml}$) in the blood of trout^a during and after different routes of exposure in a flow-through system.

Time (hr)	Exposure Route		
	Whole-Fish ^b	Whole-Fish ^c	Head-Only
0.08	4.84±2.29	NS	4.37±2.05
0.17	5.70±2.69	NS	5.34±2.17
0.25	6.19±2.61	NS	6.20±1.77
1.00	6.85±1.91	5.12±1.18	6.71±1.99
2.00	9.83±1.70	7.18±2.11	8.45±2.79
3.00	12.33±2.67	8.34±1.50	11.59±4.12
4.00	14.25±4.11	9.09±1.52	12.10±3.79
4.08	10.68±2.60	8.08±1.07	10.34±3.23
4.17	9.67±1.94	7.02±1.01	8.09±3.59
4.25	8.60±1.17	5.46±1.07	7.22±2.89
4.50	7.66±0.86	4.41±1.00	6.37±2.09
4.75	6.66±1.21	NS	5.00±1.40
5.00	6.20±1.10	3.91±0.79	4.52±1.34
5.50	NS	3.45±0.83	NS
6.00	4.64±0.66	2.63±0.69	3.45±0.97
7.00	NS	2.21±0.66	NS
8.00	NS	1.67±0.32	1.98±0.82
10.0	NS	0.93±0.49	NS
14.0	NS	0.59±0.36	NS
22.0	1.91±0.99	0.30±0.10	0.70±0.24
28.0	0.67±0.28	0.15±0.06 ^d	0.48±0.14
44.0	NS	NS	0.37±0.10
52.0	0.44±0.16	0.08±0.08	0.32±0.11
76.0	0.35±0.12	ND	0.13±0.12
100.0	0.21±0.20	ND	ND

a) Trout exposed to 8 mg/l of pyrene for 4 hr. Each value represents the mean±sd of three fish.

b) Chambered-Fish

c) Free-Swimming

d) Mean±sd of two fish

Table A.2. Concentration of pyrene ($\mu\text{g/ml}$) in the blood of trout^a during and after static body-only exposure.

Time (hr)	Static Body-Only
1.00	0.16 \pm 0.13
2.00	0.20 \pm 0.11
3.00	0.26 \pm 0.12
4.00	0.37 \pm 0.11
4.50	0.32 \pm 0.09
9.00	0.19 \pm 0.09
12.0	0.07 \pm 0.08
20.0	0.04 \pm 0.05 ^b
28.0	0.03 \pm 0.03

a) Trout exposed in a compartmentalized chamber containing 24 mg/l of pyrene for 4 hr. Each value represents the mean \pm sd of three fish.

b) Mean \pm sd of two fish.

Table A.3. Concentration of OTC in tissues of chinook salmon^a maintained at 9°C water temperature during and after medication.

Time	Tissue				
	Liver	Kidney	Muscle	Skin	Bone
2 ^b	2.68±0.86	1.07±0.23	0.16±0.18	0.34±0.17	0.19±0.17
4	6.44±3.84	2.80±1.63	0.19±0.12	0.47±0.19	0.55±0.25
6	8.63±4.90	3.00±3.30	0.55±0.23	1.61±0.93	1.19±0.48
9	22.49±14.9	4.23±3.94	0.78±0.65	3.55±1.66	2.35±0.37
13	6.31±1.96	2.35±0.91	0.69±0.35	2.73±1.66	3.75±0.51
15	5.17±1.66	1.80±0.91	0.56±0.27	1.00±0.57	4.81±1.11
17	2.44±0.52	1.22±0.18	0.53±0.21	0.87±0.69	3.80±1.54
22	1.25±1.03	0.85±0.47	0.45±0.18	0.62±0.10	2.69±1.13
29	1.04±0.80	0.85±0.81	0.46±0.41	0.59±0.06	1.97±1.31
36	0.65±0.39	0.54±0.37	0.33±0.11	0.75±0.10	1.21±0.57
43	0.40±0.11	0.35±0.27	0.10±0.07	0.19±0.14	0.94±0.42

a) Salmon received OTC-medicated feed at the rate of 100 mg/kg fish/day for 10 days at Farm A.

b) Indicate the second day of medication.

Table A.4. Concentration of OTC in tissues of chinook salmon^a maintained at 12°C water temperature during and after medication.

Time	Tissue				
	Liver	Kidney	Muscle	Skin	Bone
2 ^b	3.04±1.34	1.22±0.61	0.18±0.15	0.33±0.21	0.08±0.10
6	13.35±9.07	4.30±2.65	0.53±0.38	1.37±1.06	0.56±0.49
9	26.91±15.5	6.12±4.45	1.04±0.48	2.85±1.01	2.49±0.39
15	4.32±1.68	1.46±0.41	0.66±0.35	1.27±0.45	4.23±1.59
17	1.96±0.95	1.06±0.51	0.55±0.40	0.80±0.61	4.06±1.87
24	1.05±0.65	0.73±0.32	0.42±0.20	0.53±0.22	2.69±0.50
35	0.65±0.41	0.62±0.32	0.30±0.24	0.36±0.15	1.86±0.64
43	0.28±0.26	0.17±0.32	0.08±0.14	0.17±0.30	1.14±1.12

a) Salmon received OTC-medicated feed at the rate of 100 mg/kg fish/day for 10 days at Farm A.

b) Indicate the second day of medication.

Table A.5. Concentration of OTC in tissues of coho salmon^a maintained at 10°C water temperature during and after medication.

Time	Tissue				
	Liver	Kidney	Muscle	Skin	Bone
7 ^b	8.97±3.27	3.76±2.26	0.95±0.44	2.62±1.12	0.22±0.09
14	10.59±1.41	3.89±1.04	1.01±0.52	4.12±1.51	2.79±2.02
21	17.55±6.09	4.45±2.65	1.69±0.35	4.46±2.84	3.33±0.97
28	17.87±5.53	5.31±2.80	1.84±0.55	4.58±2.98	3.78±1.80
42	21.37±5.76	5.39±1.48	1.99±0.74	4.89±1.51	5.12±1.50
49	4.84±3.01	2.35±1.64	1.78±1.65	4.05±2.25	7.01±7.60
56	1.04±0.45	0.47±0.45	0.53±0.18	2.10±0.33	5.13±2.63
63	0.53±0.53	0.32±0.33	0.20±0.20	0.99±0.43	3.84±3.52

a) Salmon kept in laboratory and fed with OTC-medicated feed at the rate of 100 mg/kg fish/day for 42 days.

b) Indicate the seventh day of medication.

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