STUDIES ON BIOLOGICAL TRANSPORT PHENOMENA OF ENVIRONMENTAL CHEMICAL POLLUTANTS

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CONTENTS

General Introduction

In recent years, a large amount of harmful chemical substances have been used and discharged into the environment. It is generally known that many organochlorine compounds will remain in the environment over a long period of time once drained out because of their stabilities and this will give rise to environmental pollution problems difficult to solve. For example, PCB widely used as industrial materials or DDT, BHC, dieldrin and chlordanes used as insecticides were prohibited on their use because of the difficulty in their degradation or their high accumulation potential and toxicity. However, they are still representative environmental chemical pollutants.

Quite recently, organotin compounds (tri-n-butyltin, triphenyltin, etc.) used as antifouling agents in ship-bottom paints or fishing nets have been noted as new environmental chemical pollutants. These compounds are known as organometallic compounds with direct bond (Sn-C) and to have toxicity such as growth inhibition, decrease in the number of white corpuscle or lymphocite. At present, the environmental contamination with these compounds is a serious problem in Japan. Many field data on the contamination of fishes and shellfishes in lake or sea by these compounds have been reported by Environment Agency[1-3] and several investigators[4-9] in Japan. These reports have revealed that the tri-n-butyltin and triphenyltin compounds in the environment tend to be highly accumulated in many species of fishes and shellfishes.

At present, the use of both compounds is regulated by a law "Kashinhou" in Japan. Further, Ministry of International Trade and Industry, Fisheries Agency and Ministry of Transport give a business circle

guidance in the decreasing or self-control use of both compounds as antifouling agents in ship-bottom paints or fishing nets. In foreign countries, the environmental contamination with organotin compounds is a serious problem. In U.S.A. and France,[10] the use of paints containing organotin compounds is not permitted on boats shorter than 25 m. In England, the sale of antifouling paints containing organotin compounds is prohibited. It is of interest how these contamination will change in the future by these counterplans.

On the other hand, the environmental contamination with pesticides is still a serious problem. At present, herbicides [benthiocarb, simetryne, oxadiazon, CNP (2,4,6-trichlorophenyl-4'-nitrophenyl ether) and chlomethoxynil], insecticides (diazinon, malathion and fenitrothion) and fungicides [IBP (S-benzyl diisopropyl phosphorothiolate)] are used in large quantities but not regulated on their use in Japan. For the structure of these pesticides, diazinon, malathion, fenitrothion and IBP are classified as organophosphorous compounds, simetryne as a triazine compound, CNP and chlomethoxynil as diphenyl ether compounds, benthiocarb as a thiocarbamate compound and oxadiazon as a diazine compound. These pesticides are known to be more easily degradated and to have lower toxicity than DDT, BHC, dieldrin, chlordanes, etc., but the chronic toxicity to man and fish or the influence on their gene is hardly known for these compounds. Field data[11-18] on the detection of these pesticides in river water and fish samples have been reported by several Japanese investigators.

Quite recently, water pollution by pesticides used in golf courses has become a serious problem in Japan. At present, a tentative plan on the concentrations of 21 pesticides in waste water from golf courses and in city water is provided by Environment Agency and Welfare Ministry in Japan.

At present, surveys on the environmental contamination with these pesticides are widely performed in Japan. However, their circulation path, ecological effect and so on are not satisfactorily researched. This is also the case for the organotin compounds and pesticides described above.

In this study, Lake Biwa and rivers flowing into the lake were selected as a global circulation model of environmental chemical pollutants. Biological transport phenomena of organotin compounds and pesticides through the medium of fishes living there were studied by means of analytical chemistry and kinetics, and each factor related to their circulation or transport was clarified.

The present thesis is based on the studies which the author has carried out at the Section of Food Sanitation and Chemistry, Shiga Prefectural Institute of Public Health and Environmental Science, from 1985 to 1989.

Chapter 1 states development of an analytical method which was used for the determination of butyltin and phenyltin compounds in several species of fishes. Chapters 2 - 4 deal with biological transport of butyltin and phenyltin compounds through the medium of freshwater-fishes. In chapter 5, the metabolism of these compounds is further studied for the freshwater-fishes. Chapter 6 deals with the differences between freshwater and marine fishes in the biological transport of tri-n-butyltin and triphenyltin compounds. It is generally known that the accumulation of environmental chemical pollutants in their biological transport through the medium of fishes is via gill and by oral intakes. Chapter 7 deals with the accumulation of tri-n-butyltin and triphenyltin compounds via gill and by oral intakes of freshwater-fish to compare their accumulation potential. Chapters 8 and 9 deal with biological transport of representative pesticides through the medium of freshwater-fishes. In chapter 10, the metabolism of CNP and its

amino derivative (CNP-amino) is further studied for carp. Chapter 11 deals with biological transport of pesticides used in golf courses through the medium of freshwater-fishes. Chapter 12 states the comparison of these experimental data with the field data in lake and river.

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CHAPTER 1

Development of Analytical Method on Butyltin and Phenyltin Compounds in Biological Samples

Introduction

In recent years, a variety of analytical methods[1-4] have been applied to the determination of organotin compounds in biological samples. Arakawa et al.[1] determined tetraalkyltin and trialkyltin compounds in rat organs by gas chromatography (GC) after silica gel cleanup. Iwai et al.[2] simultaneously determined butyltin compounds and inorganic tin in the same samples by atomic absorption spectrometry. Further, Yu and Arakawa[3] developed a high-performance liquid chromatographic (HPLC) method for simultaneous determination of dialkyltin compounds in samples. On the other hand, Maguire and Tkacz[4] analyzed butyltin compounds and inorganic tin in oligochaetes by gas chromatography with a flame photometric detector (GC-FPD) after pentylation.

Since 1987, a large number of analytical methods[5-11] have been developed by Japanese investigators for the determination of butyltin and phenyltin compounds in biological samples.

In 1987, Takeuchi et al.[5] determined tri-n-butyltin compounds in fish and shellfish samples by gas chromatography with an electron capture detector (GC-ECD) after converting to tri-n-butyltin chloride. Takami et al.[6] also developed a GC-ECD method to determine traces of di- and tri-n-butyltin compounds in fish by use of an innovative cleanup cartridge.

In 1988, Ohsawa et al.[7] simultaneously determine tri- and di-n-butyltin compounds in marine products by GC-ECD. Sasaki et al.[8] simultaneously determined the same compounds in fish samples by GC-FPD after methylation with Grignard reagent. Chikamoto et al.[9] developed GC methods with ECD and mass spectrometry (MS) to determine tri-n-butyltin compounds in fish samples. Further, Takami et al.[10] simultaneously determined triphenyltin and tri-n-butyltin compounds in fish and shellfish samples by a combination of cleanup with ion exchange resins and capillary GC-FPD.

In 1989, Takeuchi et al.[11] also developed a GC-ECD method using a GC column pretreated with hydrobromic acid for the simultaneous determination of tri-n-butyltin and triphenyltin compounds in fish and shellfish samples.

To reveal environmental contamination by organotin compounds and their metabolism, rapid and sensitive methods applicable to various organotin compounds are required. At present, the GC methods by Takami et al. and Takeuchi et al. are very useful for the simultaneous determination of tri-n-butyltin and triphenyltin compounds in fish and shellfish samples obtained from sea or lake. These methods are accurate and sensitive, but time consuming.

We have developed a sensitive and rapid GC method[12,13] for the determination of butyltin and phenyltin compounds in fish and shellfish samples. Our proposed method could be applied to analyses of environmental samples (fish and shellfish) and studies on the accumulation, excretion and metabolism of butyltin and phenyltin compounds by fishes.

Experimental Section

Reagents

Di-n-butyltin dichloride (Bu_2SnCl_2), di-n-butyltin dilaurate [$Bu_2Sn(O_2CC_{11}H_{23})_2$], tri-n-butyltin chloride (Bu_3SnCl), tri-n-butyltin acetate (Bu_3SnOAc), bis(tri-n-butyltin)oxide [$(Bu_3Sn)_2O$] and triphenyltin chloride (Ph_3SnCl), each analytical grade, were obtained from Tokyo Chemical Industry (Tokyo, Japan). Mono-n-butyltin trichloride ($BuSnCl_3$), monophenyltin trichloride ($PhSnCl_3$) and diphenyltin dichloride (Ph_2SnCl_2), each analytical grade, were obtained from Strem Chemicals (Newburyport, MA, U.S.A.). The 5% water-deactivated silica gel (Kieselgel 60, 70 - 230 mesh; Merck) was used for column cleanup. Pesticide grade solvents and analytical grade chemicals were used throughout.

Standard solution

Standard stock solutions (1000 μg/m*l*) were prepared by dissolving 100 mg of each butyltin chloride and phenyltin chloride in 100 m*l* of ethanol. Working standards (0.0625 - 2.0 μg/m*l*) were prepared by diluting these standard stock solutions in ethanol prior to use.

Apparatus

(1) Gas chromatograph - Model G180 (Yanagimoto, Kyoto, Japan) equipped with ^{63}Ni electron capture detector and GC-9AM (Shimadzu, Kyoto,Japan) equipped with flame photometric detector were used. Two 1.5 m x 2 mm ϕ glass column packed with 20% PEG 20M and 10% OV1 on Chromosorb W AW DMCS were used for G180 and capillary GC column DB1 (30 m x 0.53 mmϕ) for GC-9AM.

(2) Rotary evaporator - Model RE 46 (Yamato Scientific, Tokyo, Japan).

(3) Centrifuge - Model 50M (Sakuma Seisakusho, Tokyo, Japan) equipped to hold 50 m*l* test tubes.

Determination

Determination (1): Determination of butyltin and phenyltin in biological samples.

A biological sample was homogenized in a commercial meat grinder. About 10 g of the homogenate were placed in a separating funnel, 100 m*l* of water, 15 g of sodium chloride and 10 m*l* of concentrated hydrochloric acid (35 %) were added and the mixture extracted with 50 m*l* ethyl acetate for 30 min in a mechanical shaker. After centrifugation of the mixture at 3000 rpm for 5 min, a measured amount (30 m*l*) of the organic layer was transferred to a 50 ml round-bottom flask.The solution was rotary-vacuum evaporated to dryness (1-2 min) at 40°C. (Caution: this step should not be prolonged.) The residue was dissolved in 1 m*l* ethanol and then hydrogenated with 2 m*l* of 2.5% sodium tetrahydroborate in ethanol for 10 min. To the reaction mixture were added 15 m*l* of water and 5 g of sodium chloride and this was extracted with 5 m*l* of hexane for 5 min. The hexane layer was passed through a 8 cm x 1 cmϕ glass cleanup column containing about 3 g of hexane-rinsed silica gel. The column was eluted with hexane. The first 5 m*l* were discarded, the next 5 - 15 m*l* were collected for $BuSnH_3$, Bu_2SnH_2, Bu_3SnH, $PhSnH_3$, Ph_2SnH_2 and the next 15 - 40 m*l* for Ph_3SnH. For the analysis of $BuSnH_3$ the eluent (10 m*l*) was injected into the gas chromatograph without concentration. For the butyltin and phenyltin hydrides except $BuSnH_3$, the eluents were rotary-vacuum evaporated to about 2 m*l*, transferred to a graduated test-tube (rinsing the flask with hexane) and adjusted to 1 - 5 m*l* under a stream of nitrogen or air at 40°C. A 5 μ*l*

volume of each sample solution was injected into gas chromatograph (GC-ECD) for analysis. Peak heights obtained from the sample injection were evaluated by the use of calibration curves.

Calibration curves: A 1 m*l* volume of each standard (0.0625 - 2.0 μg/m*l*) was hydrogenated and extracted with hexane as described above. A 5 μ*l* volume of the hexane layers was injected into the gas chromatograph, and calibration curves were constructed by plotting the peak heights against the concentrations for $BuSnCl_3$ (0.05 - 0.4 μg/m*l*), Bu_2SnCl_2 (0.0125 - 0.1 μg/m*l*), Bu_3SnCl (0.025 - 0.2 μg/m*l*), $PhSnCl_3$ (0.05 - 0.4 μg/m*l*), Ph_2SnCl_2 (0.025 - 0.2 μg/m*l*) and Ph_3SnCl (0.05 - 0.4 μg/m*l*).

Determination (2): Simultaneous determination of tri-n-butyltin and triphenyltin in biological samples.

Analysis was carried out by the above method modified as follows. The eluate from silica gel cleanup was collected in a volume of 0 - 50 m*l* and concentrated to 0.5 - 5 m*l*. The Bu_3SnH and Ph_3SnH in the concentrate were determined simultaneously by GC-FPD.

Calibration curves: A 1 m*l* volume of each standard (0.125 - 2.0 μg/m*l*) was hydrogenated and extracted with hexane as described above. A 5 μ*l* volume of the hexane layers was injected into the gas chromatograph, and calibration curves were constructed by plotting the peak heights against the concentrations for Bu_3SnCl (0.025 - 0.2 μg/m*l*) and Ph_3SnCl (0.05 - 0.4 μg/m*l*).

Results and Discussion

Extraction of butyltin and phenyltin from biological samples

Efficiency of extraction of di-n-butyltin and tri-n-butyltin compounds from fish muscle was investigated for the following solvents:(1) hexane; (2) ethyl acetate-hexane(3+2); (3) ethyl acetate. Investigations were carried out with about 10 g sample spiked with 10 μg each of Bu_2SnCl_2 and Bu_3SnCl according to the above procedure. In the course of our investigations, we found that the addition of NaCl was necessary to enhance extraction efficiency and to facilitate phase separation. When hexane was used for extraction, little organic layer was obtained in spite of centrifugation due to the formation of an emulsified layer. In case of extraction with both ethyl acetate-hexane(3+2) and ethyl acetate, good recoveries were obtained without emulsification. Since ethyl acetate-hexane (3+2) extracted less impurities than ethyl acetate and no difference in the extraction efficiency between the two solvents could be detected, ethyl acetate-hexane (3+2) was selected.

However, for $BuSnCl_3$ or phenyltin chlorides , the extraction efficiency was not good, especially at low levels (0.1 μg/g). For example, the average recovery of Ph_3SnCl after silica gel cleanup was 75.9% (n=3) for 1.0 μg/g spiked, but 33.3% (n=3) at 0.1 μg/g.

Here ethyl acetate was again tried for extraction. The average recovery was 81.5% (n=5) for samples spiked at a level of 1.0 μg/g and 86.3% (n=5) at 0.1 μg/g. Ethyl acetate, which extracted more impurities than ethyl acetate-hexane (3+2), was used because of the high extraction efficiency and satisfactory silica gel cleanup.

Silica gel cleanup

Elution patterns of Bu_2SnH_2 and Bu_3SnH were investigated for 0 - 20% water-deactivated silica gel column chromatography. Recoveries of the butyltin hydrides eluted with 50 m*l* hexane were (1) 70.3 and 63.3%; (2) 97.9 and 92.9%; (3) 100 and 90.0%; (4) 90.0 and 100% for 0, 5, 10, 20% water-deactivated silica gel, respectively. The recoveries were constant in the range of 5 - 20% water, so 5% water-deactivated silica gel with the highest adsorptive activity was chosen for cleanup. The elution of Bu_2SnH_2 and Bu_3SnH was in the range of 5 - 15 m*l* for silica gel column chromatography, so the eluate was collected from 0 to 20 m*l*. Silica gel cleanup of a spiked fish sample is shown in Fig.1. Effective cleanup was observed, particularly at the position of Bu_2SnH_2.

Here, elution patterns of butyltin and phenyltin hydrides were again investigated for 5% water-deactivated silica gel. All the hydrides except Ph_3SnH (15 - 30 m*l*) were eluted from 5 to 15 m*l* (Fig.2). As shown in Fig.3, interference was observed at the Ph_3SnH peak position in the gas chromatogram (GC-ECD) of the eluent (0 - 15 m*l*) for a fish sample. In case of GC-ECD analysis, eluent should be separately collected from 5 to 15 m*l* for the hydrides except Ph_3SnH, and from 15 to 40 m*l* for Ph_3SnH. However, the eluent (0 - 40 m*l*) could be together collected in the GC-FPD analysis because of no interference in the gas chromatogram (Fig.4).

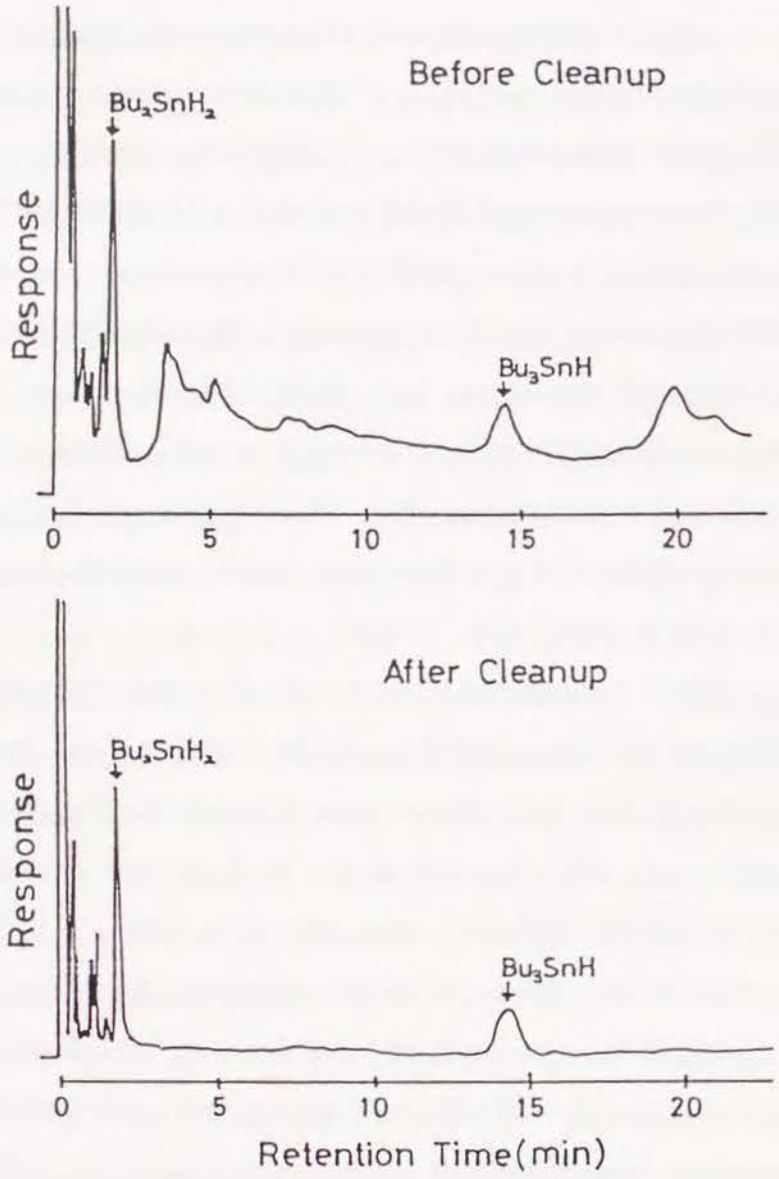

Fig.1 Cleanup of extract from spiked fish muscle after hydrogenation by silica gel column chromatography.
GC column: 2% OV17; temperature: column 90°C, detector and injection 200°C; carrier: N_2 40 m*l*/min; detector: ECD; spiked: 1 μg each; sample volume: 5 m*l*.

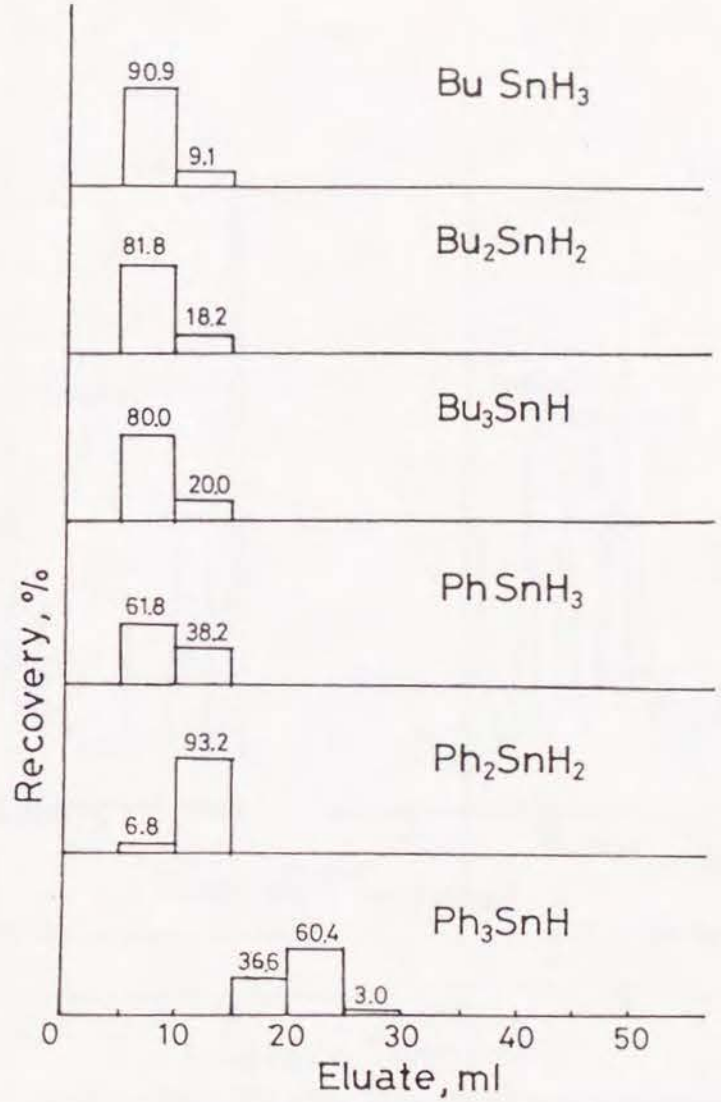

Fig.2 Elution patterns of butyltin and phenyltin hydrides from a 5% water-deactivated silica gel column.

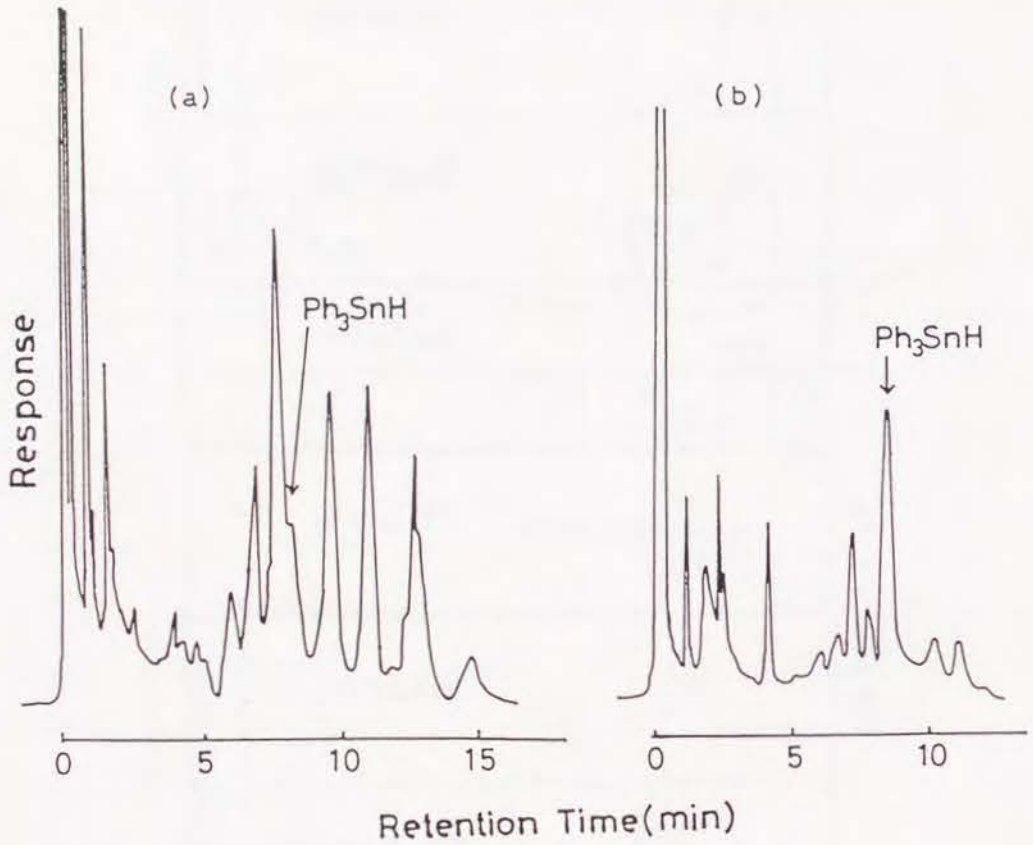

Fig.3 Gas chromatograms of Ph_3SnH after silica gel cleanup. Crucian carp muscle was spiked with 10 μg of Ph_3SnCl.
GC column: 10% OV1; temperature: column 250°C, detector and injection 275°C; carrier: N_2 40 m*l*/min; detector: ECD.
(a) Sample volume: 5 m*l*; eluate: 0 - 15 m*l*.
(b) Sample volume: 5 m*l*; eluate: 15 - 40 m*l*.

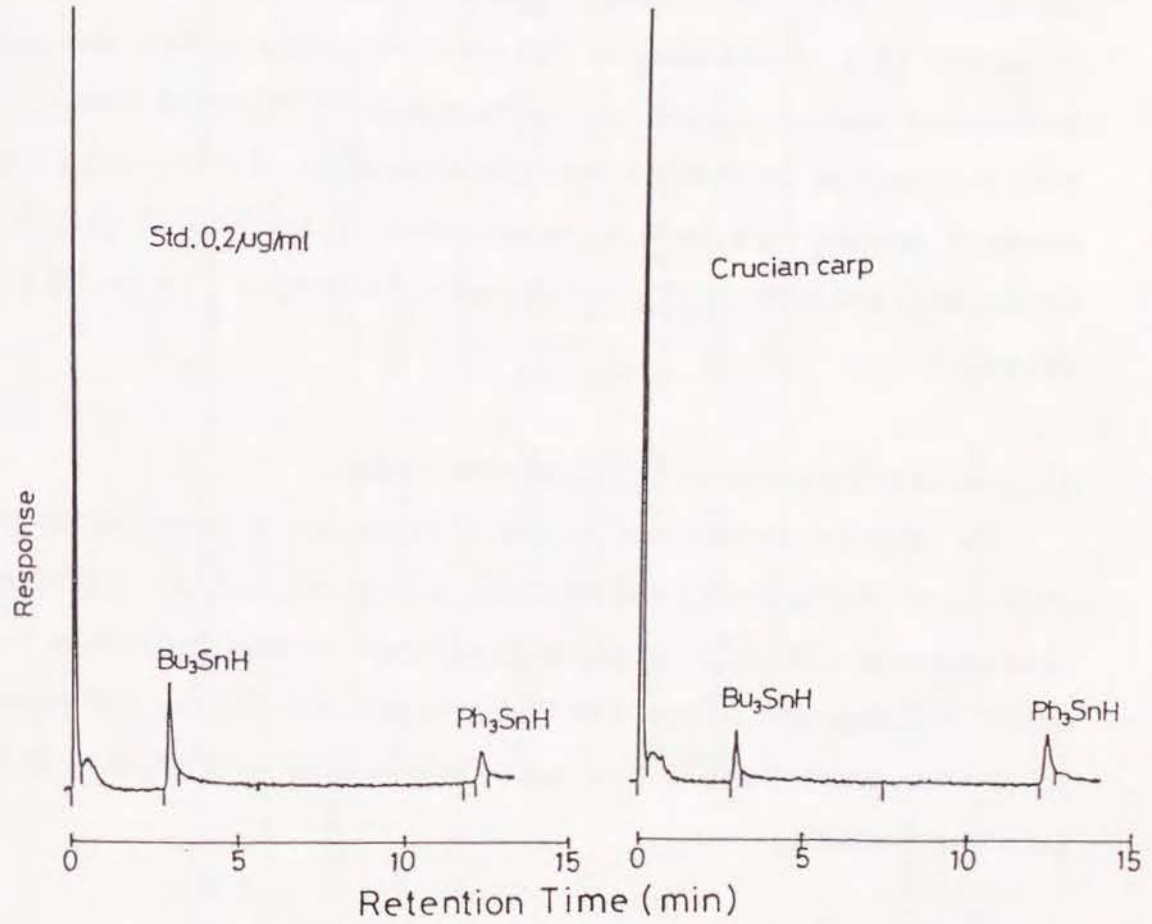

Fig.4 Analysis of crucian carp obtained from a harbor of Lake Biwa.
GC column: DB1; temperature: column 140°C(1 min) 8°C/min to 240°C, detector and injection 280°C; carrier: N_2 30 m*l*/min; detector: FPD; sample volume: 1m*l*.

Recoveries of butyltin and phenyltin from biological samples

The recoveries of butyltin and phenyltin chlorides added to fish muscle were evaluated. The results on determination (1) by GC-ECD are given in Table 1. The recoveries varied from 86.2 to 99.8% for butyltin chlorides and from 75.0 to 88.0% for phenyltin chlorides spiked at the levels of 1 and 10 μg per 10 g of fish sample. The precision of the method was also evaluated by replicate analysis (n=5). The reproducibility varied from 1.1 to 5.6% expressed as the standard deviation at the levels of 1 and 10 μg. The results of recovery tests (n=3) on determination (2) by GC-FPD were 88% for Bu_3SnCl and 83% for Ph_3SnCl spiked at the level of 1 μg per 10 g of fish sample.

Application to accumulation and metabolism studies

The proposed method was applied to the studies of accumulation and metabolism of $(Bu_3Sn)_2O$ and Ph_3SnCl in carp. Fig.5 shows typical gas chromatograms (GC-ECD) of phenyltin chlorides as their hydrides in carp muscle exposure for 7 days. For Ph_2SnH_2 and $PhSnH_3$, the GC sample solution was concentrated to 1 ml, but no interferences were observed on the gas chromatograms.

Detection limits

The detection limits by GC-ECD were 20 ng/g for $BuSnCl_3$ (GC sample: 10 ml), 1.0 ng/g for Bu_2SnCl_2, 2 ng/g for Bu_3SnCl and Ph_2SnCl_2, and 5 ng/g for $PhSnCl_3$ and Ph_3SnCl (GC sample: each 1 ml). Similarly, those by GC-FPD were 2 ng/g for Bu_3SnCl and 5 ng/g for Ph_3SnCl.

Table 1. Recovery of butyltin and phenyltin compounds from biological and sediment samples.

Sample	Amount of tin compound added (μg)	Recovery[a] (%)					
		$BuSnCl_3$	Bu_2SnCl_2	Bu_3SnCl	$PhSnCl_3$	Ph_2SnCl_2	Ph_3SnCl
Fish[b]	1	86.6±4.7	86.2±5.6	88.5±4.7	75.0±4.1	86.0±3.3	86.3±3.7
	10	95.3±3.7	99.3±3.1	99.8±1.1	85.3±4.4	88.0±1.6	81.5±1.8
Sediment[c]	1	70.3±3.7	90.0±3.2	90.1±3.3	71.7±2.4	76.7±3.3	74.1±3.1
	10	73.3±2.4	96.6±2.1	95.9±2.1	85.7±3.5	85.2±2.6	80.8±3.0

[a] Mean±SD (n=5)

[b] Round crucian carp muscle, 10 g

[c] Wet sediment, 20 g; water, 46.2%; ignition loss, 10.0%

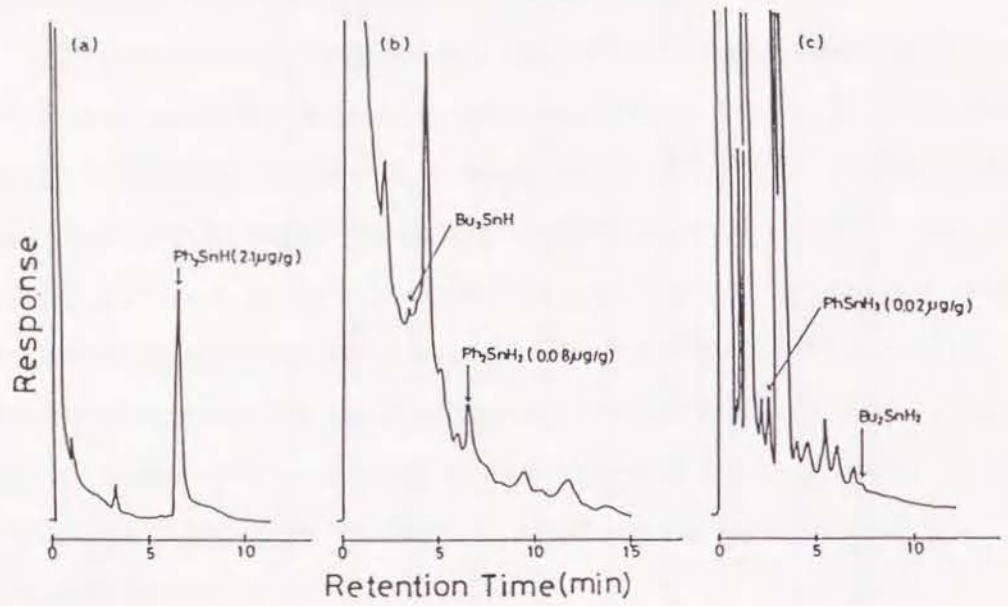

Fig.5 Gas chromatograms of Ph_3SnCl and its metabolites, Ph_2SnCl_2 and $PhSnCl_3$, as their hydrides.

GC column: 10% OV1; detector: ECD.

(a) Column temperature: 250°C; sample volume: 10 m*l*.

(b) Column temperature: 170°C; sample volume: 1 m*l*.

(c) Column temperature: 100°C; sample volume: 1 m*l*.

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CHAPTER 2

Accumulation of Butyltin and Phenyltin Compounds in Freshwater-Fishes

Introduction

In recent years, a number of organotin compounds, such as tri-n-butyltin and triphenyltin, have been produced and discharged into the environment (lake or sea). In view of the increasing environmental pollution by tributyltin (Bu_3Sn^+), triphenyltin (Ph_3Sn^+) and their degradation products (Bu_2Sn^{2+}, $BuSn^{3+}$, Ph_2Sn^{2+} and $PhSn^{3+}$), the data on their accumulation are very useful for the evaluation of their contamination of the fishes in the environment.

However, few reports have been published for the accumulation of these organotin compounds in fishes.[1]

In this study,[2-5] we measured the bioconcentration factor (BCF) of butyltin compounds such as $(Bu_3Sn)_2O$, Bu_2SnCl_2 and $BuSnCl_3$ and phenyltin compounds such as Ph_3SnCl, Ph_2SnCl_2 and $PhSnCl_3$ in carp, crucian carp and goldfish. Further, partition coefficients between n-octanol and water (P_{ow}) were measured for these compounds as reference data for discussion. From these data, we revealed the accumulation potential of these organotin compounds.

Experimental Section

Chemicals

$(Bu_3Sn)_2O$, Bu_3SnCl, Bu_2SnCl_2 and Ph_3SnCl were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Ph_2SnCl_2, $PhSnCl_3$ and $BuSnCl_3$ from Strem Chemicals (Newburyport, U.S.A.). These chemicals, each of reagent grade, were used without further purification.

Test fish

Carp (*Cyprinus carpio* L.) were obtained from Nango Suisan Center (Shiga Prefecture, Japan), round crucian carp (*Carassius carassius grandoculis*) from Shiga Prefectural Fisheries Experimental Station and goldfish (*Carassius aurapus*) from Yamato Kohriyama (Nara Prefecture, Japan). The body length and weight were 9.5 - 12.0 cm and 20.0 - 36.1 g for carp, 12.0 - 14.0 cm and 43.0 - 70.0 g for round crucian carp, and 2.8 - 3.5 cm and 0.9 - 1.7 g for goldfish. Lipid contents by Folch method were 0.75 - 0.80% (n=6) in muscle of carp, 1.5 - 2.0% (n=5) in muscle of round crucian carp and 2.1 - 2.6% (n=5) in whole body of goldfish. Butyltin and phenyltin compounds were not detected in the fishes before exposure to these compounds.

Test system

The study was carried out under the continuous flow through system. The peristeric micropump (Cole-Parmer Master Flex PA-21A type) was used to obtain a continuous flow of test solution. Aquaria tanks made of acrylic plastic holding about 50 *l* were used. Aqueous stock solutions of butyltin and phenyltin compounds (0.6 - 1.5 μg/m*l*) were diluted continuously 100 or 150 times with dechlorinated city water and supplied to

aquaria tanks containing fishes (Fig.1). During the tests, the flow rate of the test water was maintained at 200 or 300 m*l*/min. The temperature was 23±1°C for carp, 22±1°C for round crucian carp and 18±1°C for goldfish. The measured concentrations of butyltin and phenyltin compounds in the test tanks are summarized in Table 1. Measurements were carried out on days 0, 1, 3, 7, 10 and 14. At these concentrations, the fishes showed no signs of tiredness or agitation during the experiments.

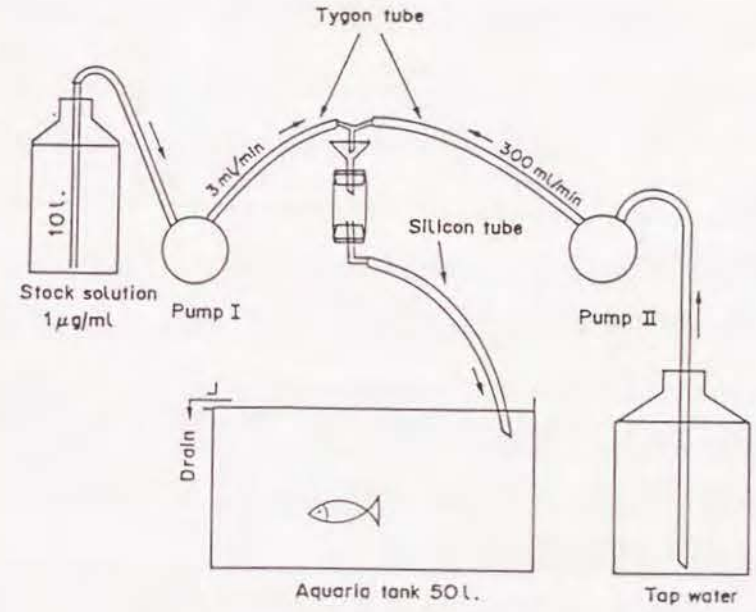

Fig.1 Experimental arrangement .

Analysis

The concentrations of the butyltin and phenyltin compounds in the test water were determined by the following procedure. A measured volume (100 m*l*) of the test water was extracted with 50 m*l* of ethyl acetate after the addition of 15 g of NaCl and 5 ml of HCl. The organic layer was rotary-

vacuum evaporated just to dryness at 40°C. The residue was hydrogenated with 2 m*l* of 2.5% $NaBH_4$ in ethanol, and extracted with 5 m*l* of hexane after the addition of 15 m*l* of water and 3 g of NaCl. The butyltin and phenyltin hydrides in the hexane layer were determined by GC-ECD.

Table 1. Measured concentrations of butyltin and phenyltin compounds in the test tank water.

	Concentrations (μg/*l*)[a]						
Fish species	$(Bu_3Sn)_2O$	Bu_3SnCl	Bu_2SnCl_2	$BuSnCl_3$	Ph_3SnCl	Ph_2SnCl_2	$PhSnCl_3$
Carp	2.1	-	4.3	6.5	5.6	2.0	6.4
RCC [b]	5.4	6.9	-	-	7.5	-	-
Goldfish	2.0	-	-	-	3.2	-	-

[a] Mean (n=6)

[b] Round crucian carp

Average recoveries (n=3) were 90% for $BuSnCl_3$, 90% for Bu_2SnCl_2, 95% for $(Bu_3Sn)_2O$, 80% for $PhSnCl_3$, 85% for Ph_2SnCl_2 and 90% for Ph_3SnCl at 0.01 μg/m*l* spiked levels.

Determination of butyltin and phenyltin compounds in the fish samples was carried out by our proposed method. In brief, homogenized fish samples were extracted with ethyl acetate after the addition of NaCl and HCl. The ethyl acetate layer was rotary-vacuum evaporated to dryness. The residue was hydrogenated and extracted with hexane in the same manner as the test water. The butyltin and phenyltin hydrides were determined by GC-ECD after being cleaned by silica gel column chromatography. At the fixed sampling times (1, 3, 7, 10 and 14 days), three fish were taken and analyzed.

Average recoveries (muscle, n=3) were 85% for Bu_3SnCl, 90% for Bu_2SnCl_2, 95% for $(Bu_3Sn)_2O$, 75% for $PhSnCl_3$, 85% for Ph_2SnCl_2 and 85% for Ph_3SnCl at 0.1 μg/g spiked levels.

Calculation of bioconcentration factor (BCF)

BCF was calculated by the following equation.

$$BCF = \frac{\text{concentration of chemical in fish}}{\text{concentration of chemical in water}}$$

The concentration of chemical in water at each sampling time (1, 3, 7, 10 and 14 days) was used for the calculation of BCF.

Measurement of partition coefficient between n-octanol and water (P_{ow})

OECD Guideline for Testing of Chemicals (1981) is not applicable to the measurements of P_{ow} for dissociative chemicals. Here, as a reference data for discussion, the P_{ow} of butyltin and phenyltin compounds was separately measured three times by a flask-shaking method according to this guide line. Measurements were carried out for n-octanol:water (volume ratio) = 1 and 1:10. The n-octanol phase was diluted by ethanol, and analyzed by GC-ECD as the corresponding hydrides. The water phase was similarly analyzed after extraction. P_{ow} was calculated by the following equation.

$$P_{ow} = \frac{\text{concentration of chemical in n-octanol}}{\text{concentration of chemical in water}}$$

Results

P_{ow} of butyltin and phenyltin

Log P_{ow} values of butyltin and phenyltin compounds are shown in Table 2. The order of butyltin compounds and phenyltin chlorides is $(Bu_3Sn)_2O$ > $BuSnCl_3 \geq Bu_2SnCl_2$ and $Ph_3SnCl > PhSnCl_3 \geq Ph_2SnCl_2$, respectively.

Table 2. Log P_{ow} of butyltin and phenyltin compounds.

	$LogP_{ow}$ [a]	
Chemical	O : W [b] = 1 : 1	O : W = 1 : 10
$BuSnCl_3$	0.09±0.01	2.2±0.05
Bu_2SnCl_2	0.05±0.01	1.0±0.02
Bu_3SnCl	-	2.1±0.01
Bu_3SnOAc	-	2.5±0.01
$(Bu_3Sn)_2O$	2.2±0.03	3.7±0.01
$PhSnCl_3$	0.2±0.01	1.7±0.01
Ph_2SnCl_2	0.1±0.01	1.4±0.04
Ph_3SnCl	2.0±0.04	2.1±0.01

[a] Mean±SD (n=3)

[b] n-Octanol:water

Accumulation of tri-n-butyltin and triphenyltin compounds in round crucian carp

Round crucian carp were separately exposed to $(Bu_3Sn)_2O$, Bu_3SnCl and Ph_3SnCl for 7 days. The concentrations of these compounds in the fish did not reach plateaus until 7 days. LogBCF of $(Bu_3Sn)_2O$, Bu_3SnCl and Ph_3SnCl in four tissues of the fish are summarized in Table 3. The orders of logBCF were liver > kidney > muscle ≥ vertebra for both tri-n-butyltin compounds and liver > vertebra, muscle > kidney for Ph_3SnCl.

Table 3. LogBCF of tri-n-butyltin and triphenyltin compounds in round crucian carp.

	LogBCF [a] (7 days)			
Chemical	Muscle	Vertebra	Liver	Kidney
Bu_3SnCl	2.6	2.5	3.5	3.4
$(Bu_3Sn)_2O$	2.8	2.7	3.7	3.5
Ph_3SnCl	1.7	1.7	2.1	1.5

[a] Mean value (n=3) for muscle, single determination value for vertebra, liver and kidney.

Accumulation of butyltin compounds in carp

Carp were separately exposed to $(Bu_3Sn)_2O$, Bu_2SnCl_2 and $BuSnCl_3$ for 14 days. Accumulation of $(Bu_3Sn)_2O$, Bu_2SnCl_2 and $BuSnCl_3$ in the four tissues of the fish is shown in Figs.2 - 4, respectively. The concentration of $(Bu_3Sn)_2O$ reached a plateau after 3 days for the gallbladder, 7 days for the liver, 10 days for the muscle, but not until 14 days for the kidney. Bu_2SnCl_2 and $BuSnCl_3$ reached plateaus more rapidly than $(Bu_3Sn)_2O$. The order of logBCF was $(Bu_3Sn)_2O$ > $BuSnCl_3$ > Bu_2SnCl_2 for muscle and all three tissues of the viscera. Further, the orders of logBCF after 14 days were kidney > gallbladder > liver > muscle for $(Bu_3Sn)_2O$, liver > gallbladder > kidney > muscle for Bu_2SnCl_2, and liver ≥ gallbladder > kidney > muscle for $BuSnCl_3$.

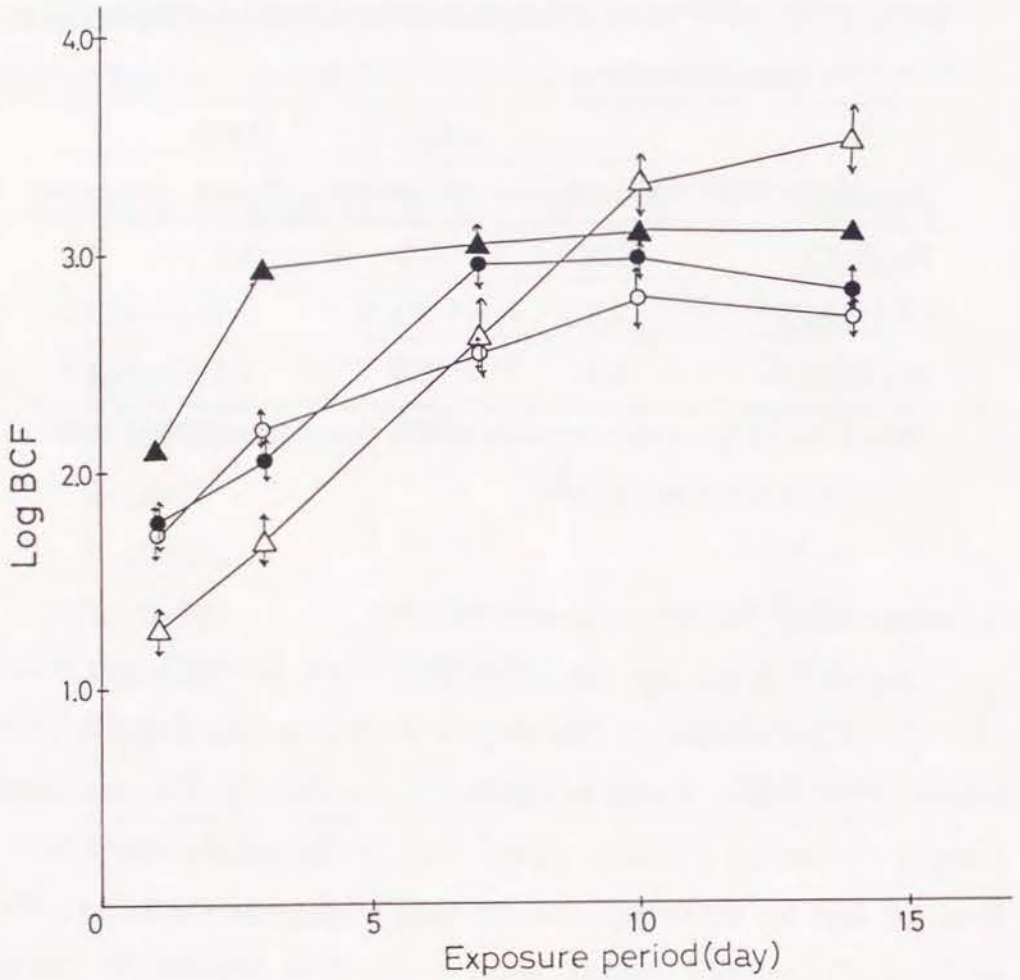

Fig.2 Accumulation of $(Bu_3Sn)_2O$ in carp.

○ Muscle ● Liver

△ Kidney ▲ Gallbladder

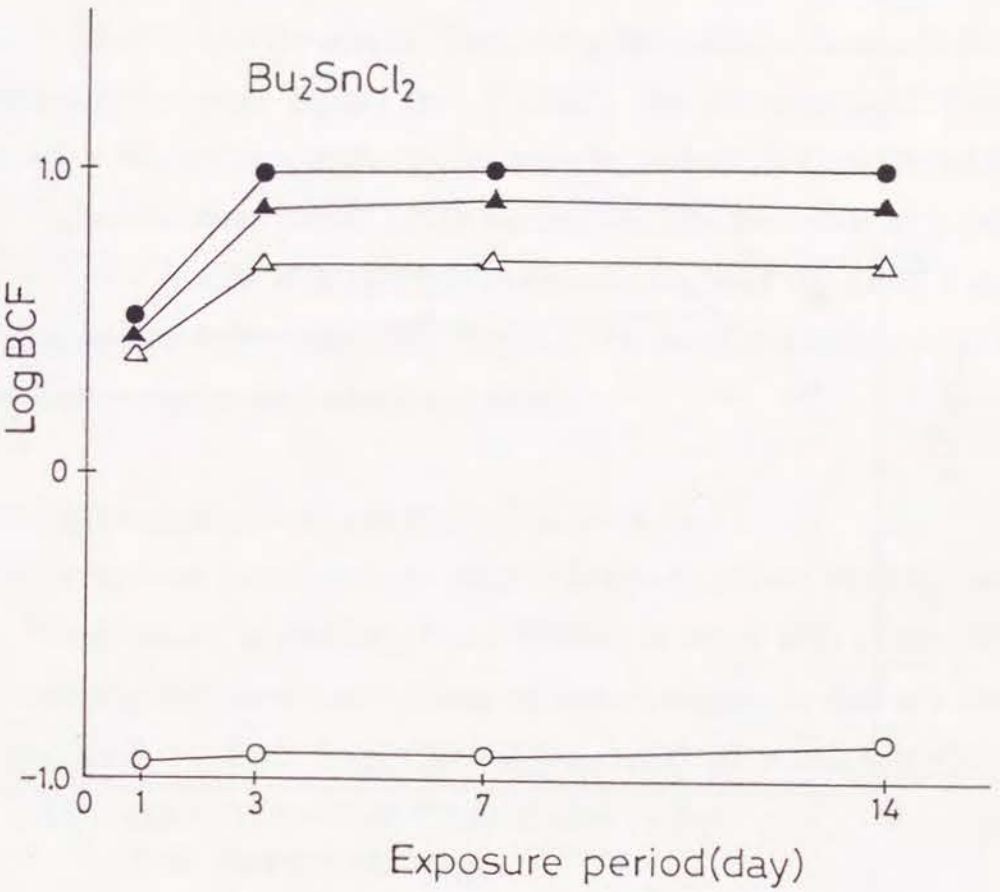

Fig.3 Accumulation of Bu_2SnCl_2 in carp.

○ Muscle ● Liver

△ Kidney ▲ Gallbladder

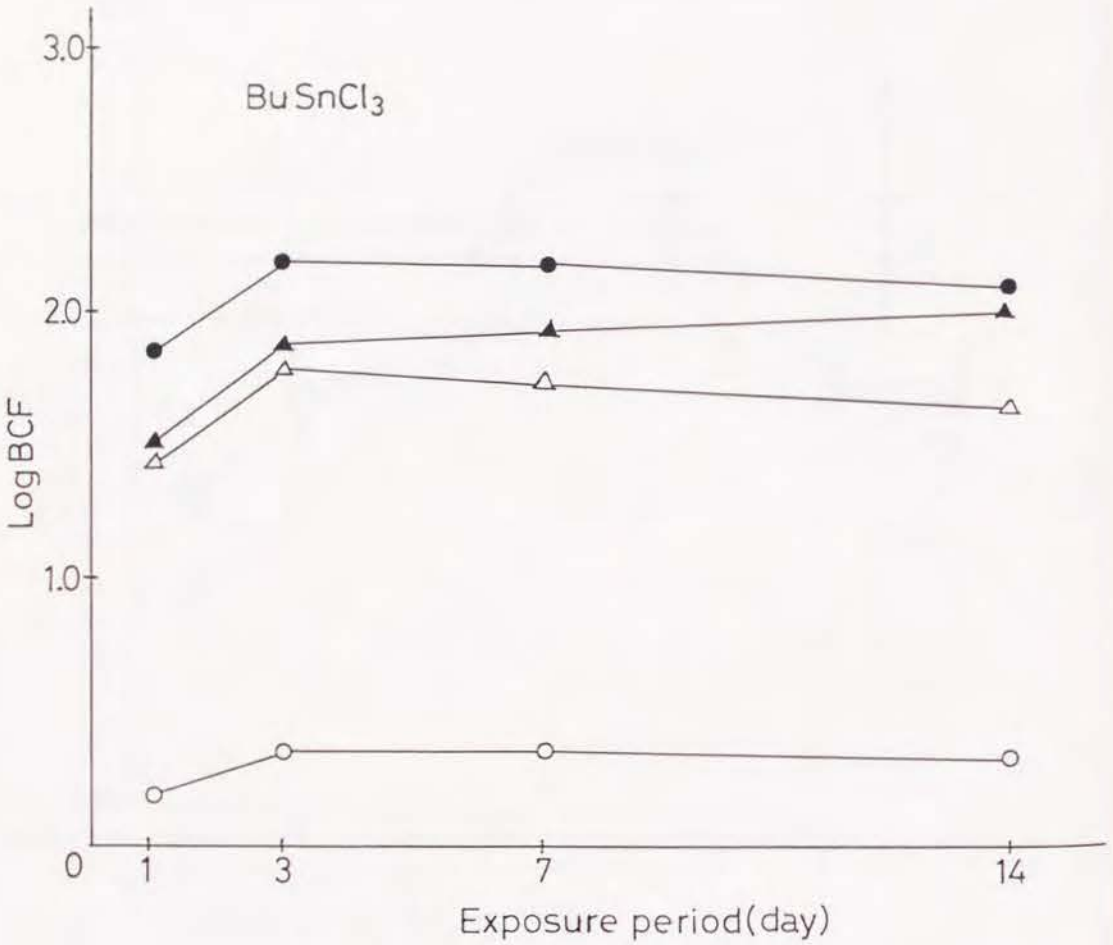

Fig.4 Accumulation of $BuSnCl_3$ in carp.

○ Muscle ● Liver

△ Kidney ▲ Gallbladder

Accumulation of phenyltin chlorides in carp

Carp were separately exposed to Ph_3SnCl (10 days), Ph_2SnCl_2(14 days) and $PhSnCl_3$ (14 days). Accumulation of Ph_3SnCl, Ph_2SnCl_2 and $PhSnCl_3$ in the four tissues of the fish is shown in Figs.5 - 7, respectively. The concentration of Ph_3SnCl reached a plateau after 3 days for gallbladder,and probably after 7 days for muscle, liver and kidney. Ph_2SnCl_2 and $PhSnCl_3$ reached plateaus more rapidly than Ph_3SnCl. The orders of logBCF were Ph_3SnCl > Ph_2SnCl_2 > $PhSnCl_3$ for muscle, and Ph_3SnCl > $PhSnCl_3$ ≥ Ph_2SnCl_2 for all three tissues of the viscera. Further, the orders of logBCF were kidney > liver > muscle ≥ gallbladder for Ph_3SnCl (10 days), liver > kidney ≥ gallbladder > muscle for Ph_2SnCl_2 (14 days), and kidney > liver > gallbladder > muscle for $PhSnCl_3$ (14 days).

Accumulation of $(Bu_3Sn)_2O$ and Ph_3SnCl in goldfish

Goldfish were simultaneously exposed to $(Bu_3Sn)_2O$ and Ph_3SnCl for 14 days. Accumulation of $(Bu_3Sn)_2O$ and Ph_3SnCl in whole body of the fish is shown in Fig.8. The concentrations of both compounds did not reach plateaus until 14 days. LogBCF was mean±SD of 3.1±0.1 (n=3) for $(Bu_3Sn)_2O$ and 2.4±0.1 (n=3) for Ph_3SnCl after 14 days.

Discussion

It is generally known that the compounds with lower P_{ow} reach plateaus more rapidly than those with higher P_{ow} in their accumulation experiments. This could be applied to the butyltin and phenyltin compounds from the results that Bu_2SnCl_2 ($logP_{ow}$=0.05) and $BuSnCl_3$ ($logP_{ow}$=0.09) or Ph_2SnCl_2

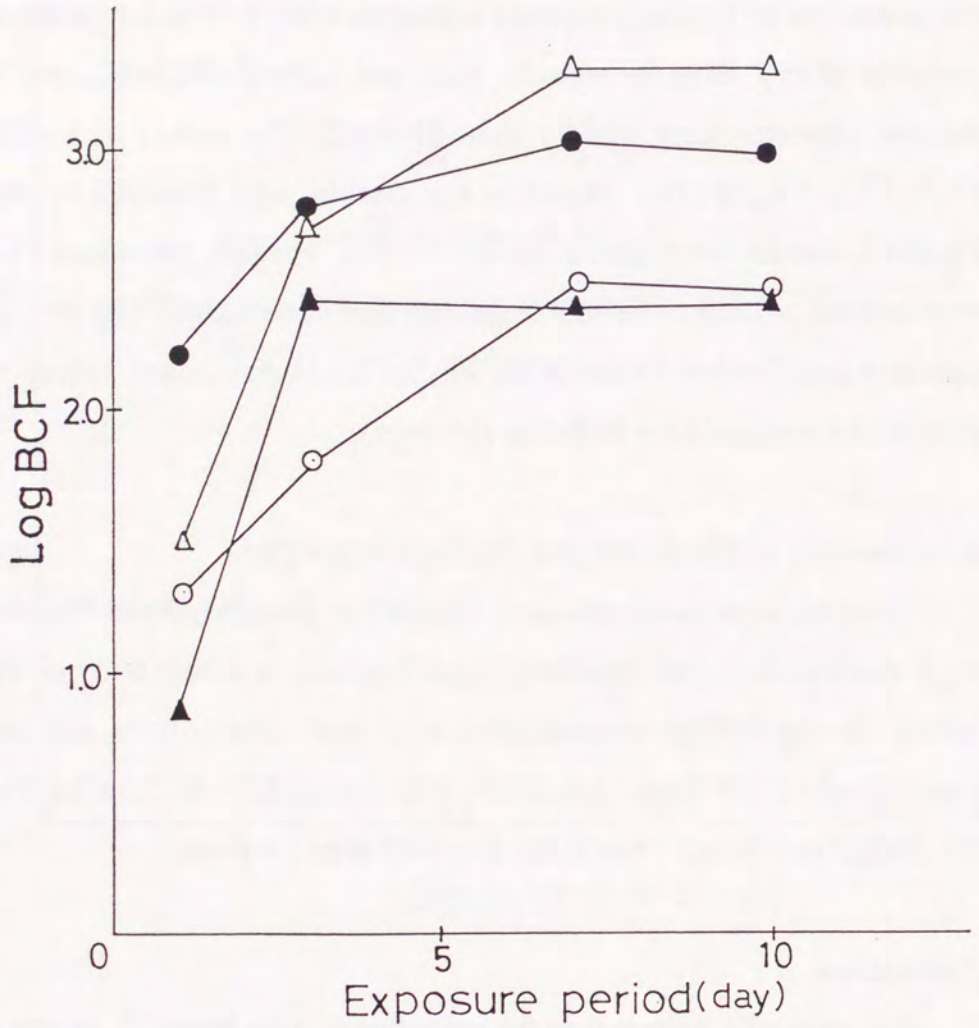

Fig.5 Accumulation of Ph_3SnCl in carp.

○ Muscle ● Liver

△ Kidney ▲ Gallbladder

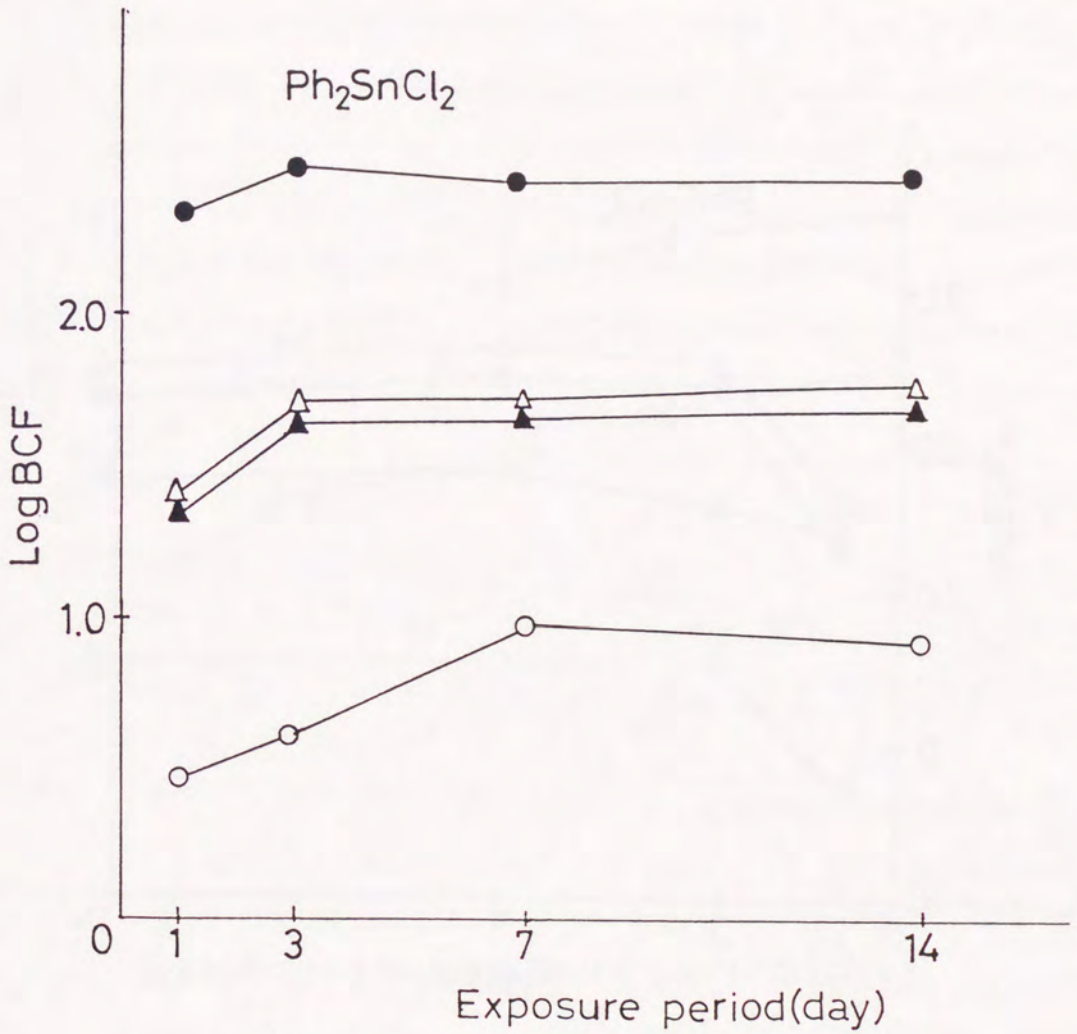

Fig.6 Accumulation of Ph_2SnCl_2 in carp.

○ Muscle ● Liver

△ Kidney ▲ Gallbladder

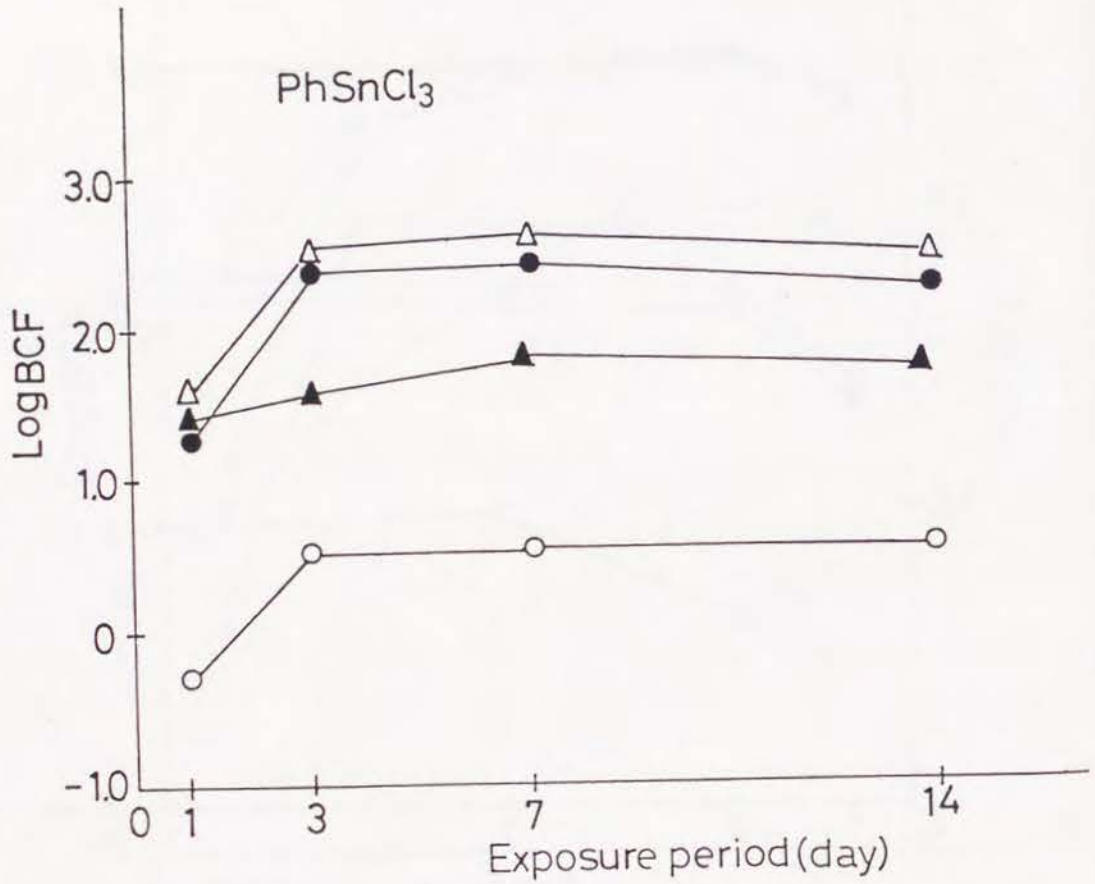

Fig.7 Accumulation of $PhSnCl_3$ in carp.

○ Muscle ● Liver

△ Kidney ▲ Gallbladder

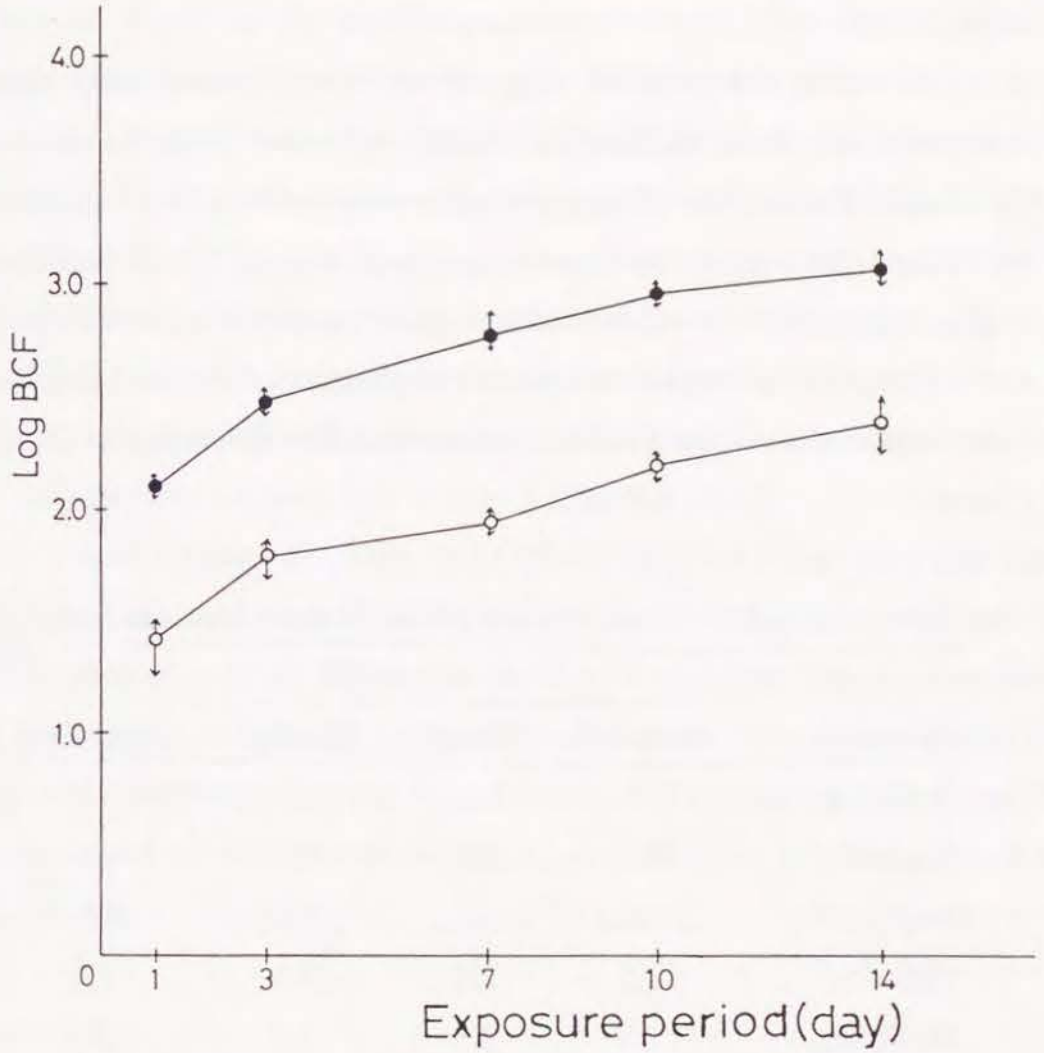

Fig.8 Accumulation of $(Bu_3Sn)_2O$ and Ph_3SnCl in goldfish.

● $(Bu_3Sn)_2O$ ○ Ph_3SnCl

($logP_{ow}$=0.11) and $PhSnCl_3$ ($logP_{ow}$=0.22) reached plateaus more rapidly than $(Bu_3Sn)_2O$ ($logP_{ow}$=2.2) or Ph_3SnCl ($logP_{ow}$=2.0) in all four tissues of carp.

The values of logBCF in carp and round crucian carp were higher in the viscera than in the muscle for all these butyltin and phenyltin compounds.

LogBCF of the butyltin and phenyltin compounds in carp is summarized in Table 4. It is generally known that there is a good correlation between $logP_{ow}$ and logBCF for nonionizable organic compounds. In this study, the order of logBCF of butyltin compounds or phenyltin chlorides in each of the four tissues of the carp (Table 4) was about the same as that of $logP_{ow}$ (Table 2).

Table 4. LogBCF of butyltin and phenyltin compounds in carp.

	LogBCF [a]			
Chemical	Muscle	Liver	Kidney	Gallbladder
$BuSnCl_3$	0.3	2.1	1.7	2.1
Bu_2SnCl_2	−0.9	1.0	0.7	0.9
$(Bu_3Sn)_2O$	2.7±0.1	2.8±0.1	3.5±0.2	3.1
$PhSnCl_3$	0.5	2.4	2.6	1.8
Ph_2SnCl_2	0.9	2.3	1.7	1.7
Ph_3SnCl	2.4±0.1	3.0±0.1	3.3±0.02	2.4

[a] Mean±SD (n=3) for $(Bu_3Sn)_2O$ and Ph_3SnCl in muscle, liver and kidney. Single determination value for $(Bu_3Sn)_2O$ and Ph_3SnCl in gallbladder and other chemicals.

The concentrations of $(Bu_3Sn)_2O$ and Ph_3SnCl in whole body of goldfish did not reach plateaus until 14 days exposure. This was different from the results of the same experiments on carp. The time required to reach plateaus was presumed to change by the difference of the fish species.

The logBCF order of $(Bu_3Sn)_2O$ and Ph_3SnCl in goldfish was $(Bu_3Sn)_2O > Ph_3SnCl$, the same as in carp and round crucian carp. LogBCF of $(Bu_3Sn)_2O$ in whole body of goldfish after 14 days exposure was 3.1, which was about the same value as estimated from that of carp (muscle 2.7, liver 2.8, kidney 3.5, gallbladder 3.1). Similarly, logBCF of Ph_3SnCl in whole body of goldfish (2.4) was about the same value as estimated from that of carp (muscle 2.4, liver 3.0, kidney 3.3, gallbladder 2.4).

The BCF values of $(Bu_3Sn)_2O$ (500 - 3200) were about two - five times as high as those of Ph_3SnCl (260 - 2100) in the four tissues of carp and the whole body of goldfish. These data could not explain the high concentrations of triphenyltin compounds compared with tri-n-butyltin compounds in sea bass and other fishes obtained from sea or lake. To explain the field data, the accumulation experiments by oral intake as well as via gill intake should be carried out.

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CHAPTER 3

Influence of pH on Accumulation of Tri-n-butyltin and Triphenyltin Compounds in Carp

Introduction

In Japan, a large amount of tri-n-butyltin compounds such as bis(tri-n-butyltin)oxide [$(Bu_3Sn)_2O$], tri-n-butyltin fluoride (Bu_3SnF), etc. have been used as antifouling agents in ship-bottom paints or fishing nets. Many field data on their contamination of fish and shellfish in lake or sea have been reported by the Japan Environmental Agency[1,2] and several investigators.[3-6] From these reports, it has been revealed that the tri-n-butyltin compounds in the environment tend to be highly accumulated in many species of fish and shellfish. Accordingly, their use in the fishing nets was made self-imposed control in 1987, and further in the ship-bottom paints they have been substituted for triphenyltin compounds such as triphenyltin fluoride (Ph_3SnF), triphenyltin chloride (Ph_3SnCl), triphenyltin hydroxide (Ph_3SnOH), etc.. At present, the contamination of the fish and shellfish in the environment with the triphenyltin compounds as well as the tri-n-butyltin compounds is a serious problem in Japan. The accumulation of $(Bu_3Sn)_2O$ and Ph_3SnCl in three species of fresh-water fish (carp, round crucian carp and goldfish) was already described in chapter 2. In this study, we revealed that the BCF values of $(Bu_3Sn)_2O$ were two - five times higher than those of Ph_3SnCl. In these accumulation experiments, the pH of the test water was maintained approximately neutral. To exactly evaluate their accumulation in the fish and shellfish which are living in their contaminated lake or sea, the

same accumulation experiments should be further carried out at various pH of the test water.

In this study,[7] we researched the influence of pH (6.0, 6.8 and 7.8) on the accumulation of Bu_3SnCl and Ph_3SnCl as representative tri-n-butyltin and triphenyltin compounds in fresh-water fish (carp).

Experimental Section

Chemicals

Tri-n-butyltin chloride (Bu_3SnCl), purity 95%, and triphenyltin chloride (Ph_3SnCl), purity 98%, were purchased from Tokyo Chemical Industry (Tokyo, Japan) and used without further purification.

Test fish

Carp (*Cyprinus carpio* L.) were purchased from Nango Suisan Center (Shiga Prefecture, Japan). The body length and body weight of the fish were 8.5 - 9.5 cm and 16.5 - 22.1 g, respectively. Tri-n-butyltin and triphenyltin compounds were not detected in the fish before exposure to these chemicals. Commercial assorted feed was given 0.5 g/fish once a day throughout the experiment period.

Test system

The study was carried out without aeration under the continuous flow through system. The experimental arrangement was the same as in The BCF values of $(Bu_3Sn)_2O$ (500 - 3200) were about two - five times as chapter 2. Aquaria tanks made of acrylic plastic holding about 50 *l*. were used. During the tests (14 days), the flow rate and temperature of each test water were maintained 18 *l*/h and 24±1°C, respectively. The concentrations of dissolved

oxygen and hardness for all tests were more than 7.0 mg/*l* and 35.4 - 39.0 mg/*l* as $CaCO_3$, respectively. Measurements were carried out once after 3 days exposure. Three stock solutions (acidic, neutral and alkaline) of Bu_3SnCl and Ph_3SnCl (each 400 μg/*l*) were respectively diluted 150 times continuously with dechlorinated city water (pH 6.8) and supplied to each of the three aquaria containing 15 fish. The neutral stock solution was prepared by diluting a 4 m*l* of ethanol solution of Bu_3SnCl and Ph_3SnCl (each 1000 μg/m*l*) with water to 10 *l*. Acidic and alkaline stock solutions were prepared by diluting the same volumes of the ethanol solution with water to 10 *l* after addition of 30 m*l* of 35% HCl and 30 m*l* of 40% NaOH, respectively. The pH of each test tank (A, B, C) water was mean, n=12 (range) of 6.0 (5.9 - 6.1), 6.8 (6.7 - 6.8) and 7.8 (7.7 - 7.9), respectively. Measurements were carried out almost every day. The concentrations of Bu_3SnCl and Ph_3SnCl were mean±SD, n=6 (range) of 1.8±0.2 (1.5 - 2.0) and 1.1±0.1 (1.0 - 1.3) μg/*l* in tank A, 1.6±0.2 (1.4 - 1.9) and 1.2±0.2 (1.0 - 1.5) μg/*l* in tank B, and 1.7±0.1 (1.7 - 1.8) and 1.2±0.1 (1.0 - 1.4) μg/*l* in tank C. Measurements were carried out at 0, 1, 3, 7, 10 and 14 days. Three fish were taken at 1, 3, 7, 10 and 14 days in each experiment. All of the fish were frozen and preserved for analysis after rinsing with distilled water.

Under these conditions, none of the fish showed signs of tiredness or agitation during the experiments.

Analysis

Determination of Bu_3SnCl and Ph_3SnCl in the water and fish samples was carried out by the same method as in chapter 2. All of the thawed fish samples were separately homogenized for whole body and analyzed as a sample for each of them.

Calculation of bioconcentration factor (BCF)

BCF was calculated by the following equation.

$$BCF = \frac{\text{chemical concentration in whole body of fish}}{\text{chemical concentration in water}}$$

The chemical concentration in the water at each sampling time was used for the calculation of BCF.

Measurement of partition coefficient between n-octanol and water (P_{ow})

Here as a reference data for discussion, P_{ow} of Bu_3SnCl and Ph_3SnCl for the pH 5.8 - 8.0 buffer solutions as water was separately measured two times by flask-shaking method. A 10 m*l* of test chemical in n-octanol (1000 μg/m*l*) was shaken with 10 m*l* of 0.1M Na_2HPO_4 - NaH_2PO_4 buffer solutions (pH 5.8 - 8.0), respectively, for 4 hr at 25±1°C in a closed centrifuge tube. After centrifugation (3000 rpm, 30 min), the two phases were separated and analyzed in the same manner as chapter 2.

Statistical analysis

One-way analysis of variance was used to detect significant differences in the BCF of Bu_3SnCl and Ph_3SnCl between the pH values of the test water.

Results and Discussion

The influence of pH on the accumulation of Bu_3SnCl and Ph_3SnCl in the whole body of carp is shown in Figs.1 and 2, respectively, over the 14 days exposure period. The BCF values of both Bu_3SnCl and Ph_3SnCl reached plateaus after 7 - 10 days exposure in all pH values of the test water. The

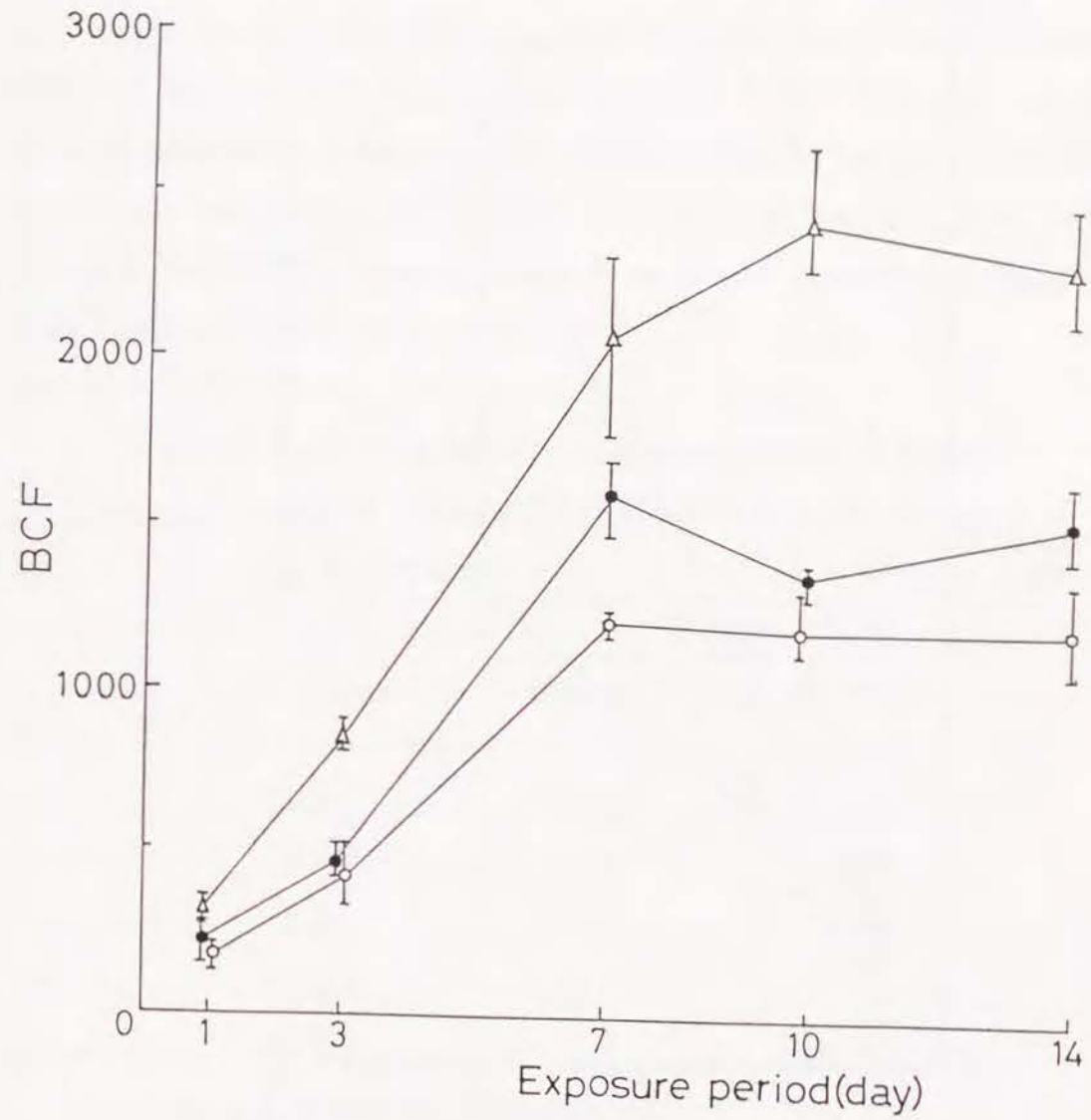

Fig.1 Influence of pH on Bu_3SnCl accumulation in whole body of carp.

○ pH 6.0 ● pH 6.8 △ pH 7.8

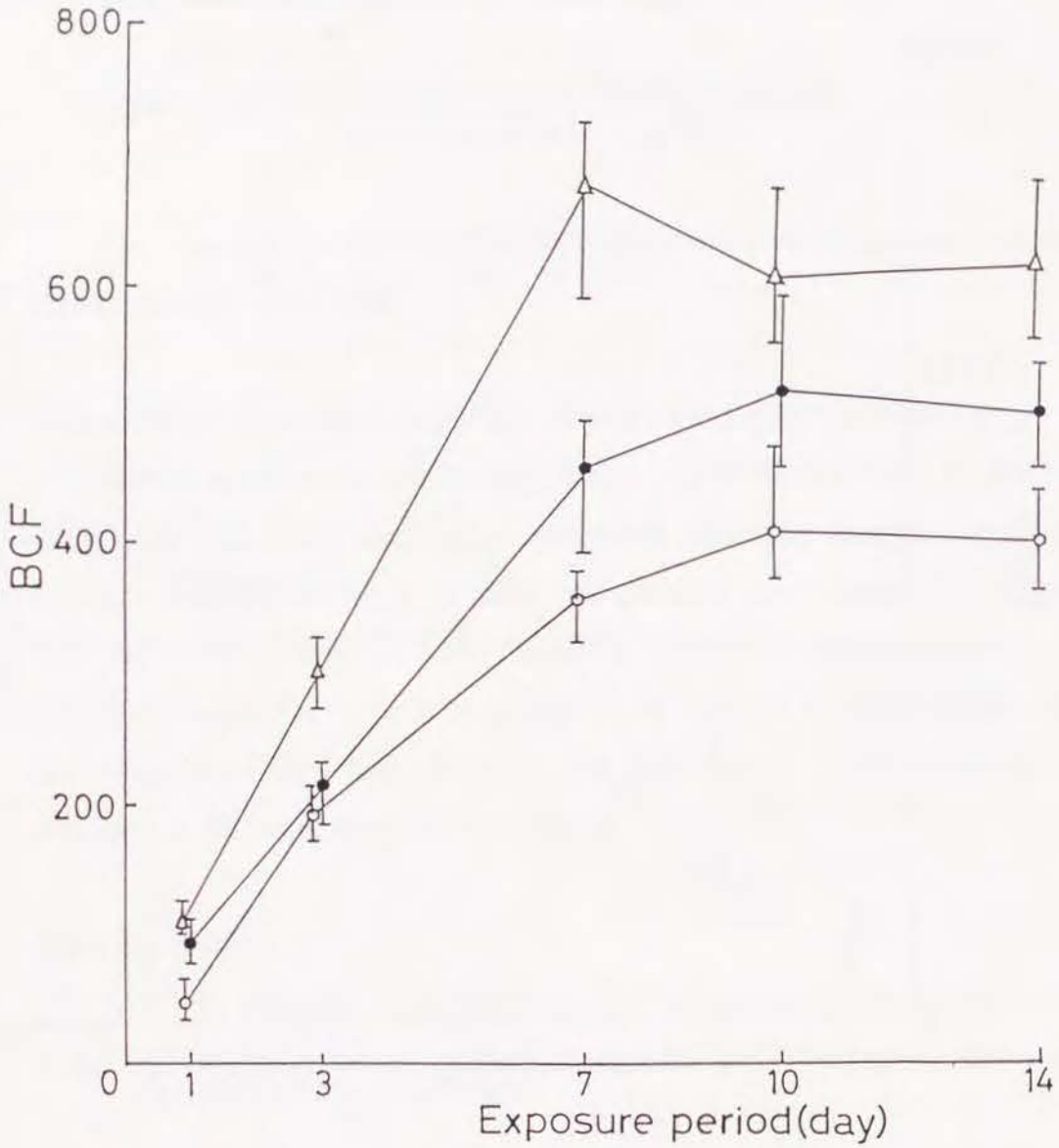

Fig.2 Influence of pH on Ph_3SnCl accumulation in whole body of carp.

○ pH 6.0 ● pH 6.8 △ pH 7.8

order of the BCF values at these pH values was pH 7.8 > pH 6.8 > pH 6.0 for both Bu_3SnCl and Ph_3SnCl over the 14 days exposure period. The average BCF ratios (n=5) of Bu_3SnCl and Ph_3SnCl at pH 6.0, 6.8 and 7.8 were 1:1.2:1.9 and 1:1.4:1.8, respectively, over the 14 days exposure period. These ratios were approximately constant throughout the period. That is, the coefficients of variation in the values 1.2 and 1.9 of Bu_3SnCl were 7.4% and 7.5%, respectively. Similarly, those in the values 1.4 and 1.8 of Ph_3SnCl were 21.6% and 19.1%, respectively.

Table 1. Analysis of variance to detect significant differences in BCF of Bu_3SnCl and Ph_3SnCl between pH values of the test water.

Exposure	F-ratio	
	Bu_3SnCl	Ph_3SnCl
1 day	6.0[a]	12.5[b]
3 day	34.2[c]	15.3[b]
7 day	17.0[b]	21.9[b]
10 day	73.3[c]	7.1[a]
14 day	39.2[c]	13.8[b]

[a] Significant at the 5% level.

[b] Significant at the 1% level.

[c] Significant at the 0.1% level.

The results of statistical analysis are shown in Table 1. This analysis was carried out at each sampling time (1, 3, 7, 10 and 14 days). There were

significant differences ($p < 0.05$ - $p < 0.001$) in the BCF values of both Bu_3SnCl and Ph_3SnCl between the pH values at every sampling time.

It is generally known that the undissociated forms of certain organic chemicals can penetrate biological membranes much more easily than the dissociated forms. Assuming that most of the Bu_3SnCl and Ph_3SnCl accumulated in carp are mainly the undissociated forms, it seems that the increase of Bu_3SnCl and Ph_3SnCl concentrations in carp is due to the change of the chemical forms of Bu_3SnCl and Ph_3SnCl from the dissociated (Bu_3Sn^+ and Ph_3Sn^+) to the undissociated forms (Bu_3SnOH and Ph_3SnOH) with an increase in pH.

As a reference data to explain the above assumption, P_{ow} of Bu_3SnCl and Ph_3SnCl for the pH 5.8 - 8.0 buffer solutions as water was measured and is shown in Table 2. The P_{ow} values of both Bu_3SnCl and Ph_3SnCl increased with an increase in pH. It is presumed that the chemical forms of Bu_3SnCl and Ph_3SnCl change from the dissociated to the undissociated ones with an increase in pH. For Ph_3SnCl, the BCF ratio (1:1.4:1.8) with pH 6.0, 6.8 and 7.8 was nearly equal to the same P_{ow} ratio (1:1.2:2.1). But for Bu_3SnCl, the BCF ratio (1:1.2:1.9) was slightly different from the P_{ow} ratio (1:2.4:3.9). Ph_3SnCl showed a higher correlation between the P_{ow} and BCF values than Bu_3SnCl in the range of pH 6.0 - 7.8. From the results, it is presumed that linear correlations exist between the P_{ow} and BCF values measured at various pH for such ionic organic chemicals as Ph_3SnCl and Bu_3SnCl.

Further, it is already well known that a linear correlation exists between the P_{ow} and BCF values particularly for similar organic chemicals.[8-10] Here, the BCF ratios (Bu_3SnCl/Ph_3SnCl) after 4 days exposure were compared with the same P_{ow} ratios at pH 6.0, 6.8 and 7.8, respectively. The BCF and P_{ow} ratios were 3.0 and 1.3 at pH 6.0, 2.5 and 2.7 at pH 6.8, and 3.7 and 2.5

Table 2. P_{ow} of Bu_3SnCl and Ph_3SnCl.

	P_{ow} [a]	
pH	Bu_3SnCl	Ph_3SnCl
5.8	1608	1182
6.0	1841	1397
6.2	2369	1490
6.4	3226	1502
6.6	3750	1592
6.8	4329	1629
7.0	5015	2058
7.2	6098	2410
7.4	6726	2415
7.6	6962	2475
7.8	7137	2890
8.0	12195	3650

[a] Mean value (n=2)

at pH 7.8. Both values agreed very closely at pH 6.8 compared with those at pH 6.0 and 7.8. It is presumed that a higher correlation exists between the P_{ow} and BCF values for these ionic organic chemicals under neutral conditions than under acidic and alkaline conditions.

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CHAPTER 4

Excretion of Tri-n-butyltin and Triphenyltin Compounds from Freshwater-Fishes

Introduction

In chapter 2, the accumulation of butyltin and phenyltin compounds in freshwater-fishes was studied to evaluate the contamination of fishes in lake and sea by these compounds. Here, the excretion of tri-n-butyltin and triphenyltin compounds from carp and goldfish was further studied to evaluate the residue of these compounds in freshwater-fishes.[1,2] The excretion rate of these compounds from the fishes was calculated by assuming the excretion process to be first-order kinetics. These data will be useful for the evaluation of their safety to man as well as their contamination of fishes in lake and sea.

Experimental Section

Reagent

$(Bu_3Sn)_2O$ and Ph_3SnCl, each reagent grade, were purchased from Tokyo Chemical Industry (Tokyo, Japan) and used without further purification.

Test fish

Carp (*Cyprinus carpio* L.) were purchased from Nango Suisan Center (Shiga Prefecture, Japan) and goldfish (*Carassius aurapus)* from Yamato Kohriyama (Nara Prefecture, Japan). The body length and weight were 10.0

- 11.0 cm and 22.9 - 30.4 g for carp, and 2.8 - 3.5 cm and 0.9 - 1.7 g for goldfish. They were fed once every day in this experiment.

Test system

The study was carried out by the same experimental arrangement as in chapter 2.

For the excretion of $(Bu_3Sn)_2O$ and Ph_3SnCl from carp (3 days), 15 fish were exposed to these compounds [$(Bu_3Sn)_2O$ 1.8 μg/*l* and Ph_3SnCl 2.1 μg/*l*] for 7 days, 12 fish except 3 fish were transferred into an aquaria tank containing clean water, and dechlorinated city water was supplied to it. During the test, the flow rate and temperature of the test water were maintained at 1 *l*/min and 22±1°C, respectively. The concentrations of these compounds excreted from carp in the test tank water were 0.12 μg/*l* as Bu_3SnCl and 0.10 μg/*l* as Ph_3SnCl after 19 h in the excretion experiment.

For the excretion of $(Bu_3Sn)_2O$ and Ph_3SnCl from goldfish (14 days), 20 fish remaining in the test tank after their accumulation experiment (chapter 2) were transferred into an aquaria tank containing clean water, and dechlorinated city water was supplied to it. During the test, the flow and temperature of the test water were maintained at 1 *l*/min and 18±1°C, respectively. The concentrations of these compounds excreted from the fish in the test tank water were less than 0.1 μg/*l* as Bu_3SnCl and Ph_3SnCl, respectively, after 18 h in the excretion experiment. From the low concentrations of these compounds in the test tanks excreted from the carp and goldfish, their accumulation in both fishes was presumed to be very little.

Analysis

Determination of $(Bu_3Sn)_2O$ and Ph_3SnCl in carp and goldfish was carried out by the same method as in chapter 2.

For carp, three fish were taken at the fixed sampling times (days 0, 1, 2 and 3). Analyses were carried out as three separate samples for muscle, and as a mixture of three samples for liver, kidney and gallbladder.

For goldfish, three fish were taken at the fixed sampling times (days 0, 1, 3, 7 and 14). Analyses were carried out as a sample for each fish.

Calculation of excretion rate constant

The following equation was used for the calculation of excretion rate constant. $C = C_0e^{-kt}$

where

C = concentration of $(Bu_3Sn)_2O$ or Ph_3SnCl in fish (μg/g)

C_0 = initial concentration of $(Bu_3Sn)_2O$ or Ph_3SnCl in fish (μg/g)

k = excretion rate constant (day^{-1})

t = time (day)

Results and Discussion

Excretion of $(Bu_3Sn)_2O$ and Ph_3SnCl from carp

Excretion of $(Bu_3Sn)_2O$ and Ph_3SnCl from the four tissues of carp is shown in Figs.1 and 2, respectively. The concentrations of $(Bu_3Sn)_2O$ and Ph_3SnCl in the muscle decreased, but those in the viscera (liver, kidney and gallbladder) did not until 3 days. The constancy of $(Bu_3Sn)_2O$ and Ph_3SnCl in the viscera was probably because the excretion of $(Bu_3Sn)_2O$ and Ph_3SnCl was equilibrated with the intake of these compounds which was derived from the muscle and other tissues except the viscera.

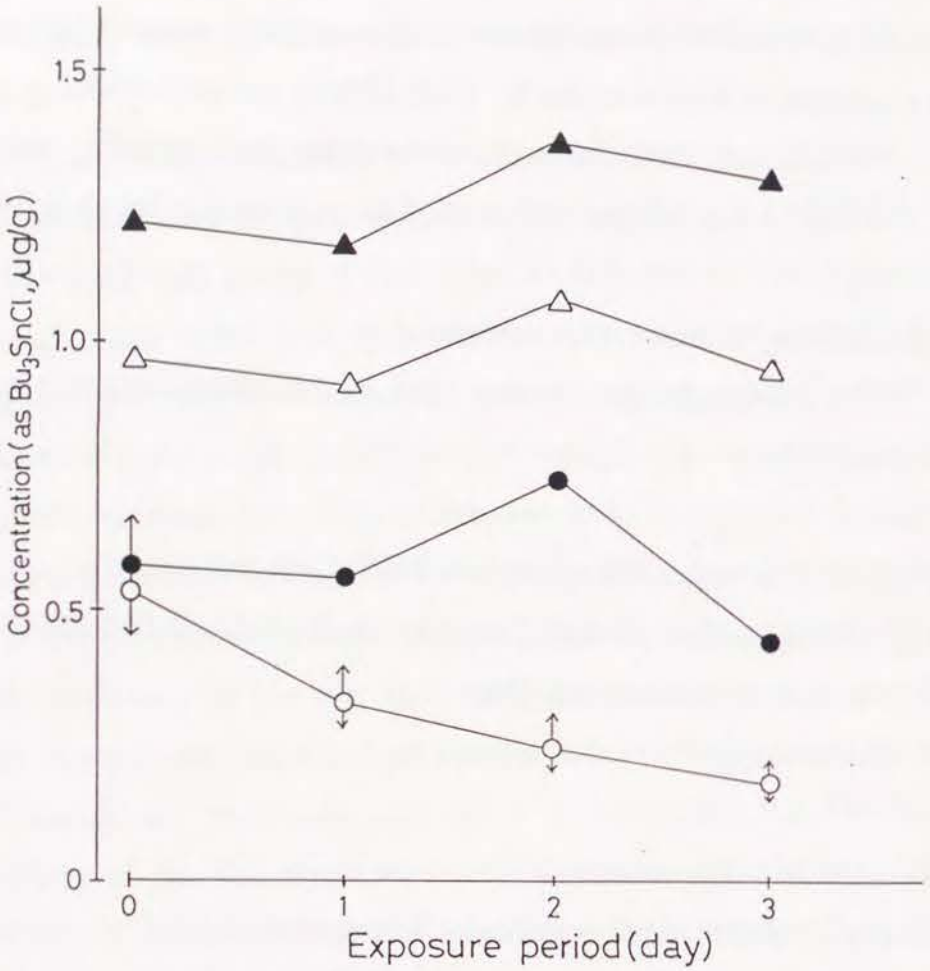

Fig.1 Excretion of $(Bu_3Sn)_2O$ from carp.

○ Muscle ● Liver

△ Kidney ▲ Gallbladder

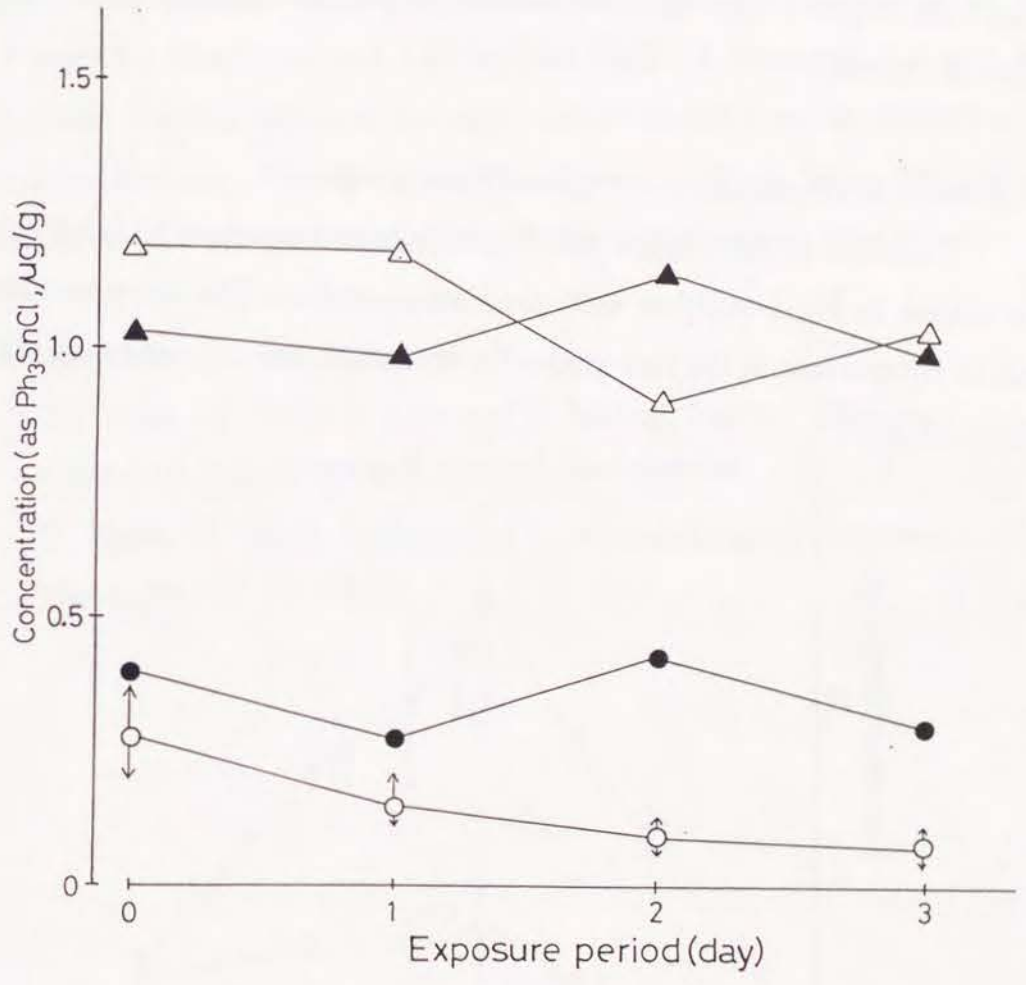

Fig.2 Excretion of Ph_3SnCl from carp.

○ Muscle ● Liver

△ Kidney ▲ Gallbladder

Excretion rate constant (k) of $(Bu_3Sn)_2O$ and Ph_3SnCl in the muscle was calculated by assuming their processes to be first-order kinetics from the data in Figs.1 and 2. The values of k were 0.4 day^{-1} for $(Bu_3Sn)_2O$ and 0.5 day^{-1} for Ph_3SnCl. The excretion rate of $(Bu_3Sn)_2O$ was slightly slower than that of Ph_3SnCl.

Excretion of $(Bu_3Sn)_2O$ and Ph_3SnCl from goldfish

Excretion of $(Bu_3Sn)_2O$ and Ph_3SnCl from the whole body of goldfish is shown in Fig.3 together with their accumulation. The concentrations of these compounds in the fish gradually decreased, but did not reach plateaus until 14 days.

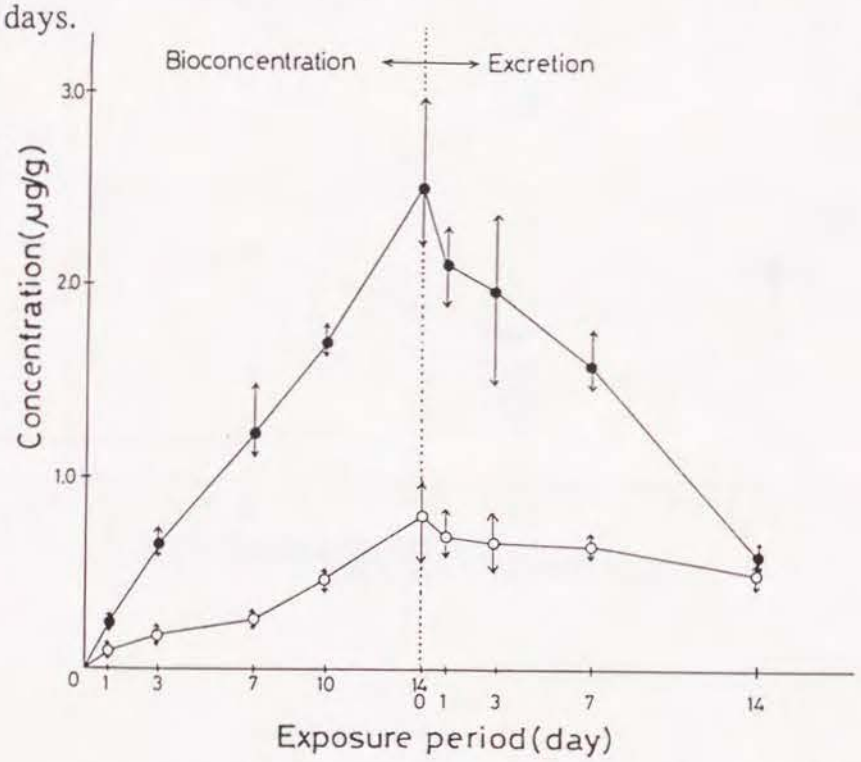

Fig.3 Excretion of $(Bu_3Sn)_2O$ and Ph_3SnCl from whole body of goldfish.

● $(Bu_3Sn)_2O$ ○ Ph_3SnCl

The rate constant (k) of these compounds was calculated by the above equation from the data in Fig.3. The values of k were 0.08 day^{-1} for $(Bu_3Sn)_2O$ and 0.04 day^{-1} for Ph_3SnCl. Their excretion rate from the whole body of goldfish was considerably slower than that from the muscle of carp, 0.4 day^{-1} for $(Bu_3Sn)_2O$ and 0.5 day^{-1} for Ph_3SnCl. However, the rate from the whole body of carp was probably as slow as that from the whole body of goldfish because the rate from the tissues such as liver, kidney and gallbladder in carp was considerably slower than that from the muscle.

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CHAPTER 5

Metabolism of Tri-n-butyltin and Triphenyltin Compounds in Freshwater-Fishes

Introduction

In chapter 2 - 4, the accumulation and excretion of butyltin and phenyltin compounds by fishes were studied to evaluate the contamination of fishes in lake and sea by these compounds.

In view of the contamination of fishes in the environment by the metabolites of tri-n-butyltin and triphenyltin compounds (Bu_2Sn^{2+}, $BuSn^{3+}$, Ph_2Sn^{2+}, $PhSn^{3+}$ and Sn^{4+}), experimental data on their metabolism in fishes are useful for the evaluation of their contamination in the fishes.

However, few reports have been published regarding the metabolism of these compounds in fishes in spite of many studies on their metabolism in mammals.[1-6]

Therefore, we studied the metabolism of $(Bu_3Sn)_2O$ and Ph_3SnCl as representative tri-n-butyltin and triphenyltin compounds in carp[7,8] and goldfish.[9]

Experimental Section

Chemicals

The same butyltin and phenyltin compounds as in chapter 2 were used without further purification.

Test system

The metabolism of $(Bu_3Sn)_2O$ and Ph_3SnCl in carp and goldfish was investigated simultaneously with the accumulation experiments on the same fishes in chapter 2. That is, analyses of fish samples at each sampling time were carried out for Bu_2Sn^{2+}, $BuSn^{3+}$, Ph_2Sn^{2+}, $PhSn^{3+}$ and inorganic tin (Sn^{4+}) which were reported to be the main metabolites of tri-n-butyltin and triphenyltin compounds in mammals.[1-6]

Analysis

Determination of the metabolites except inorganic tin in fish samples was carried out by our proposed method described in chapter 1. Inorganic tin in the muscle of carp was analyzed as Sn^{4+} by the method of Iwai et al.[10] Analysis of Bu_2Sn^{2+}, $BuSn^{3+}$, Ph_2Sn^{2+} and $PhSn^{3+}$ was carried out as a mixture of three samples at each sampling time for muscle, liver, kidney and gallbladder of carp and whole body of goldfish.

Results

Metabolism of $(Bu_3Sn)_2O$ and Ph_3SnCl in carp

The concentrations of metabolites in the four tissues of carp are shown in Figs.1 - 4 for $(Bu_3Sn)_2O$ and in Figs.5 - 8 for Ph_3SnCl during the exposure time [$(Bu_3Sn)_2O$ 14 days, Ph_3SnCl 10 days].

The concentrations of Bu_2Sn^{2+} and $BuSn^{3+}$ were much lower than that of $(Bu_3Sn)_2O$ in the muscle of carp. But in the viscera, the concentrations of Bu_2Sn^{2+} and particularly $BuSn^{3+}$ were higher than those in the muscle. Bu_2Sn^{2+} and $BuSn^{3+}$ did not reach a plateau in any of the four tissues of the carp during the exposure time. For inorganic tin, the concentration of Sn^{4+} in the muscle was constant during the exposure time.

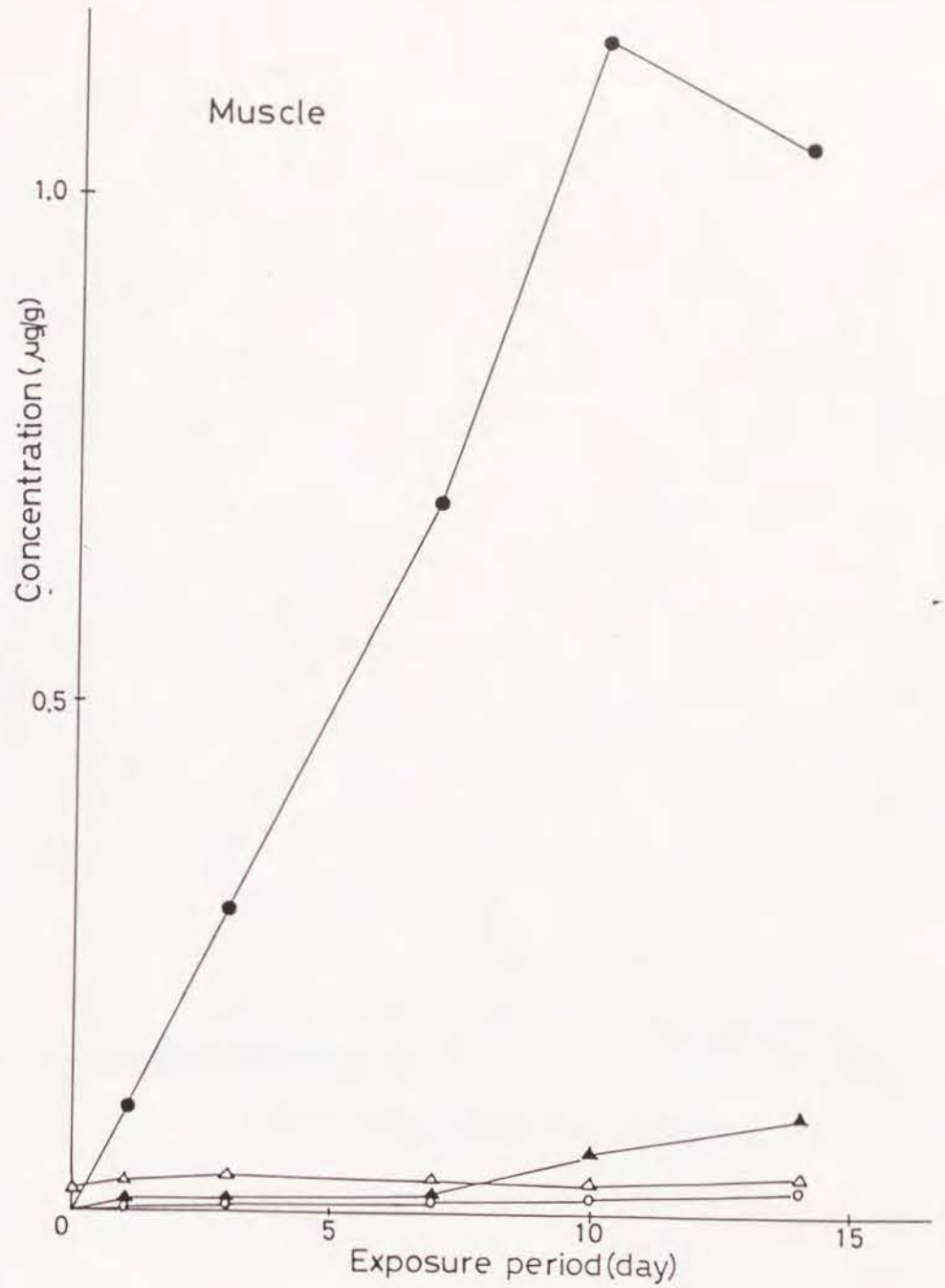

Fig.1 Contents of $(Bu_3Sn)_2O$ and its metabolites in the muscle of carp.

● $(Bu_3Sn)_2O$ (as Bu_3SnCl)　　○ Bu_2Sn^{2+} (as Bu_2SnCl_2)

▲ $BuSn^{3+}$ (as $BuSnCl_3$)　　△ Sn^{4+}

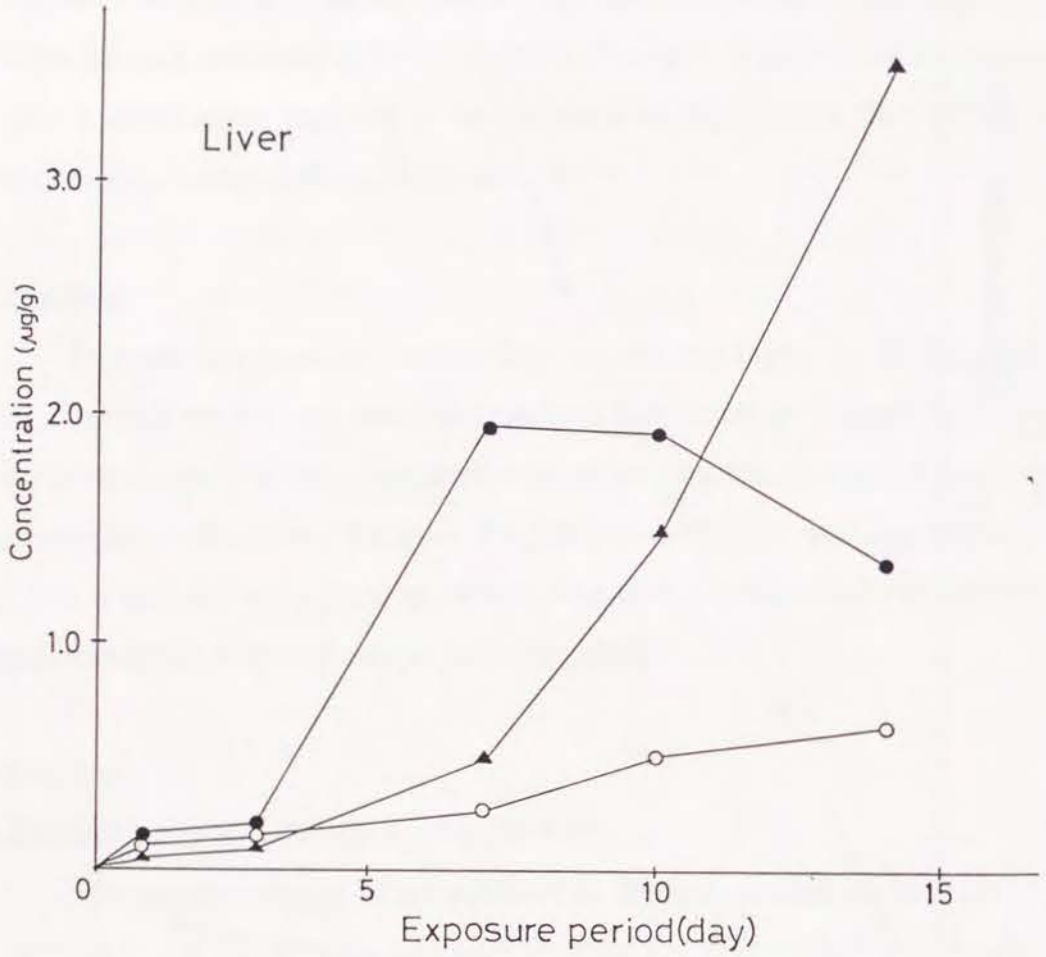

Fig.2 Contents of $(Bu_3Sn)_2O$ and its metabolites in the liver of carp.

● $(Bu_3Sn)_2O$ (as Bu_3SnCl)　○ Bu_2Sn^{2+} (as Bu_2SnCl_2)

▲ $BuSn^{3+}$ (as $BuSnCl_3$)

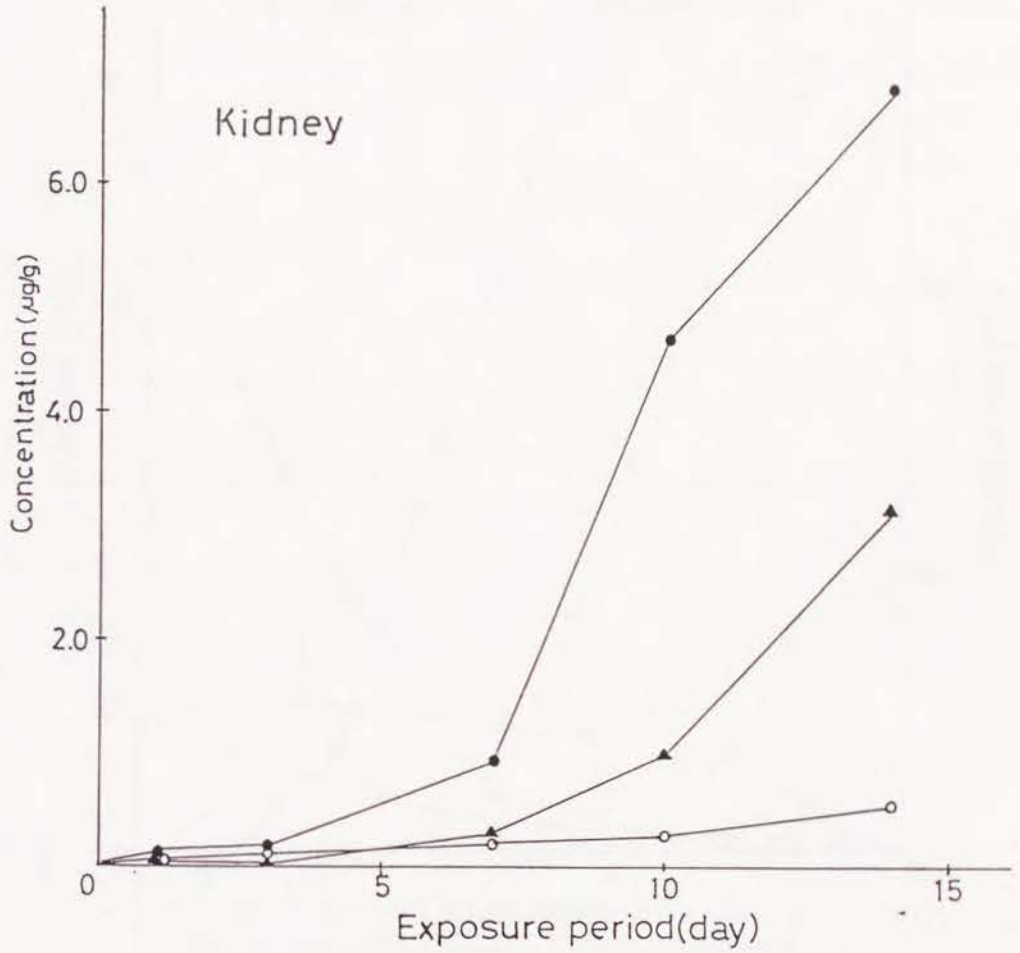

Fig.3 Contents of $(Bu_3Sn)_2O$ and its metabolites in the kidney of carp.

● $(Bu_3Sn)_2O$ (as Bu_3SnCl)　○ Bu_2Sn^{2+} (as Bu_2SnCl_2)

▲ $BuSn^{3+}$ (as $BuSnCl_3$)

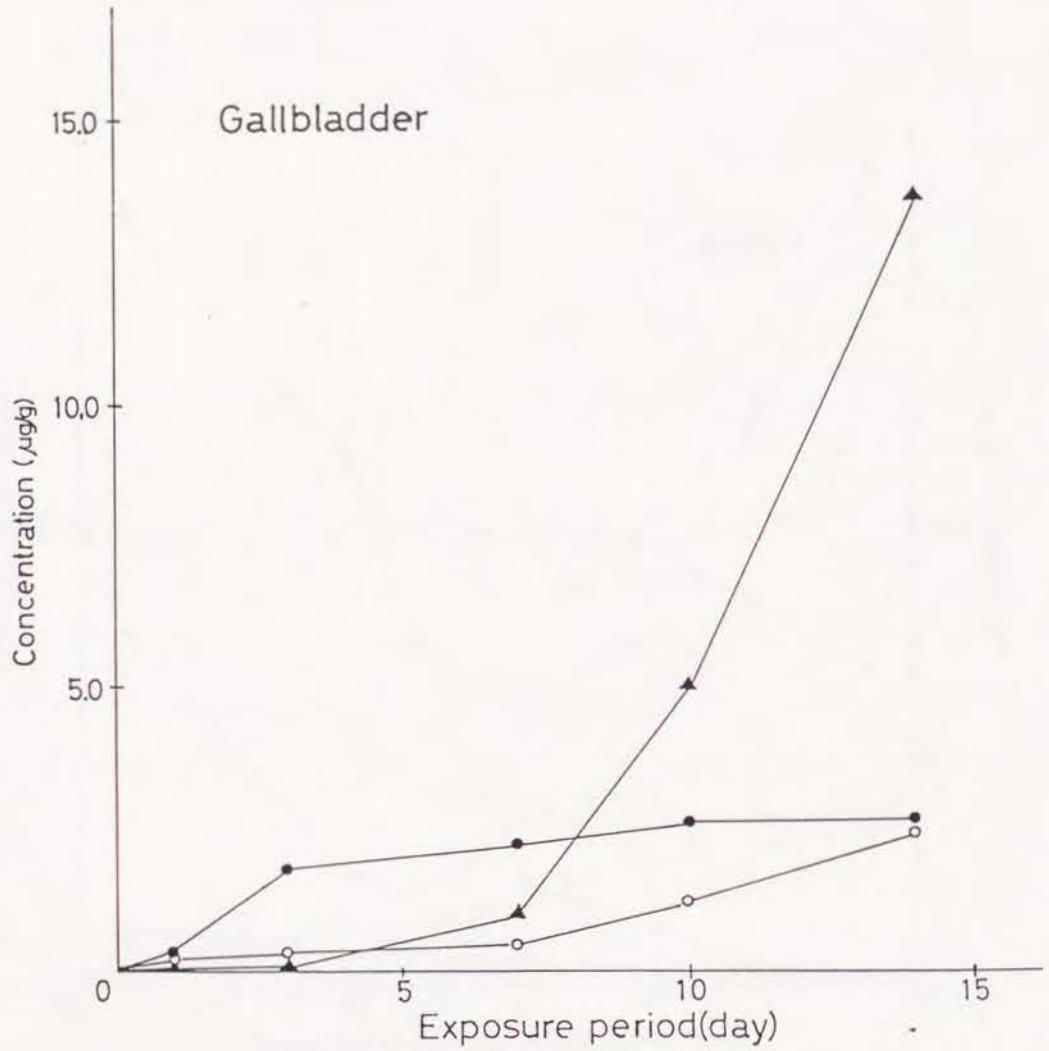

Fig.4 Contents of $(Bu_3Sn)_2O$ and its metabolites in the gallbladder of carp.

● $(Bu_3Sn)_2O$ (as Bu_3SnCl)　　○ Bu_2Sn^{2+} (as Bu_2SnCl_2)

▲ $BuSn^{3+}$ (as $BuSnCl_3$)

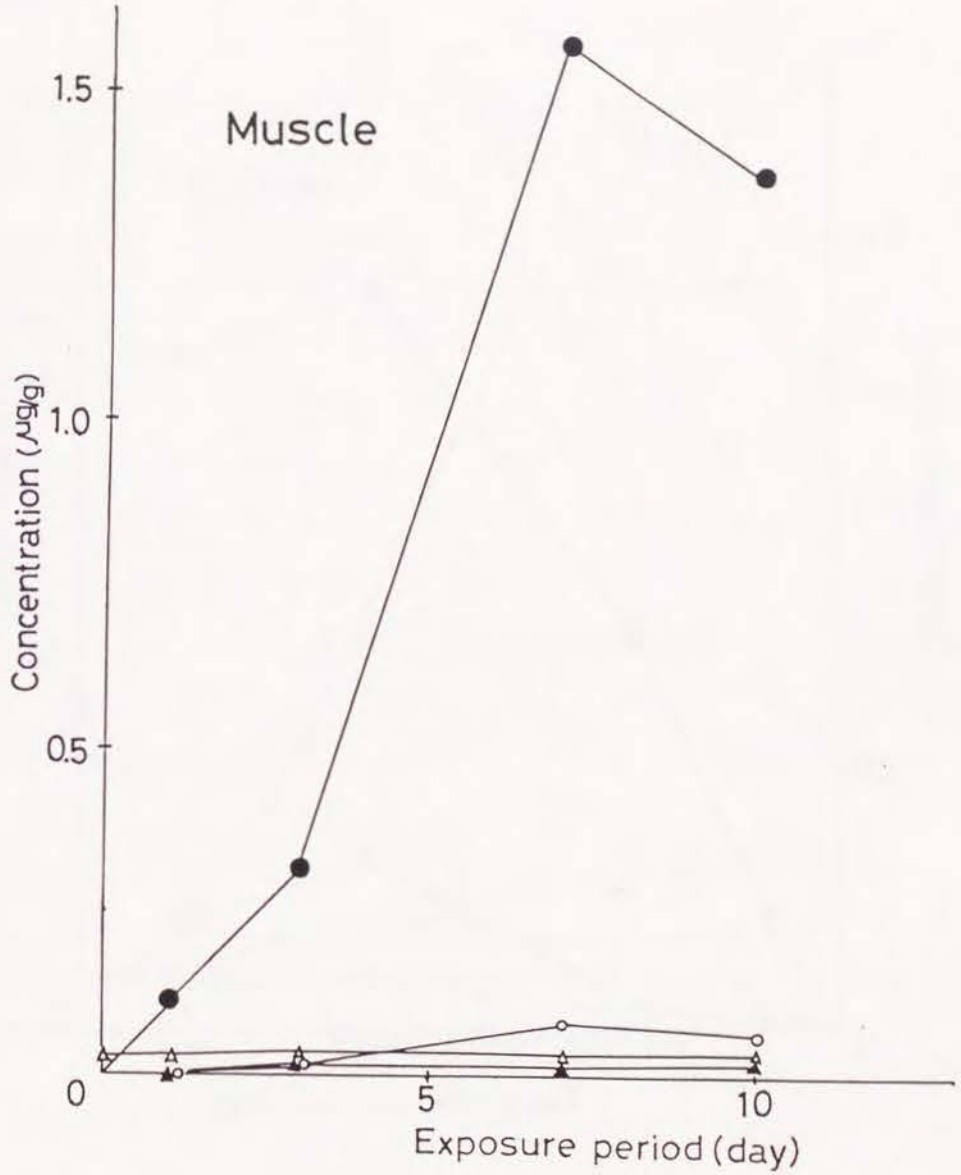

Fig.5 Contents of Ph_3SnCl and its metabolites in the muscle of carp.

● Ph_3SnCl　　○ Ph_2Sn^{2+} (Ph_2SnCl_2)

▲ $PhSn^{3+}$ ($PhSnCl_3$)　　△ Sn^{4+}

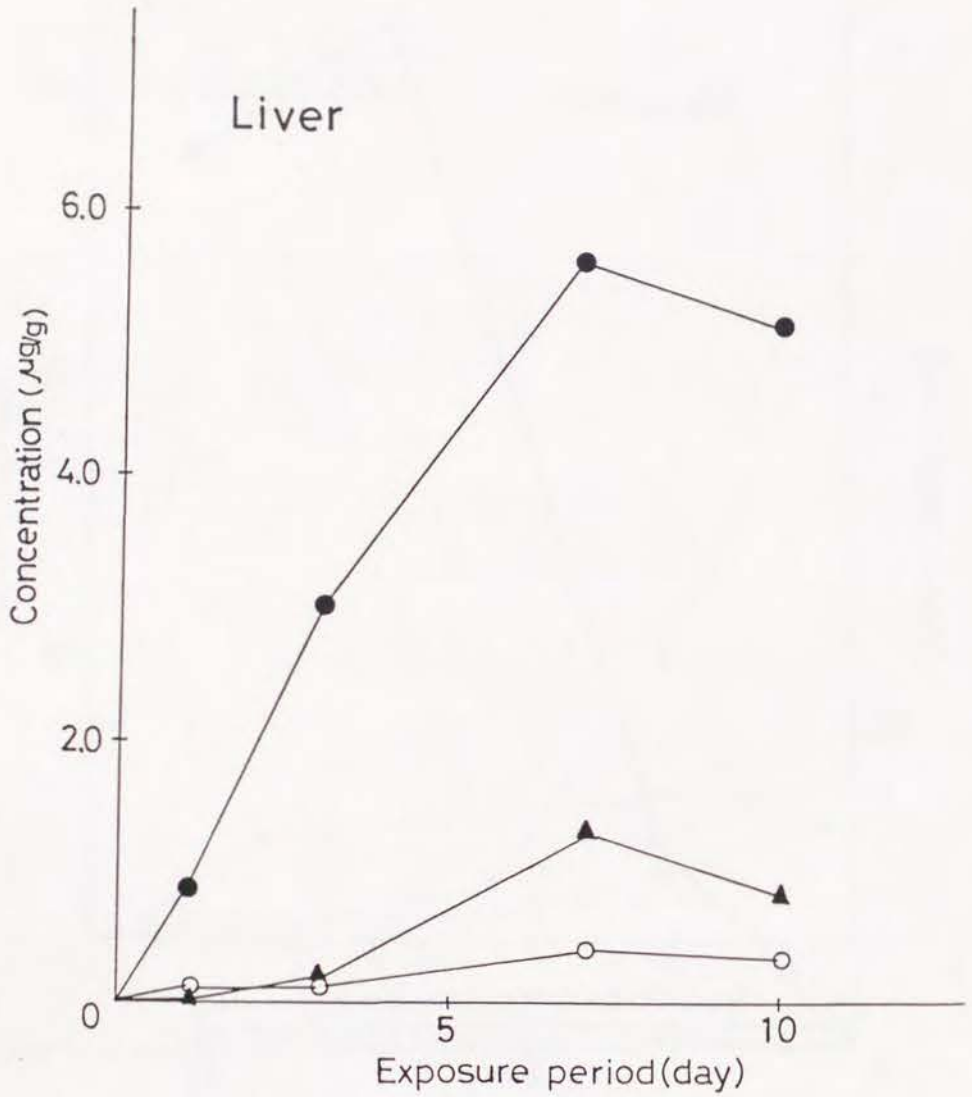

Fig.6 Contents of Ph_3SnCl and its metabolites in the liver of carp.

● Ph_3SnCl ○ Ph_2Sn^{2+} (Ph_2SnCl_2)

▲ $PhSn^{3+}$ ($PhSnCl_3$)

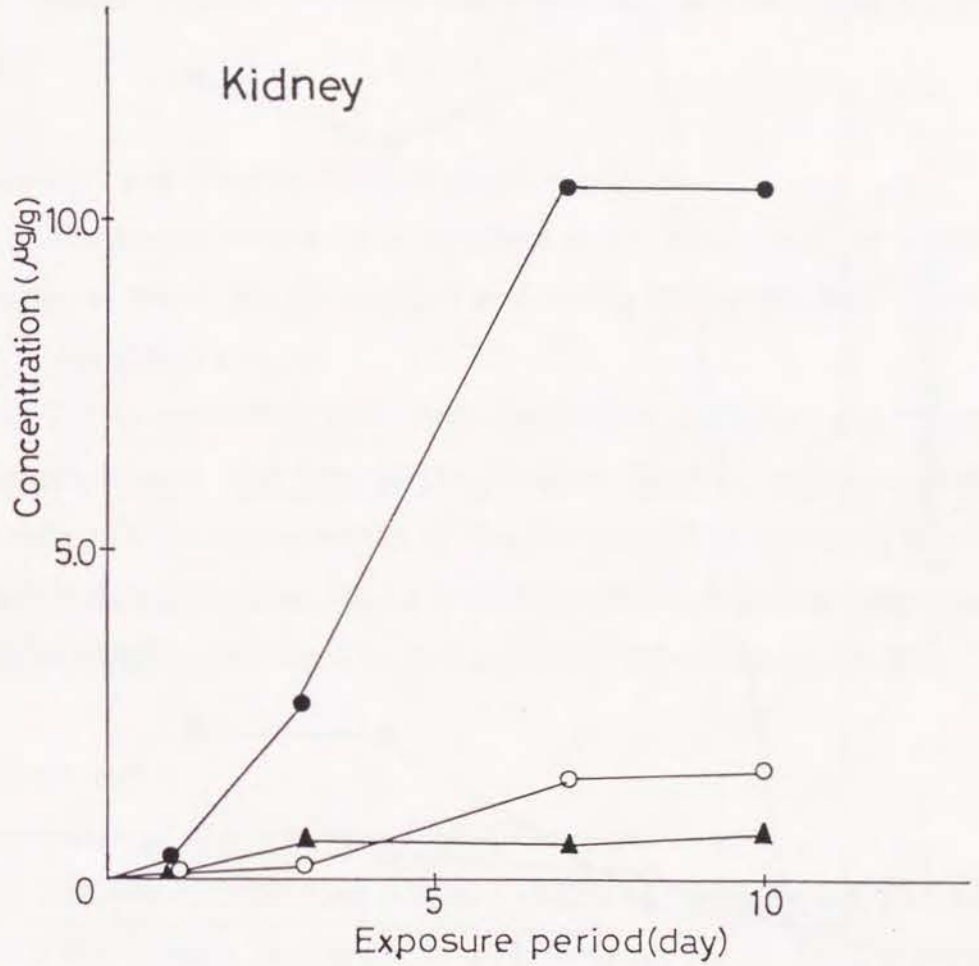

Fig.7 Contents of Ph_3SnCl and its metabolites in the kidney of carp.

● Ph_3SnCl ○ Ph_2Sn^{2+} (Ph_2SnCl_2)

▲ $PhSn^{3+}$ ($PhSnCl_3$)

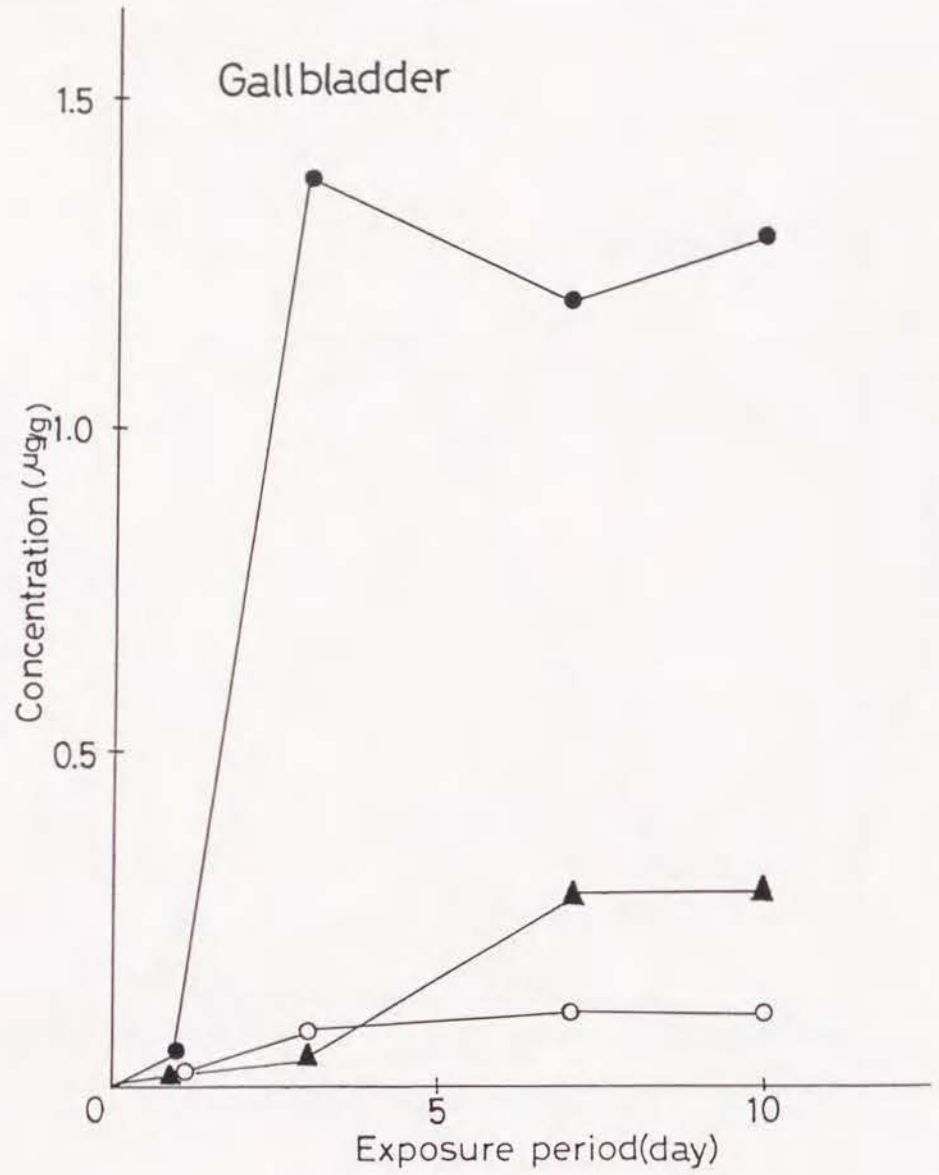

Fig.8 Contents of Ph_3SnCl and its metabolites in the gallbladder of carp.

● Ph_3SnCl ○ Ph_2Sn^{2+} (Ph_2SnCl_2)

▲ $PhSn^{3+}$ ($PhSnCl_3$)

The concentrations of Ph_2Sn^{2+} and $PhSn^{3+}$ were much lower than those of Ph_3SnCl in all of them. But Ph_2Sn^{2+} and $PhSn^{3+}$ reached plateaus after approximately the same period (7 - 10 days) as Ph_3SnCl. For inorganic tin, the concentration of Sn^{4+} in the muscle was also constant during the exposure time.

Metabolism of $(Bu_3Sn)_2O$ and Ph_3SnCl in goldfish

The concentrations of metabolites in the whole body of goldfish are shown in Fig.9 for $(Bu_3Sn)_2O$ and in Fig.10 for Ph_3SnCl during the exposure time (14 days).

The concentrations of Bu_2Sn^{2+} and $BuSn^{3+}$ or Ph_2Sn^{2+} and $PhSn^{3+}$ were also much lower than those of $(Bu_3Sn)_2O$ or Ph_3SnCl. In the metabolism of $(Bu_3Sn)_2O$, the concentration of Bu_2Sn^{2+} was slightly higher than that of $BuSn^{3+}$ during the time. For the metabolism of Ph_3SnCl, the concentration of Ph_2Sn^{2+} slightly increased but that of $PhSn^{3+}$ did not during the time.

Discussion

Metabolism of $(Bu_3Sn)_2O$ and Ph_3SnCl in carp

The low accumulation potential (BCF) of Ph_2SnCl_2 and $PhSnCl_3$ (see Table 4 in chapter 2) is probably an explanation for the low concentrations of metabolized Ph_2Sn^{2+} and $PhSn^{3+}$ in the four tissues of carp. For example, the concentration ratio of Ph_3SnCl, Ph_2Sn^{2+} (Ph_2SnCl_2) and $PhSn^{3+}$ ($PhSnCl_3$) in the muscle was 1:0.043:0.014 in the metabolism experiment of Ph_3SnCl. On the other hand, the BCF ratio of these phenyltin chlorides in the muscle was 1:0.030:0.012. From the agreement of both ratios, it was presumed that the concentrations of metabolized Ph_2Sn^{2+} and $PhSn^{3+}$ in the muscle were ruled mainly by their accumulation potential. This could

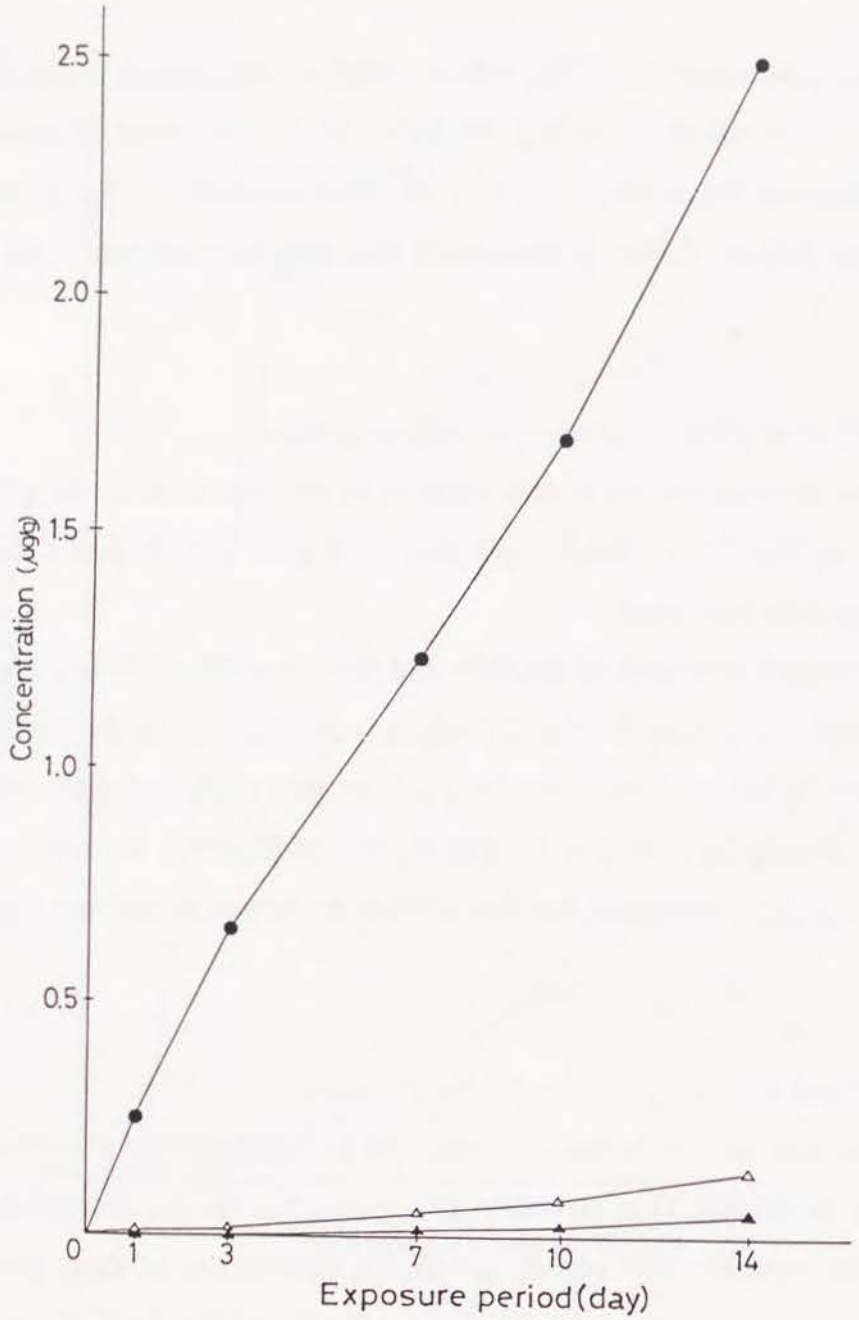

Fig.9 Metabolism of $(Bu_3Sn)_2O$ in goldfish.

● $(Bu_3Sn)_2O$ (Bu_3SnCl) △ Bu_2Sn^{2+} (Bu_2SnCl_2) ▲ $BuSn^{3+}$ $(BuSnCl_3)$

Fig. 10 Metabolism of Ph_3SnCl in goldfish.

○ Ph_3SnCl □ Ph_2Sn^{2+} (Ph_2SnCl_2) ■ $PhSn^{3+}$ $(PhSnCl_3)$

be similarly applied to the viscera such as liver and gallbladder. Further, the constancy of inorganic tin (Sn^{4+}) in the muscle was probably because the metabolized Sn^{4+} was little accumulated during the exposure time.

The concentrations of metabolized Bu_2Sn^{2+} and $BuSn^{3+}$ in the muscle were low as were those of Ph_2Sn^{2+} and $PhSn^{3+}$ in the similar metabolism experiment with Ph_3SnCl. However, those of metabolized Bu_2Sn^{2+} and $BuSn^{3+}$ in the viscera were high, which differed to that of Ph_3SnCl. In particular, the concentration of $BuSn^{3+}$ was greater than that of $(Bu_3Sn)_2O$ in the liver and gallbladder after 10 - 14 days exposure.

The accumulation potential (BCF) of Ph_2SnCl_2 and $PhSnCl_3$ could be used as an explanation for the concentrations of metabolized Ph_2Sn^{2+} and $PhSn^{3+}$ in the carp. However, the same potential of Bu_2SnCl_2 and $BuSnCl_3$ (see Table 4 in chapter 2) cannot be applied to the metabolism of $(Bu_3Sn)_2O$, because the concentrations of Bu_2Sn^{2+} and $BuSn^{3+}$ formed by the metabolism in all tissues of the carp are higher than those estimated by the BCF of the Bu_2SnCl_2 and $BuSnCl_3$.

To clarify the facts of the metabolism of $(Bu_3Sn)_2O$ in carp, the minute structures and accumulation potentials of metabolized Bu_2Sn^{2+} and $BuSn^{3+}$, and the metabolism and excretion rate of these butyltin compounds should be revealed.

Metabolism of $(Bu_3Sn)_2O$ and Ph_3SnCl in goldfish

The concentrations of Bu_2Sn^{2+} and $BuSn^{3+}$ (Fig.9) or Ph_2Sn^{2+} and $PhSn^{3+}$ (Fig.10) in the whole body of goldfish were about the same as or lower than the values estimated from those in the muscle, liver, kidney and gallbladder of carp. The metabolism rate of $(Bu_3Sn)_2O$ and Ph_3SnCl in

goldfish was presumed to be about the same as or slower than that in the carp.

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CHAPTER 6

Differences between Freshwater and Seawater-Acclimated Guppies in Accumulation and Excretion of Tri-n-butyltin and Triphenyltin Compounds

Introduction

In Japan, many field data on the contamination of freshwater and marine fish and shellfish with triphenyltin compounds and tri-n-butyltin compounds have been reported by Environment Agency.[1] These reports have revealed that both types of compounds in the environment tend to be highly accumulated in several species of fish and shellfish.

The accumulation and excretion of these compounds in two species of freshwater fish (carp and goldfish) were already described in chapters 2 - 4. However, for marine fish these experimental data were scarce.[2] To precisely evaluate the contamination of fish in the environment by these compounds, experimental data for both freshwater and marine fish are necessary. Since there are differences between freshwater and marine fish in their physiological regulation of osmotic pressure, their accumulation and excretion of organotin compounds might also differ.

To study these differences, accumulation and excretion experiments on Bu_3SnCl and Ph_3SnCl were carried out using freshwater and seawater acclimated guppies.[3]

Experimental Section

Chemicals

Tri-n-butyltin chloride (Bu_3SnCl), purity 95%, and triphenyltin chloride (Ph_3SnCl), purity 98%, were purchased from Tokyo Chemical Industry (Tokyo, Japan) and used without further purification.

Artificial seawater was prepared by dissolving NaCl 1197 g, $MgCl_2$ 254 g, Na_2SO_4 200 g, $CaCl_2$ 56.2 g, KCl 33.4 g, KBr 4.9 g, H_3BO_3 1.4 g, $SrCl_2$ 1.2 g, NaF 0.15 g and $NaHCO_3$ 9.8 g in 50 *l* dechlorinated city water with reference to the studies by Tachikawa et al.[4,5]

Test fish

All female guppies (*Lebistes reticulatus*) were purchased from a local fish market. The body length and body weight of the fishes were 2.4 - 2.7 cm and 0.41 - 0.55 g, respectively. Whole-body lipid content (n=7) was 2.7% by the Folch method. For the accumulation and excretion experiments in seawater, freshwater guppies were gradually acclimated to artificial seawater (7 days) by supplying the seawater at 1.2 *l*/h to the aquaria (freshwater, 50 *l*) containing 40 fish. These fish were kept for a further 7 days in the seawater. Tri-n-butyltin and triphenyltin compounds were not detected in the fish before exposure to these chemicals. A commercial assorted feed, TetraMin (Tetra Werke, West Germany), was given at 10 mg/fish once a day throughout the accumulation and excretion experiments.

Test system

The study was carried out with aeration under a continuous flow-through system. The experimental arrangement was the same as in chapter 2.

Glass tanks holding about 6 *l* and 3 *l* were used for the accumulation and excretion experiments, respectively.

For the accumulation experiments (14 days), two stock solutions of Bu_3SnCl and Ph_3SnCl mixtures (each 10 μg/*l*) were respectively diluted 10 times continuously with dechlorinated city water and artificial seawater, and supplied to each of the two aquaria containing 30 fish. Each stock solution was prepared by diluting 0.1 m*l* of an ethanol solution of Bu_3SnCl and Ph_3SnCl mixture (each 1000 μg/m*l*) with water to 10 *l*. These concentrations were considered to be appropriate from data LC_{50} (7 days) for guppy (TBTO 40 μg/*l*).[6] During the tests, the flow rate and temperature of water for each exposure were maintained at 1.2 *l*/h and 25±1°C, respectively. The concentrations of Bu_3SnCl and Ph_3SnCl were [mean±SD,n=6(range)] 0.54±0.08 (0.43 - 0.63) and 0.90±0.07 (0.83 - 1.0) μg/*l* in freshwater, and 0.28±0.03 (0.22 - 0.30) and 0.71±0.07 (0.62 - 0.81) μg/*l* in seawater. The low concentrations of these chemicals in the exposure water were probably due to their adsorption to the tubes of the experimental arrangement or to the walls of the tanks. Measurements of Bu_3SnCl and Ph_3SnCl in the exposure water were carried out at 0, 1, 3, 7, 10 and 14 days. The estimated Cl^- concentration in seawater was 19 g/*l*. The pH of each exposure water was 7.1 - 7.3 for freshwater and 8.0 - 8.2 for seawater. The concentration of dissolved oxygen was more than 7 mg/*l* for both types of water, and hardness was 37 mg/*l* as $CaCO_3$ in freshwater. Measurements were carried out every day for pH and once after 3 days for dissolved oxygen and hardness. Three fish were taken at 1, 3, 7, 10 and 14 days in the accumulation experiments. All of the fish were frozen and preserved for analysis after rinsing with distilled water.

For excretion experiments (14 days), 15 fish remaining in the freshwater and seawater tanks were separately transferred into each of the two test tanks, and dechlorinated city water and artificial seawater were supplied to each tank. During the tests, the flow rate and temperature of water in each test tank were maintained at 1.2 *l*/h and 25±1°C, respectively. The concentrations of Bu_3SnCl and Ph_3SnCl excreted from the fish in the test tanks were < 0.05 μg/*l* and < 0.10 μg/*l*, respectively, after 20 h in both excretion experiments. The estimated Cl^- concentration in seawater was 19 g/*l*. The pH of each test tank water was 7.2 - 7.4 for freshwater and 8.1 - 8.3 for seawater. The concentration of dissolved oxygen was more than 7 mg/*l* for both types of water, and hardness was 35 mg/*l* as $CaCO_3$ in freshwater. Measurements were carried out every day for pH and once after 3 days for dissolved oxygen and hardness. Three fish were taken at 1, 3, 7 and 14 days in the excretion experiments. All of the fish were frozen and preserved for analysis after rinsing with distilled water.

Under these conditions, none of the fish showed any signs of tiredness or agitation during the accumulation and excretion experiments.

Analysis

The concentrations of Bu_3SnCl and Ph_3SnCl in the freshwater and seawater were determined using the method similar to that in chapter 2. That is, a 200 m*l* of water sample was shaken with 80 m*l* of hexane after addition of 5 g of NaCl and 10 m*l* of HCl. For seawater, NaCl was not added. The hexane layer was rotary-vacuum evaporated to 5 m*l* at 40°C.The concentrate was hydrogenated with 3 m*l* of 2.5% $NaBH_4$ in ethanol, and slightly shaken after addition of 15 m*l* of water. The tributyltin and triphenyltin hydrides (Bu_3SnH and Ph_3SnH) in the hexane layer were simultaneously determined

by a gas chromatograph equipped with a flame photometric detector (FPD-GC). Average recoveries (n=3) from freshwater were 100% for Bu_3SnCl and 92% for Ph_3SnCl at a spiked level of 1.0 μg/*l*.

Determination of Bu_3SnCl and Ph_3SnCl in the fish samples was carried out by the method of Tsuda et al.[7] modified as follows. The eluate from silica gel cleanup was collected in a volume of 0 - 50 m*l* and concentrated to 0.5 - 5 m*l*. The Bu_3SnH and Ph_3SnH in the concentrate were determined simultaneously by FPD-GC. Average recoveries (whole body, n=3) were 88% for Bu_3SnCl and 83% for Ph_3SnCl at a spiked level of 100 ng/g. The GC (Shimadzu GC-9AM) operating conditions were as follows:

GC column: J&W DB 1 (0.53 mmϕ x 30 m, film thickness 1.5 μm)

Carrier: N_2 30 m*l*/min Air: 50 m*l*/min H_2: 100m*l*/min

Temperatures: injection and detector 280°C; column 140°C (1min)

8°C/min to 240°C

The thawed fish samples were homogenized as a mixture of three samples for each sampling time. Analyses were carried out as a single sample for each.

Calculation of bioconcentration factor (BCF) and excretion rate constant (k)

BCF was calculated from the following equation.

$$BCF = \frac{\text{chemical concentration in whole body of fish}}{\text{chemical concentration in water}}$$

The chemical concentration in the water at each sampling time was used for the calculation of BCF. The excretion rate constant (k) of the chemicals from the whole body of fish was calculated assuming that the excretion process follows first-order kinetics.

Results and Discussion

Accumulation of Bu_3SnCl and Ph_3SnCl

The concentrations of Bu_3SnCl and Ph_3SnCl in the seawater were considerably lower than those in the freshwater. This was probably because these chemicals in seawater (pH 8) have chemical forms that are adsorbed more easily to the tubes of the experimental arrangement or to the walls of the tanks, than those in freshwater (pH 7). The BCF of Bu_3SnCl in the whole body of freshwater and seawater-acclimated guppies is shown in Fig.1 over the 14 day exposure period. The BCF of Bu_3SnCl in both types of fish reached a plateau level after 7 days of exposure. The average BCF value of Bu_3SnCl (460, n=3) in the freshwater fish was about twice as high as that (240, n=3) in the seawater-acclimated fish after 7 - 14 days of exposure.

Similarly, the BCF of Ph_3SnCl in the whole body of both types of fish is shown in Fig.2. The BCF of Ph_3SnCl in both types of fish reached a plateau level after 10 days of exposure. The average BCF value of Ph_3SnCl (1100, n=2) in the freshwater fish was also about twice as high as that (530, n=2) in the seawater-acclimated fish after 10 - 14 days of exposure. This tendency was equal to the results of similar accumulation experiments by Tachikawa et al.,[8,9] which showed that the BCF values of pentachlorophenol (PCP) in the whole body of freshwater and seawater-acclimated killifishes were 1000 and 500, respectively.[8] Similarly, those of 1,2,3-trichlorobenzene (1,2,3-TCB) were 26000 and 20000, respectively.[9] However, for α-hexachloro-cyclohexane (α-HCH), the order of the BCF values (145 and 500) in freshwater and seawater-acclimated guppies[10,11] was reversed compared with the order described above.

These differences between the two types of fish in accumulation of these chemicals are probably due to the differences in their chemical forms

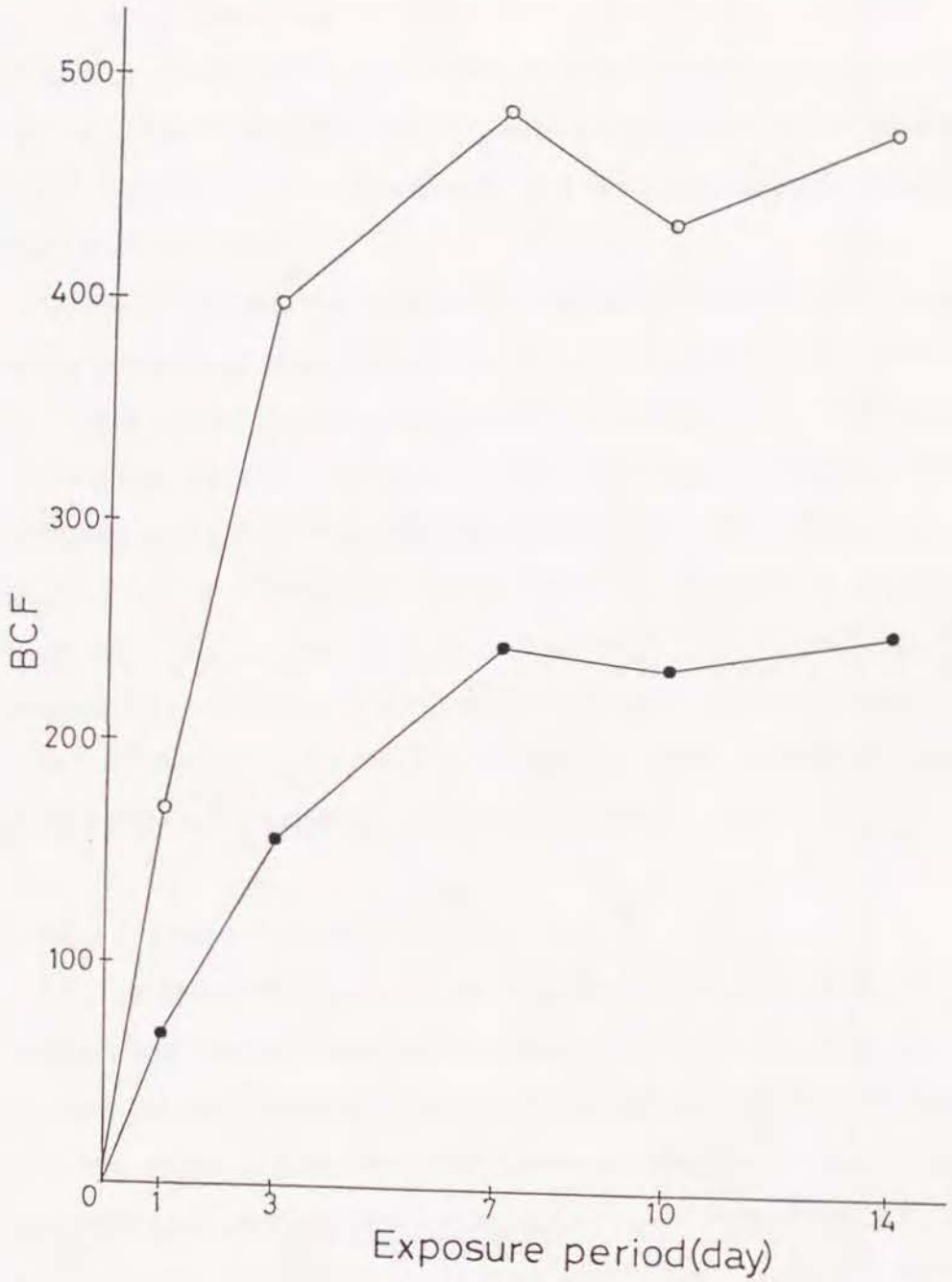

Fig.1 Bioconcentration of Bu_3SnCl in the whole body of guppy.

○ freshwater ● seawater

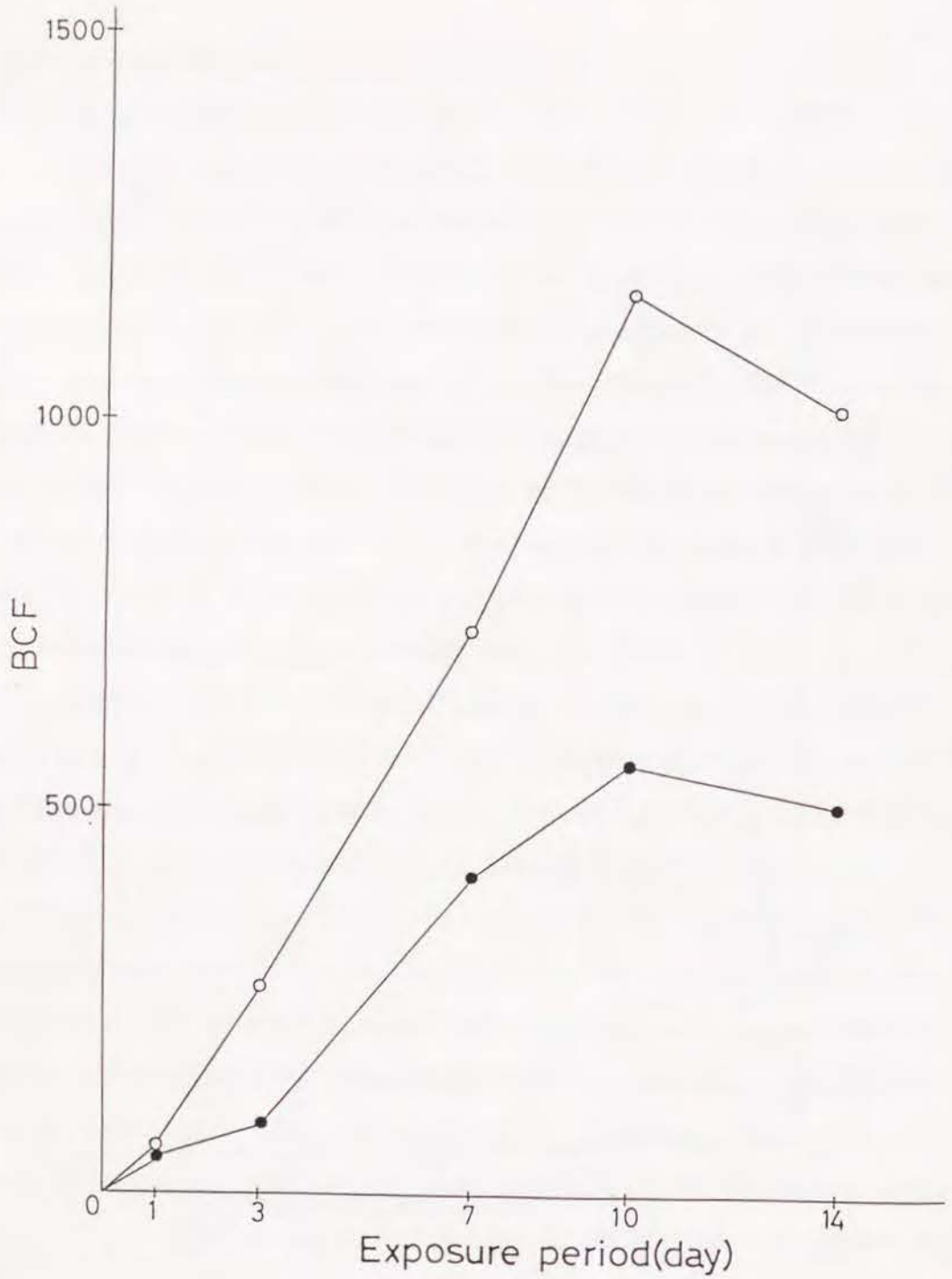

Fig.2 Bioconcentration of Ph_3SnCl in the whole body of guppy.

○ freshwater ● seawater

between freshwater and seawater areas, as well as the physiological mechanisms operating for regulation of osmotic pressure in the fish. The chemical forms of Bu_3SnCl and Ph_3SnCl are presumed to be dissociated (Bu_3Sn^+ and Ph_3Sn^+) in freshwater and undissociated (Bu_3SnOH and Ph_3SnOH) in seawater.

On the other hand, the average BCF values for Ph_3SnCl (n=2) after 10 - 14 days were about twice as high as those for Bu_3SnCl (n=3) after 7 - 14 days in both freshwater and seawater-acclimated guppies.

This was opposite to the results obtained in carp in chapter 3, where the BCF values of Bu_3SnCl were about three times higher than those of Ph_3SnCl. The order of the BCF values for Bu_3SnCl and Ph_3SnCl is presumed to change according to the fish species examined. This is probably due to corresponding variation in the function of their gills. To confirm this possibility, similar accumulation experiments are scheduled for other freshwater fish such as goldfish and willow shiner.

Excretion of Bu_3SnCl and Ph_3SnCl

The excretion of Bu_3SnCl and Ph_3SnCl from the whole body of freshwater and seawater-acclimated guppies is shown in Figs.3 and 4 over the 14 day period. The concentrations of Bu_3SnCl and Ph_3SnCl in the fish at day 0 were equal to those at 14 days in the accumulation experiments. The excretion rate constants (k) and biological half-lives were calculated, and are shown in Table 1. The excretion rate of Ph_3SnCl from the seawater-acclimated fish (0.30 day^{-1}) was about twice as rapid as that from the freshwater fish (0.13 day^{-1}), but there was no difference between the fish in the rates for Bu_3SnCl (0.35 and 0.32 day^{-1}).

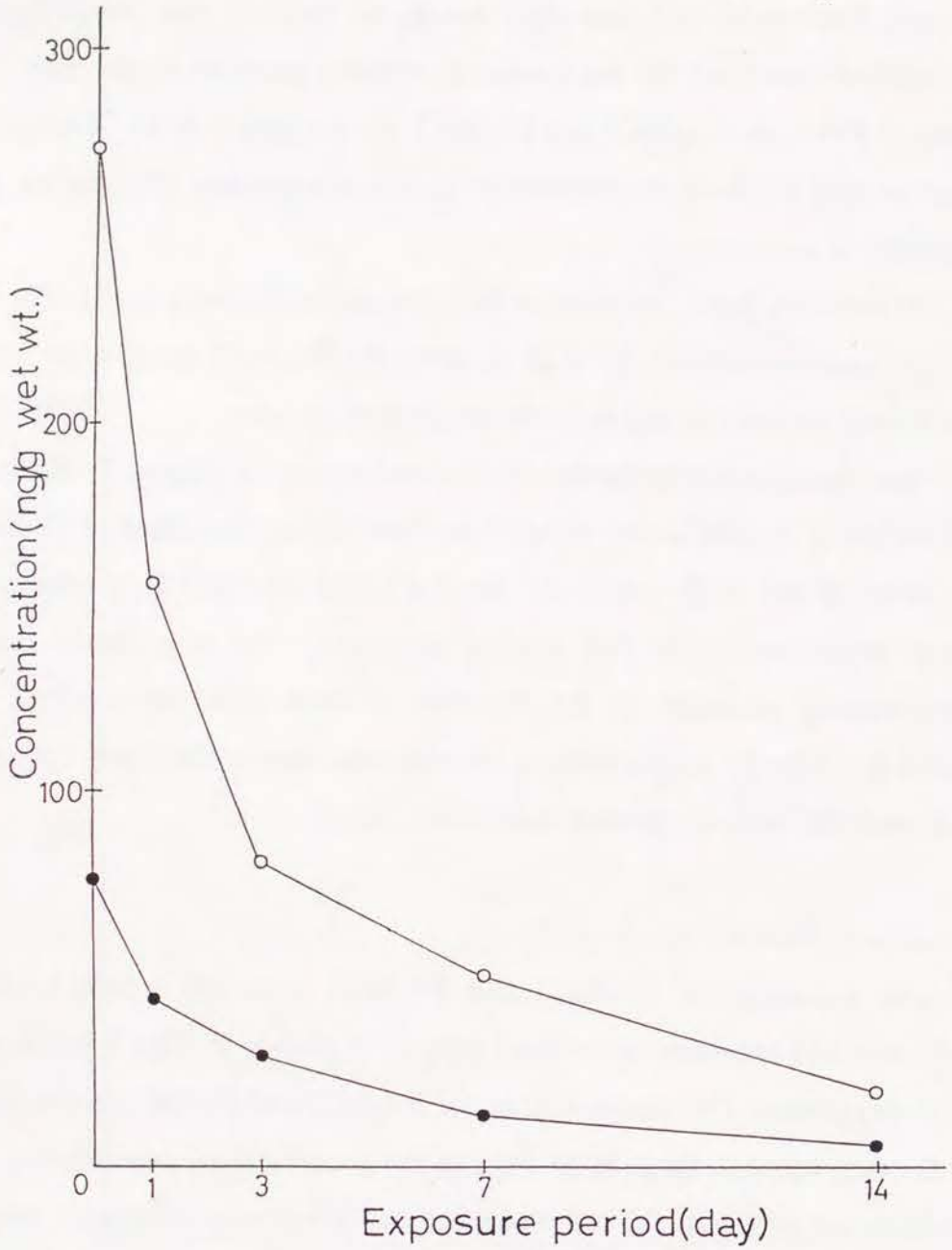

Fig.3 Excretion of Bu_3SnCl from the whole body of guppy.

○ freshwater ● seawater

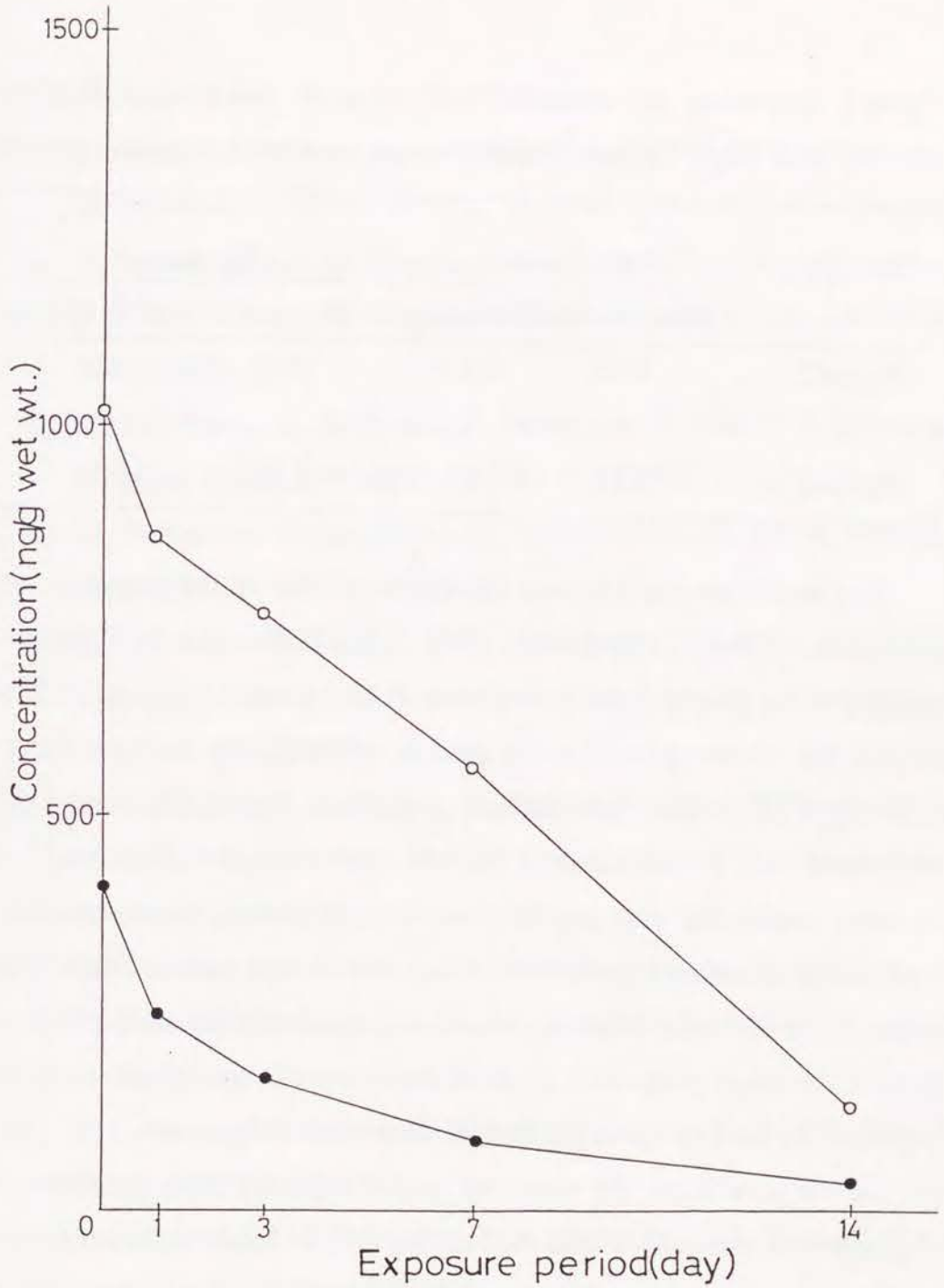

Fig.4 Excretion of Ph_3SnCl from the whole body of guppy.

○ freshwater ● seawater

Table 1. Excretion rate constants and biological half-lives of Bu_3SnCl and Ph_3SnCl from freshwater and seawater-acclimated guppies.

Chemicals	Freshwater		Seawater	
	k(day-1)	Half-lives(day)	k(day-1)	Half-lives(day)
Bu_3SnCl	0.35	2.0	0.32	2.2
Ph_3SnCl	0.13	5.3	0.30	2.3

Similar differences between both types of fish in the excretion rate of environmental chemical pollutants (PCP, 1,2,3-TCB and α-HCH) were also reported in the studies described above.[8-11] The excretion rate of PCP from killifish was slower in freshwater than in seawater, but the rate for 1,3,5-TCB and α-HCH from killifish and guppy was conversely more rapid in freshwater than in seawater. It is thus presumed that differences exist between freshwater and seawater-acclimated fish in their excretion of environmental chemical pollutants. These differences between the two types of fish in the excretion of these chemicals are probably due to the differences in their chemical properties as well as in the physiological mechanisms employed by the fish for regulation of their osmotic pressure.

On the other hand, the excretion rate of Bu_3SnCl from the whole body of guppy was more rapid than that of Ph_3SnCl in freshwater, but there was no difference in seawater. This result is also probably due to the differences between the two types of fish in the physiological regulation of their osmotic pressure.

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CHAPTER 7

Accumulation of Tri-n-butyltin and Triphenyltin Compounds via Gill and by Oral Intakes of Goldfish

Introduction

In Japan, many field data on the contamination of freshwater and marine fish and shellfish with tri-n-butyltin and triphenyltin compounds have been reported by Environment Agency (Japan)[1-3] and several investigators.[4-9] These reports have revealed that both types of compound in the environment tend to be highly accumulated in several species of fish and shellfish. Many experimental data on the accumulation of these compounds via gill intake in several species of fish were already described in chapters 2, 3, and 4. To precisely evaluate the contamination of fish and shellfish in the environment by tri-n-butyltin and triphenyltin compounds, their accumulation experiments by oral intake as well as via gill intake are necessary.

In this study,[10] both experiments were performed using goldfish and the accumulation potential of Bu_3SnCl and Ph_3SnCl by oral intake was compared with the potential via gill intake. Further, the contamination of freshwater fish by these compounds was discussed using the results of these experiments.

Experimental Section

Chemicals

Tri-n-butyltin chloride (Bu_3SnCl), purity 95%, and triphenyltin chloride (Ph_3SnCl), purity 98%, were purchased from Tokyo Chemical Industry (Tokyo, Japan) and used without further purification.

Test fish

Goldfish (*Carassius aurapus)* were purchased from a local fish market. The body length and body weight of the fish were 3.5 - 4.0 cm and 1.6 - 2.9 g, respectively. Tri-n-butyltin and triphenyltin compounds were not detected in the fish (Detection limits: 1 ng/g as Bu_3SnCl and 2 ng/g as Ph_3SnCl) before experiments.

Feed

For the via gill intake experiments, commercial assorted feed was used. Tri-n-butyltin and triphenyltin compounds were not detected in this feed (Detection limits: 2 ng/g as Bu_3SnCl and 4 ng/g as Ph_3SnCl). For the oral intake experiment, the feed containing Bu_3SnCl and Ph_3SnCl was prepared by adding these chemicals (each 2 μg/m*l* in acetone, 150 m*l*) to 150 g of the commercial assorted feed, mixing adequately and standing overnight under room temperature. The concentrations of Bu_3SnCl and Ph_3SnCl in this feed (mean±SD, n=3) were 1890±77 and 1670±115 ng/g, respectively.

Test system

For the oral intake experiment (35 days), 50 fishes were thrown into the aquaria (acrylic plastic, 50 *l*) containing clean water and dechlorinated city water was continuously supplied to it. The flow rate and temperature of

the test water were maintained at 60 *l*/h and 23±1°C, respectively. The pH of the test water was 6.9 - 7.0. Measurements were carried out every day. The concentration of dissolved oxygen was more than 7 mg/*l* and hardness was 36 mg/*l* as $CaCO_3$. Measurements were carried out once after 3 days. The feed containing chemicals was given 20 mg/fish twice a day (9:00 *a.m.* and 4:00 *p.m.*) throughout the experimental period. Six fish were taken 8:30 *a.m.* at 1, 3, 7, 14, 21 28 and 35 days. All of the fish were frozen and preserved for analysis.

For the via gill intake experiment (28 days), the experimental arrangement was the same as in chapter 2. A stock solution of Bu_3SnCl and Ph_3SnCl mixtures (each 30 μg/*l*) were diluted 100 times continuously with dechlorinated city water and supplied to the aquaria (acrylic plastic, 50 *l*) containing 25 fishes. The flow rate and temperature of the test water were maintained at 12 *l*/h and 23±1°C, respectively. The stock solution was prepared by diluting 0.3 m*l* of ethanol solution of Bu_3SnCl and Ph_3SnCl mixtures (each 1000 μg/m*l*) with water to 10 *l*. The concentrations of Bu_3SnCl and Ph_3SnCl were [mean±SD, n=8 (range)] 0.13±0.01 (0.11 - 0.14) and 0.14±0.01 (0.13 - 0.17) μg/*l*, respectively. These concentrations were fixed by reference to the data of 48 h LC_{50} to rainbow trout and 24 h LC_{100} to goldfish (tri-n-butyltin compounds: 21 and 75 μg/*l*).[11] Measurements were carried out at 0, 1, 3, 7, 10, 14, 21 and 28 days. The low concentrations of these chemicals in the test water were probably due to their adsorption to the tubes of the experimental arrangement or to the walls of the aquaria. The pH of the test water was 7.1 - 7.2. Measurements were carried out every day. The concentration of dissolved oxygen was more than 7 mg/*l* and hardness was 39 mg/*l* as $CaCO_3$. Measurements were carried out once after 3 days of exposure. The feed without chemicals was given at 20

mg/fish twice a day in the same manner for the oral intake experiment. Three fish were taken at 1, 3, 7, 14, 21 and 28 days. All of the fish were frozen and preserved for analysis after rinsing with distilled water.

Under these conditions, none of the fish showed any signs of tiredness or agitation during the oral and via gill intake experiments.

Analysis

The concentrations of Bu_3SnCl and Ph_3SnCl in the water and fish samples were determined by the same method as in chapter 6. Analyses of the fish samples (whole body) were carried out as a sample for every two fish in the oral intake experiment, and a sample for each fish in the via gill intake experiment.

Calculation of bioconcentration factor (BCF) and retention percent (RP)

The BCF by oral intake (BCF_o) and via gill intake (BCF_g) were calculated from the following equations.

$$BCF_o = \frac{\text{chemical concentration in whole body of fish}}{\text{chemical concentration in feed}}$$

The retention percent (RP) was calculated as the percentage of the absolute amount of chemical in fish to the absolute amount of it in the feed taken.

$$BCF_g = \frac{\text{chemical concentration in whole body of fish}}{\text{chemical concentration in water}}$$

For the calculation of BCF_g, the chemical concentration at each sampling time was used.

Results and Discussion

Accumulation of Bu_3SnCl and Ph_3SnCl via gill intake

The BCF_g of Bu_3SnCl and Ph_3SnCl via gill intake in whole body of goldfish is shown in Fig.1 over the 28 days exposure period. The BCF_g values of both Bu_3SnCl and Ph_3SnCl reached plateaus after 21 days exposure. This period was longer than the periods 7 - 10 days in carp (chapters 2 and 3). The average BCF_g values of Bu_3SnCl and Ph_3SnCl (n=2) were 1976 and 1384, respectively, after 21 - 28 days of exposure. The order of these BCF_g values (Bu_3SnCl > Ph_3SnCl) in goldfish was the same as round crucian carp and carp (chapters 2 and 3). But the order was reversed in both of the freshwater and seawater-acclimated guppies (chapter 6). The order of the BCF values for Bu_3SnCl and Ph_3SnCl is presumed to change according to the fish species examined. This is probably due to corresponding variation in the function of their gills.

Accumulation of Bu_3SnCl and Ph_3SnCl by oral intake

The BCF_o of Bu_3SnCl and Ph_3SnCl by oral intake in whole body of gold fish is shown in Fig.2 over the 35 days period. Their BCF_o values reached plateaus after 28 days for Bu_3SnCl, but did not for Ph_3SnCl. For Ph_3SnCl, the experiment should have been further continued. The average BCF_o value of Ph_3SnCl (0.10) was 2.5 times higher than the value of Bu_3SnCl (0.04) after 35 days. This result was reasonable when considered with the data in chapter 2 that the excretion rate of Ph_3SnCl from goldfish (0.04 day^{-1}) was slower than that of $(Bu_3Sn)_2O$ (0.08 day^{-1}). RP was calculated from the data

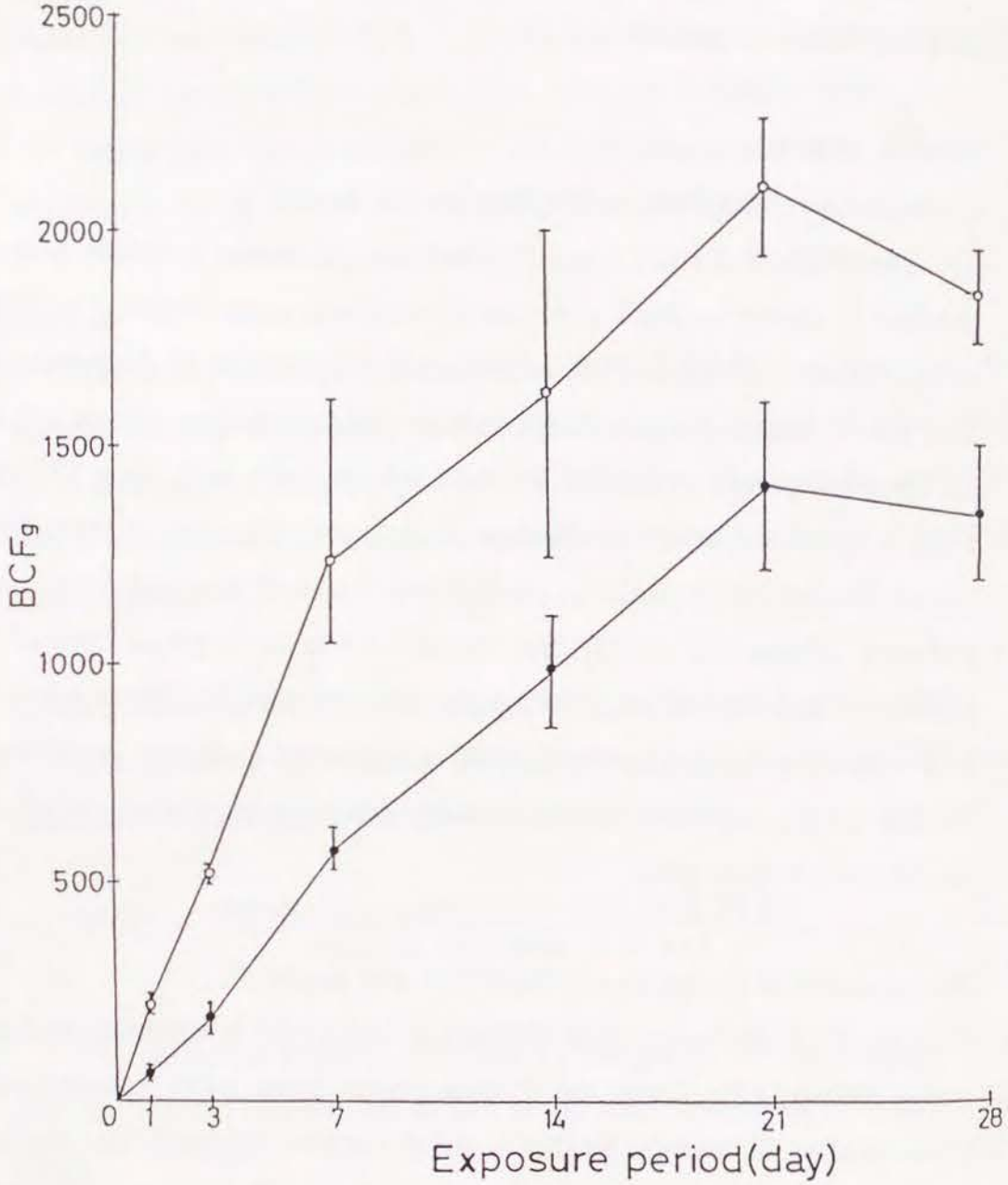

Fig.1 Accumulation of Bu_3SnCl and Ph_3SnCl via gill intake of gold fish.

○ Bu_3SnCl ● Ph_3SnCl

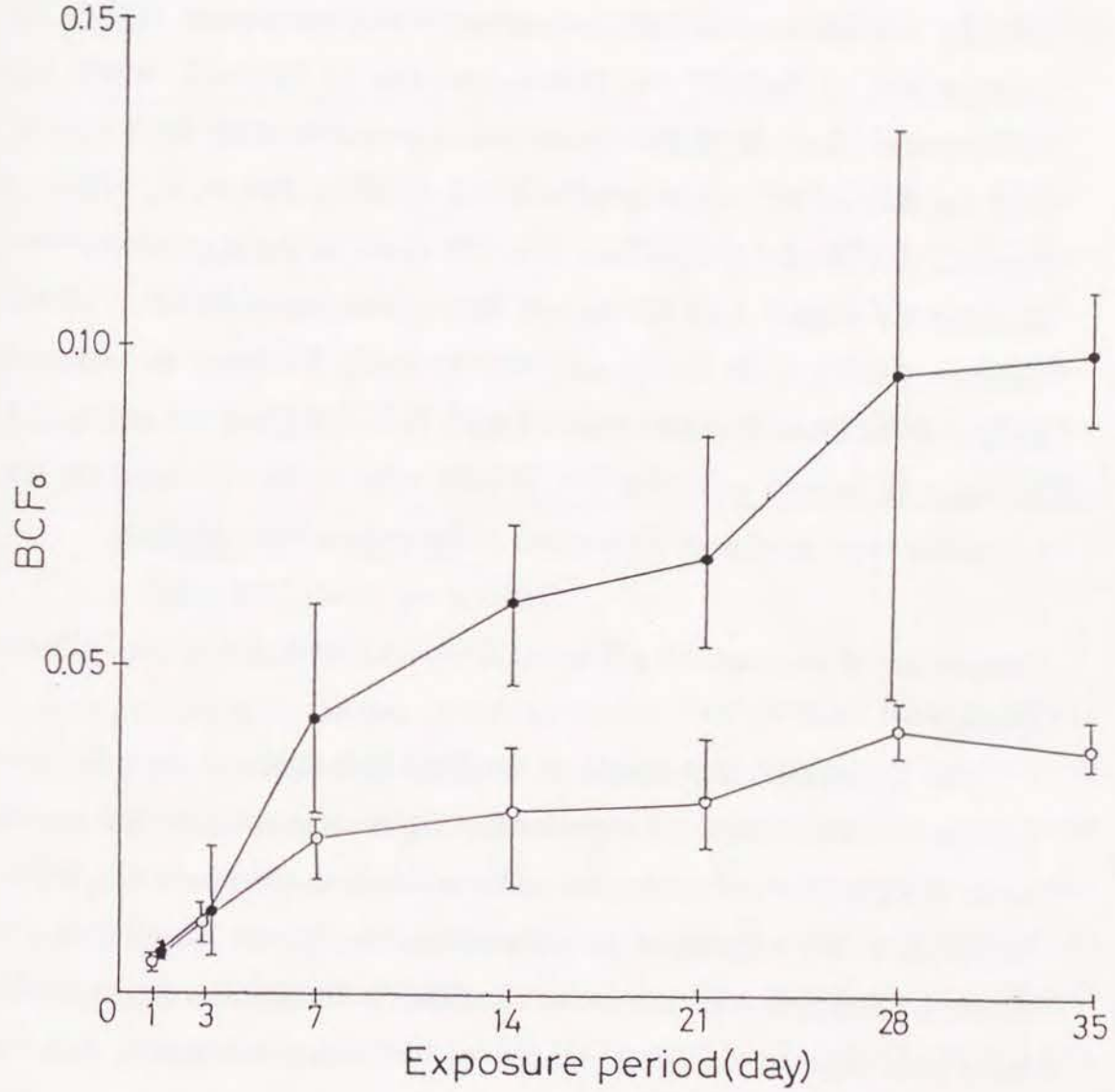

Fig.2 Accumulation of Bu_3SnCl and Ph_3SnCl by oral intake of gold fish.

○ Bu_3SnCl ● Ph_3SnCl

of oral intake experiment and are shown in Table 1. The RP values of both chemicals gradually decreased with the elapse of the experiment period. This tendency was the same as the pesticides such as diazinon and CNP.[12] The decrease rate of Ph_3SnCl was slower than that of Bu_3SnCl, which was considered to show the higher accumulation potential of Ph_3SnCl together with the data of RP values (Ph_3SnCl > Bu_3SnCl). The BCF_o values of Bu_3SnCl and Ph_3SnCl (0.04 and 0.10, 35 days) in our experiment were considerably higher than the values of fenitrothion (0.00009, 7 days), diazinon (0.00017, 14 days) and CNP (0.0207, 14 days) in topmouth gudgeon[12] but lower than the values of DDT (1.2, 266 days) and endrin (0.8, 56 days) in fathead minnows.[13,14] For RP values, these comparison was impossible because of large differences of the experimental periods.

Comparison of oral and via gill intake in the accumulation of Bu_3SnCl and Ph_3SnCl

The accumulation potential of Bu_3SnCl and Ph_3SnCl via gill intake (BCF_g=1976 and 1384) was considerably higher than the potential by oral intake (BCF_o=0.04 and 0.10). The order of of these chemicals (Ph_3SnCl > Bu_3SnCl) in the accumulation potential by oral intake of goldfish was reversed compared with the order (Bu_3SnCl > Ph_3SnCl) via gill intake. Many field data on the contamination of freshwater and marine fish and shellfish by these compounds have been reported by Environment Agency (Japan)(1988)[3] and several investigators.[8,9] These data have revealed that the concentrations of triphenyltin compounds in marine fish were considerably higher than those of tri-n-butyltin compounds. For freshwater fish,

Table 1. RP of Bu_3SnCl and Ph_3SnCl by oral intake of gold fish.

Days	Body weights (g)[a]	Concentrations(ng/g)[b]		RP(%)	
		Bu_3SnCl	Ph_3SnCl	Bu_3SnCl	Ph_3SnCl
1	2.0±0.2	8± 1	9± 2	21.2%	26.9%
3	1.7±0.1	20± 5	21±13	15.0%	17.8%
7	2.1±0.4	45± 9	72±23	17.9%	32.3%
14	1.7±0.2	53±17	102±17	8.5%	18.5%
21	2.1±0.2	57±14	113±25	7.5%	16.9%
28	2.7±0.2	78± 7	162±63	9.9%	23.4%
35	2.7±0.1	73± 6	169±15	7.4%	19.5%

a Mean±SD (n=6)

b Mean±SD (n=3), per g fish

their concentrations in two species of crucian carp obtained from harbors of Lake Biwa are shown in Table 2. Analyses were carried out by the same method as in chapter 6. The concentrations of triphenyltin compounds (Ph_3Sn^+) were higher than those of tributyltin compounds (Bu_3Sn^+) in round crucian carp, but the order was reversed in deep crucian carp (Harbor B). The concentrations of Ph_3Sn^+ in the water of these harbors (< 0.10 μg/*l*) were probably equal to or lower than those of Bu_3Sn^+ (0.05 - 0.10 μg/*l*). These concentrations (Ph_3Sn^+ > Bu_3Sn^+) in round crucian carp were presumed to be accumulated mainly by oral intake when considered with these concentrations in the harbors and the result (BCF_g: Bu_3SnCl > Ph_3SnCl) of the via gill intake experiment on the same fish (chapter 2). For deep crucian carp, these concentrations (Bu_3Sn^+ > Ph_3Sn^+) were presumed

Table 2. Concentrations of tributyltin and triphenyltin compounds in muscle of crucian carp obtained from harbors of Lake Biwa.

Sample [a]	Location	Body length (cm)	Weight (g)	Bu_3Sn^+ (ng/g)[b]	Ph_3Sn^+ (ng/g)[c]
	A	22.0	307.7	11	41
RCC [d]	A	18.5	205.2	19	95
	B	15.0	103.9	46	148
	A	22.5	370.5	18	69
DCC [e]	B	13.5	82.7	17	5
	B	13.5	82.7	17	13

[a] Collected in June and July (1989).

[b] Expressed as Bu_3SnCl in wet weight.

[c] Expressed as Ph_3SnCl in wet weight.

[d] Round crucian carp.

[e] Deep crucian carp.

to be accumulated mainly via gill intake when considered with the results of the via gill intake experiment on carp in chapter 3 and goldfish in this study.

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CHAPTER 8

Accumulation of Pesticides in Freshwater-Fishes

Introduction

In recent years, a large number of pesticides such as herbicides or insecticides have been produced and discharged into the environment. The data on their accumulation in fishes are useful for the evaluation of the contamination of fishes by pesticides in lakes or rivers.

Accumulation of various pesticides in fishes have been reported by several investigators,[1-3] but there are still many pesticides that have not been studied in relation to their accumulation in fishes.

In this chapter, the accumulation of pesticides in freshwater-fishes is described. That is, the bioconcentration factor (BCF) in willow shiner[4-6] and carp[7,8] was measured for herbicides (benthiocarb, simetryne, oxadiazon, CNP and chlomethoxynil), insecticides (diazinon, malathion and fenitrothion) and fungicides (IBP).

Experimental Section

Chemicals

Benthiocarb (S-p-chlorobenzyl N,N-diethylthiocarbamate), simetryne [2,4-bis(ethylamino)-6-methylthio-1,3,5-triazine], Oxadiazon [5-tert-butyl-3-(2,4-dichloro-5-isopropoxy phenyl)-1,3,4-oxadiazolin-2-on], CNP (2,4,6 - trichlorophenyl-4'-nitrophenyl ether), chlomethoxynil (2,4-dichlorophenyl 3-methoxy-4-nitrophenyl ether), diazinon (diethyl 2-isopropyl-4-methyl-6-pyrimidinyl phosphorothionate), malathion [S-1,2-

bis(ethoxycarbonyl)ethyl dimethyl phosphorothionate], fenitrothion (dimethyl 4-nitro-m-tolyl phosphorothionate) and IBP (S-benzyl diisopropyl phosphorothiolate) were purchased from Wako Pure Chemical Industries Ltd (Osaka Japan). These chemicals, each reagent grade (more than 98.0%), were used without further purification.

Test fish

Willow shiner (*Gnathopogon caerulescens)* were obtained from Shiga Prefecture Fisheries Experimental Station. Carp (*Cyprinus carpio* L.) were purchased from Nango Suisan Center (Shiga Prefecture, Japan). The body length and body weight of the fishes were 4.7 - 5.4 cm and 1.70 - 2.53 g for willow shiner, and 9.0 - 11.0 cm and 19.0 - 34.9 g for carp. Lipid contents by Folch method were 8.0 - 9.2 % (n=5) in the whole body of willow shiner and 0.7 - 0.8 % (n=5) in the muscle of carp. These pesticides were not detected in the fishes before exposure to them. Commercial assorted feed was given 50 mg/fish for willow shiner and 500 mg/fish for carp once a day throughout the experiments.

Test system

The study was carried out for 3 - 14 days under the continuous flow through system by using the same experimental arrangement as in chapter 2. Aqueous stock solutions of these pesticides (0.5 - 2.0 μg/m*l*) were diluted continuously 100 or 150 times with dechlorinated city water and supplied to aquaria tanks containing fishes. During the test, the flow rate of the test water was maintained at 200 or 300 m*l*/min and the temperature was 20±1 or 24±1°C for willow shiner and 19±1 or 25±1°C for carp. The measured concentrations of these pesticides in the test tanks were summarized in Table

1. At these concentrations, none of the fishes showed any signs of tiredness and agitation during the experiments. At the fixed sampling times, three fish were taken.

Table 1. Measured concentrations of pesticides in the test tanks.

Pesticides	Concentrations (μg/*l*)[a]	
	willow shiner	carp
Benthiocarb	2.8±0.2	4.5 - 5.0
Simetryne	8.1±0.3	8.0 - 8.9
Oxadiazon	2.0 - 2.7	-
CNP	1.7 - 2.0	-
Chlomethoxynil	1.7 - 2.1	-
Diazinon	2.4 - 3.3	1.5 - 2.4
Malathion	5.2 - 6.2	4.0 - 6.2
Fenitrothion	2.5 - 3.5	1.8 - 2.9
IBP	5.8 - 6.5	5.0 - 8.3

[a] Range (n=6) or mean±SD (n=6)

Analysis

The concentrations of these pesticides in the water were determined by the following procedure.[4-8] A measured volume (100 m*l*) of the water was shaken with 50 m*l* of hexane after addition of 5 g of NaCl. The organic layer was injected into gas chromatograph equipped with electron capture detector (GC-ECD) for oxadiazon, CNP and chlomethoxynil and gas chromatograph equipped with flame thermionic detector (GC-FTD) for benthiocarb, simetryne, diazinon, malathion, fenitrothion and IBP. Average recoveries

(n=3) were 91 % for benthiocarb and 89 % for simetryne at 10 μg/*l* spiked levels, and 99 % for oxadiazon, 98 % for CNP, 100 % for chlomethoxynil, 96 % for diazinon, 97 % for malathion, 93 % for fenitrothion and 92 % for IBP at 5 μg/*l* spiked levels.

Determination of these pesticides in fish samples was carried out by the following method.[4-8]

For oxadiazon, CNP and chlomethoxynil, a fish sample was homogenized with 30 m*l* of acetonitrile by high-speed homogenizer after addition of 5 g of anhydrous Na_2SO_4 and the organic layer was filtrated. The residue was again homogenized and filtrated in the same manner. The combined filtrate was rotary-vacuum evaporated just to dryness at 40°C and the residue was dissolved in 10 m*l* of hexane. The hexane solution was shaken with 30 m*l* of acetonitrile saturated with hexane (twice), and the combined acetonitrile layer was rotary-vacuum evaporated just to dryness at 40°C. The residue was dissolved with 5 m*l* of hexane and the hexane solution was passed through a SEP-PAK florisil cartridge (Waters Associates, USA). Each pesticide was eluted with 10 m*l* of hexane. Each pesticide concentration of the eluate was measured by GC-ECD after rotary-vacuum evaporation to 5 - 20 m*l*. Average recoveries (whole body 5 g, n=3) were 87 % for oxadiazon, 97 % for CNP and 98 % for chlomethoxynil at 50 ng/g spiked levels.

For benthiocarb, simetryne, diazinon, malathion, fenitrothion and IBP, a fish sample was homogenized with 30 m*l* of acetonitrile and the organic layer was filtrated. The residue was rinsed with 10 m*l* of acetonitrile and the rinse was again filtrated. The combined filtrate was shaken twice with 50 m*l* of dichloromethane after addition of 100 m*l* of 20 % NaCl. The combined dichloromethane layer was dehydrated with anhydrous Na_2SO_4 and rotary-

vacuum evaporated just to dryness at 40°C. The residue was dissolved with 5 m*l* of acetone and passed through a column (a mixture of activated charcoal 0.3 g and microcrystalline cellulose 2.7 g). These pesticides were eluted with 100 m*l* of acetone. Each pesticide concentration of the eluate was measured by GC-FTD after rotary-vacuum evaporation to 1 - 5 m*l*. Average recoveries (whole body 5 g, n=3) were 72 % for benthiocarb, 75 % for simetryne, 87 % for diazinon, 90 % for malathion, 75 % for fenitrothion and 90 % for IBP.

Analyses were carried out as a sample for whole body of each fish (willow shiner), and as three separate samples for muscle and as a mixture of three samples for liver, kidney and gallbladder (carp) at each sampling time.

Calculation of bioconcentration factor (BCF)

BCF was calculated by the following equation.

$$BCF = \frac{\text{chemical concentration in fish}}{\text{chemical concentration in water}}$$

The concentration of chemical in the water at each sampling time was used for the calculation of BCF.

Results and Discussion

Accumulation of pesticides in willow shiner

Willow shiner were exposed to benthiocarb and simetryne for 14 days (experiment 1), diazinon, IBP , malathion and fenitrothion for 7 days

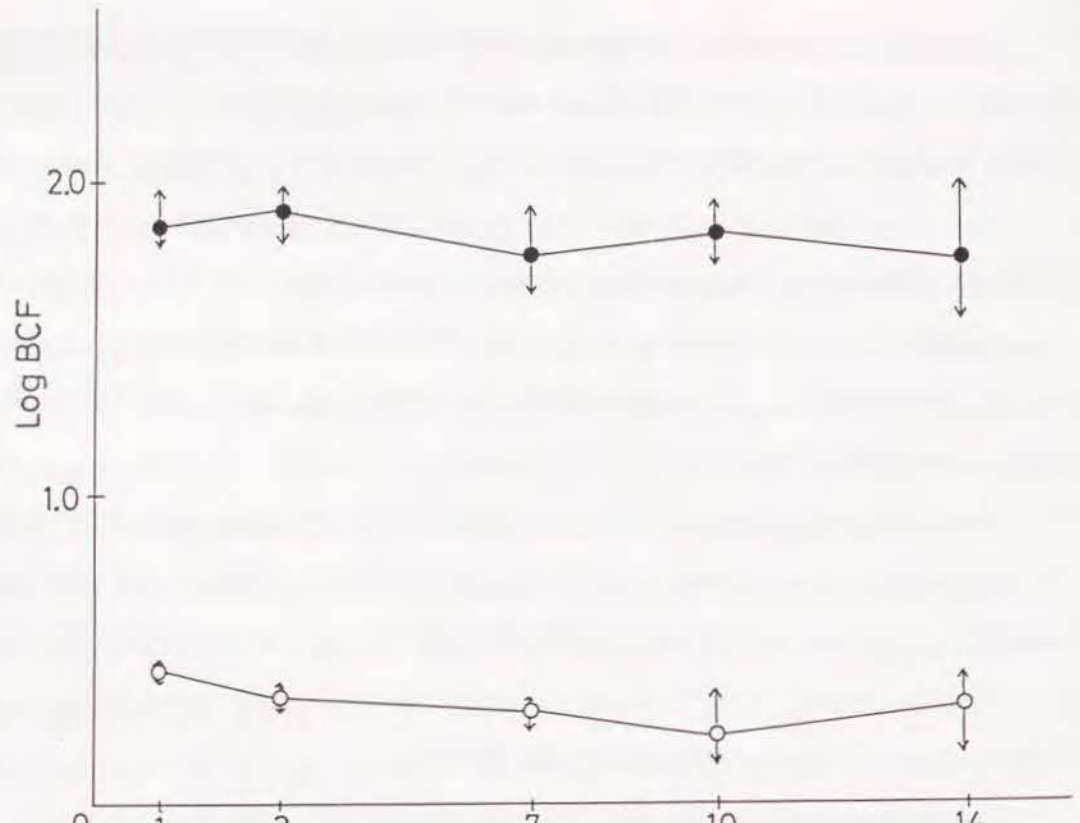

Fig.1 Accumulation of benthiocarb and simetryne in willow shiner.

● Benthiocarb ○ Simetryne

(experiment 2), and oxadiazon, CNP and chlomethoxynil for 14 days (experiment 3).

Fig.1 shows logBCF of benthiocarb and simetryne in whole body of the fish after 1, 3, 7, 10 and 14 days (experiment 1). The values of both chemicals reached plateaus in 24 h exposure. The average values of logBCF for 14 days were 1.82 (BCF=65) for benthiocarb and 0.31 (BCF=2) for simetryne. For benthiocarb, the value of 1.82 was slightly lower than that of 2.23 by freshwater-fish, topmouth gudgeon.[3]

The experimental results (experiment 2) are shown in Table 2. The concentrations of the chemicals in whole body of willow shiner reached plateaus in 6 - 48 h exposure (IBP 6 h, fenitrothion and malathion 24 h, diazinon 48 h). This was similar to the results of the same experiment on topmouth gudgeon (IBP and fenitrothion 24 h).[3,9] The average values of BCF were 33 (n=6, 6 - 168 h) for IBP, 399 (n=4, 24 - 168 h) for fenitrothion, 34 (n=4, 24 - 168 h) for malathion and 248 (n=3, 48 - 168 h) for diazinon through the 168 h exposure period. The order of BCF was fenitrothion > diazinon > malathion ≥ IBP. This was different from that of P_{ow} (fenitrothion > IBP ≥ diazinon > malathion).[3,10] It is generally known that there is a linearity between logP_{ow} and logBCF, but the BCF value of IBP was considerably lower than the value estimated from P_{ow}.

Table 2. BCF of pesticides (experiment 2) in willow shiner.

	BCF [a] in whole body of fish					
Pesticides	6h	12h	24h	48h	72h	168h
Diazinon	131±25	148±30	192±23	248±23	221±26	274±18
IBP	38±7	26±2	26±3	38±2	32±1	39±4
Fenitrothion	158±29	287±25	387±15	394±55	449±37	364±98
Malathion	10±2	25±12	37±10	31±7	33±12	38±15

[a] Mean value±SD (n=3)

However, the BCF value of 33 for IBP was considerably higher than the value of 4 in topmouth gudgeon.[3] For fenitrothion and diazinon, the values of 399 and 248 were slightly higher than those of 246 and 152 in the same fish,[3] respectively. However, the order of BCF values for these pesticides was fenitrothion > diazinon > IBP in both fishes.

Table 3. BCF of pesticides (experiment 3) in willow shiner.

	BCF [a] in whole body of fish				
Pesticides	1 day	3 days	7 days	10 days	14 days
Oxadiazon	464±100	872±243	1351±791	1390±668	938±100
CNP	1430±116	3250±176	5320±1071	5788±450	5960±592
Chlomethoxynil	1166±200	2244±494	4268±2153	4461±2116	3061±750

[a] Mean value±SD (n=3)

The experimental results (experiment 3) are shown in Table 3. The concentrations of these pesticides in the whole body of willow shiner reached plateaus in 7 days exposure. For CNP, this period was equal to the result of the same experiment in topmouth gudgeon.[11] The average BCF values (n=3) in the whole body of willow shiner were 1226 for oxadiazon, 5689 for CNP and 3930 for chlomethoxynil after 7 - 14 days exposure. The BCF value of CNP was considerably higher than the values 1109 and 950 in the whole body of topmouth gudgeon[3] and goldfish,[12] respectively. Further, those in muscle and viscera (liver, kidney and gallbladder) of carp were also low (334 - 862) in our previous study.[13] The BCF values of CNP were presumed to change by the difference of the fish species or the environment (temperature, concentration, etc.). On the other hand, Watanabe et al.[14] estimated the BCF values of CNP in several fishes to be 420 - 8000 from the field data. That is, these values were calculated by comparing the CNP concentrations in the fishes with those in water. The experimental data agreed closely with the field data. For oxadiazon and chlomethoxynil, the BCF values in the fishes estimated from field data were not reported because these pesticides were not detected in any fish examined.[14,15]

The accumulation potential (BCF) of these pesticides in whole body of willow shiner was low for simetryne, IBP, malathion and benthiocarb (BCF: 2 - 65), middle for diazinon and fenitrothion (BCF: 248 and 399) and high for oxadiazon, chlomethoxynil and CNP (BCF: 1226 - 5689).

Accumulation of pesticides in carp

Carp were exposed to benthiocarb for 7 days and to simetryne for 3 days(experiment 4), and to diazinon, IBP, malathion and fenitrothion for 7 days (experiment 5).

Table 4. BCF of benthiocarb and simetryne (experiment 4) in carp.

	BCF [a] of benthiocarb				BCF [a] of simetryne			
Organs	12h	24h	72h	168h	12h	24h	48h	72h
Muscle	26.8	25.9	21.3	28.1	2.4	2.8	2.6	1.9
Liver	82.5	84.5	50.5	33.3	14.6	16.5	11.8	11.1
Kidney	94.4	69.0	54.3	73.2	9.2	10.0	6.7	6.4
Gallbladder	72.4	79.8	61.1	39.8	11.9	11.3	13.5	6.8

[a] Mean value (n=3) for muscle and single determination value for liver, kidney and gallbladder.

The experimental results (experiment 4) are shown in Table 4. The concentrations of the chemicals in all four tissues of the carp reached plateaus after 12 h exposure. This was similar to the results of the same experiment on willow shiner described above. BCF values of benthiocarb were 25.5 in muscle, 62.7 in liver, 72.7 in kidney and 63.3 (mean, n=4) in gallbladder over the 168 h exposure period. Similarly, those of simetryne were 2.4 in muscle, 13.5 in liver, 8.1 in kidney and 10.9 in gallbladder over

the 72 h exposure period. The order of the BCF values in the four tissues of the carp for benthiocarb (kidney > gallbladder ≥ liver > muscle) was slightly different to that of simetryne (liver > gallbladder > kidney > muscle). But for both chemicals, the values of BCF in the viscera were higher than those in the muscle.

The experimental results (experiment 5) are shown in Table 5. The concentrations of the chemicals in all four tissues of the carp reached plateaus in 12 - 48 h exposure (diazinon 24 - 48 h, IBP 12 - 24 h, malathion 12 - 24 h and fenitrothion 12 - 24 h). This was similar to the results of the same experiments on topmouth gudgeon (IBP and fenitrothion 24 h)[3,9] or willow shiner (diazinon 48 h, IBP 6 h, malathion and fenitrothion 24 h) described above. The average BCF values for diazinon were 20.9 (n=3, 48 - 168 h) in muscle, 60.0 (n=3, 48 - 168 h) in liver, 111.1 (n=4, 24 - 168 h) in kidney and 32.2 (n=4, 24 - 168 h) in gallbladder over the 168 h exposure period. Similarly, those values were 4.3, 18.2, 26.7 and 17.5 for IBP, 6.0, 3.2, 17.3 and 2.7 for malathion, and 36.0, 101.9, 157.1 and 65.1 for fenitrothion over the same exposure period. The order of the BCF values was kidney > liver > gallbladder > muscle for diazinon, IBP and fenitrothion. For malathion, the order was kidney > muscle > liver ≥ gallbladder. The BCF value of malathion in the muscle was higher than those in the liver and gallbladder, which was different from the results of the other pesticides. On the other hands, the order of the BCF values were fenitrothion > diazinon > malathion ≥ IBP in the muscle and viscera. The BCF values of IBP in all four tissues of the carp were considerably lower than those estimated from the values of octanol-water partition coefficients (fenitrothion > IBP ≥ diazinon > malathion).[3,10] But these results were similar to those of the same experiment on willow shiner described above.

Table 5. BCF of pesticides (experiment 5) in carp.

Pesticides	Organs	BCF [a] 12h	24h	48h	72h	168h
Diazinon	Muscle	14.0	11.5	25.3	25.3	12.1
	Liver	33.0	37.7	57.3	71.3	51.3
	Kidney	2.9	149.2	88.7	156.0	50.4
	Gallbladder	14.0	36.2	36.7	44.0	11.7
IBP	Muscle	3.8	2.8	5.3	6.3	3.4
	Liver	17.2	15.3	18.1	25.2	15.4
	Kidney	6.6	33.5	23.5	33.8	16.1
	Gallbladder	14.0	21.5	15.8	25.5	10.6
Malathion	Muscle	4.6	3.2	8.3	9.3	4.5
	Liver	2.3	2.1	3.8	5.0	2.6
	Kidney	0.6	21.0	19.3	20.0	8.7
	Gallbladder	1.4	1.3	3.0	6.7	1.3
Fenitrothion	Muscle	31.7	26.5	40.5	48.3	33.1
	Liver	75.8	138.8	90.5	110.6	93.8
	Kidney	22.5	205.3	107.0	222.8	93.1
	Gallbladder	104.2	85.9	38.5	66.7	30.3

[a] Mean value (n=3) for muscle and single determination value for liver, kidney and gallbladder.

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CHAPTER 9

Excretion of Pesticides from Freshwater-Fishes

Introduction

In chapter 8, the accumulation of pesticides in freshwater-fishes was studied to evaluate the contamination of fishes in lakes and rivers by the chemicals.

Here, the excretion of pesticides from willow shiner[1-3] and carp[4,5] was further studied to evaluate the residue of the chemicals in freshwater-fishes. The excretion rate of the chemicals from the fishes was calculated by assuming the excretion process to be first-order or second-order kinetics. These data will be useful for the evaluation of their safety to man as well as their contamination of fishes in lakes and rivers.

Experimental Section

Reagent

Pesticides were the same as in chapter 8.

Test fish

Willow shiner and carp were obtained from the same places as in chapter 8. The body length and weight of the fishes were about the same as in chapter 8. Commercial assorted feed was given 50 mg/fish for willow shiner and 500 mg/fish for carp once a day throughout the experiment.

Test system

The study was carried out by the same experimental arrangement in chapter 2.

For the excretion of these pesticides (7 - 14 days) from willow shiner, 15 - 30 fish remaining in the test tanks after their accumulation experiment (chapter 8) were transferred into aquaria tanks containing clean water, and dechlorinated city water was supplied to them. During the test, the flow rate and temperature of the test water were maintained at 1 *l*/min and 20±1 or 24±1 °C, respectively. The concentrations of these pesticides excreted from the fish in each test tank water were 0.3 μg/*l* for benthiocarb, less than 0.1 μg/*l* for simetryne, 0.02 μg/*l* for diazinon, less than 0.02 μg/*l* for IBP, less than 0.01 μg/*l* for malathion and fenitrothion, 0.1 μg/*l* for oxadiazon and chlomethoxynil, and 0.2 μg/*l* for CNP after 10 - 24 h in these excretion experiments.

Excretion experiments (24 - 72 h) of these pesticides from carp were carried out in dechlorinated city water after accumulation of the chemicals in the same manner for the accumulation experiments in chapter 8. In each experiment, 15 or 18 fish were exposed to the chemicals for 3 - 7 days, 12 fish except 3 fish or 15 fish except 3 fish were transferred into each aquaria and dechlorinated city water was supplied to them. During the test, the flow rate and temperature of the test water were maintained at 1.0 - 1.5 *l*/min and 19±1°C or 23±1°C, respectively. The concentrations of these pesticides excreted from the fish in each test water were less than 0.2 μg/*l* for benthiocarb, 0.1 μg/*l* for simetryne and less than 0.05 μg/*l* for diazinon, IBP, malathion and fenitrothion after 8 h in these excretion experiments. From the low concentrations of these pesticides in the test tanks excreted

from willow shiner and carp, their accumulation in both fishes was presumed to be very little.

For both willow shiner and carp, three fish were taken at appropriate intervals throughout the experiments.

Under these conditions, none of the fishes showed any signs of tiredness or agitation during the experiments.

Analysis

Determination of these pesticides in willow shiner and carp was carried out by the same method as in chapter 8. Analyses were carried out as a sample for whole body of each fish in willow shiner, and as three separate samples for muscle and as a mixture of three samples for liver, kidney and gallbladder in carp.

Calculation of excretion rate constant

The following equations were used for the calculation of excretion rate constants of chemicals from fish.

$C = C_o e^{-kt}$ (1) or $1/C - 1/C_o = kt$ (2)

where

C = chemical concentration in fish (ng/g) at time, t

C_o = chemical concentration in fish (ng/g) initially

k = excretion rate constant {h^{-1} or $(g/ng)h^{-1}$}

t = time (h)

The experimental data were plotted on $\ln C_o/C$ vs. t (1) and $1/C - 1/C_o$ vs. t (2). The equation that gave a more linear plot was selected for each chemical.

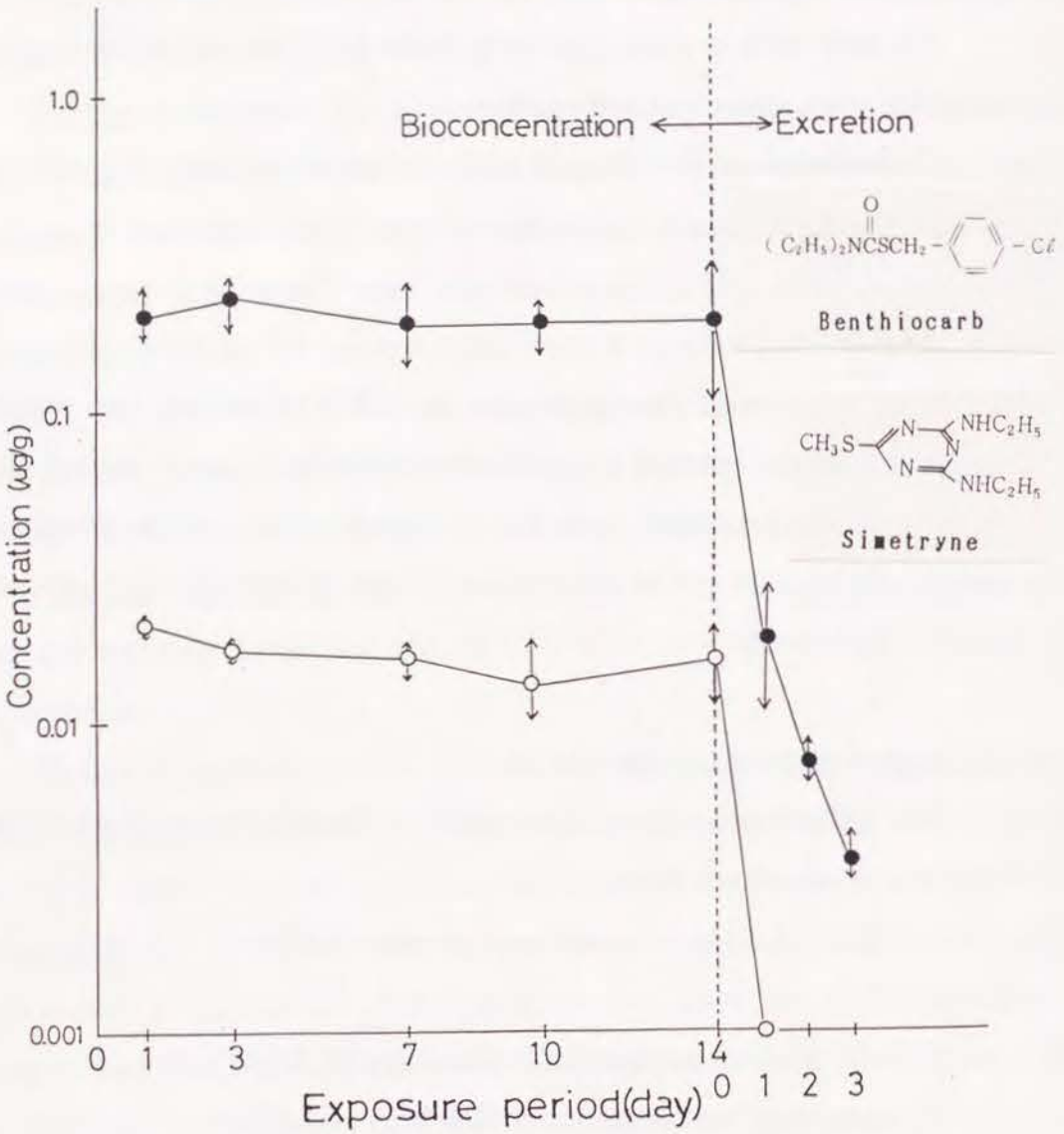

Fig.1 Excretion of benthiocarb and simetryne from willow shiner.

● Benthiocarb ○ Simetryne

Results and Discussion

Excretion of pesticides from willow shiner

Excretion of pesticides from whole body of willow shiner is shown in Figs.1 - 3.

The experimental results of benthiocarb and simetryne are shown in Fig.1 together with their accumulation. The concentrations of these chemicals in the fish rapidly decreased, so could not be measured by GC-FTD after 7 days for benthiocarb and after 2 - 7 days for simetryne. The excretion rate constants of benthiocarb and simetryne were calculated by assuming their process to be first-order kinetics from the data of Fig.1.

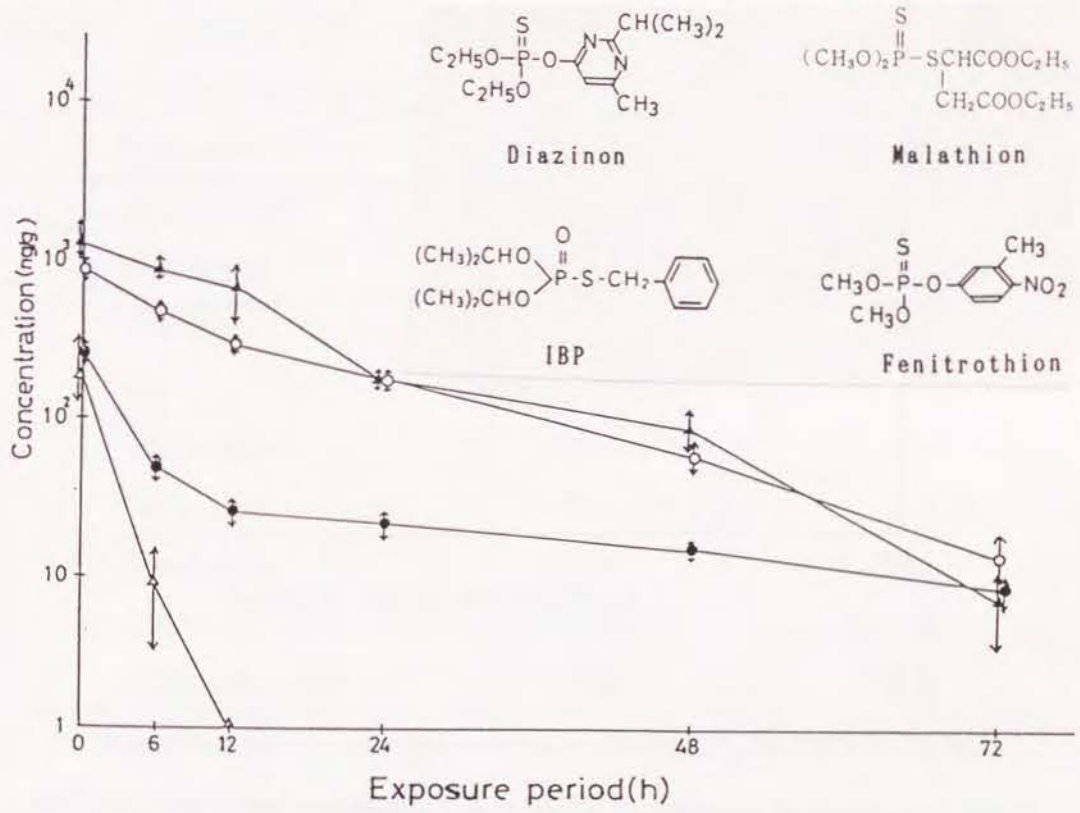

Fig.2 Excretion of diazinon, IBP, malathion and fenitrothion from willow shiner. ○ Diazinon ● IBP △ Malathion ▲ Fenitrothion

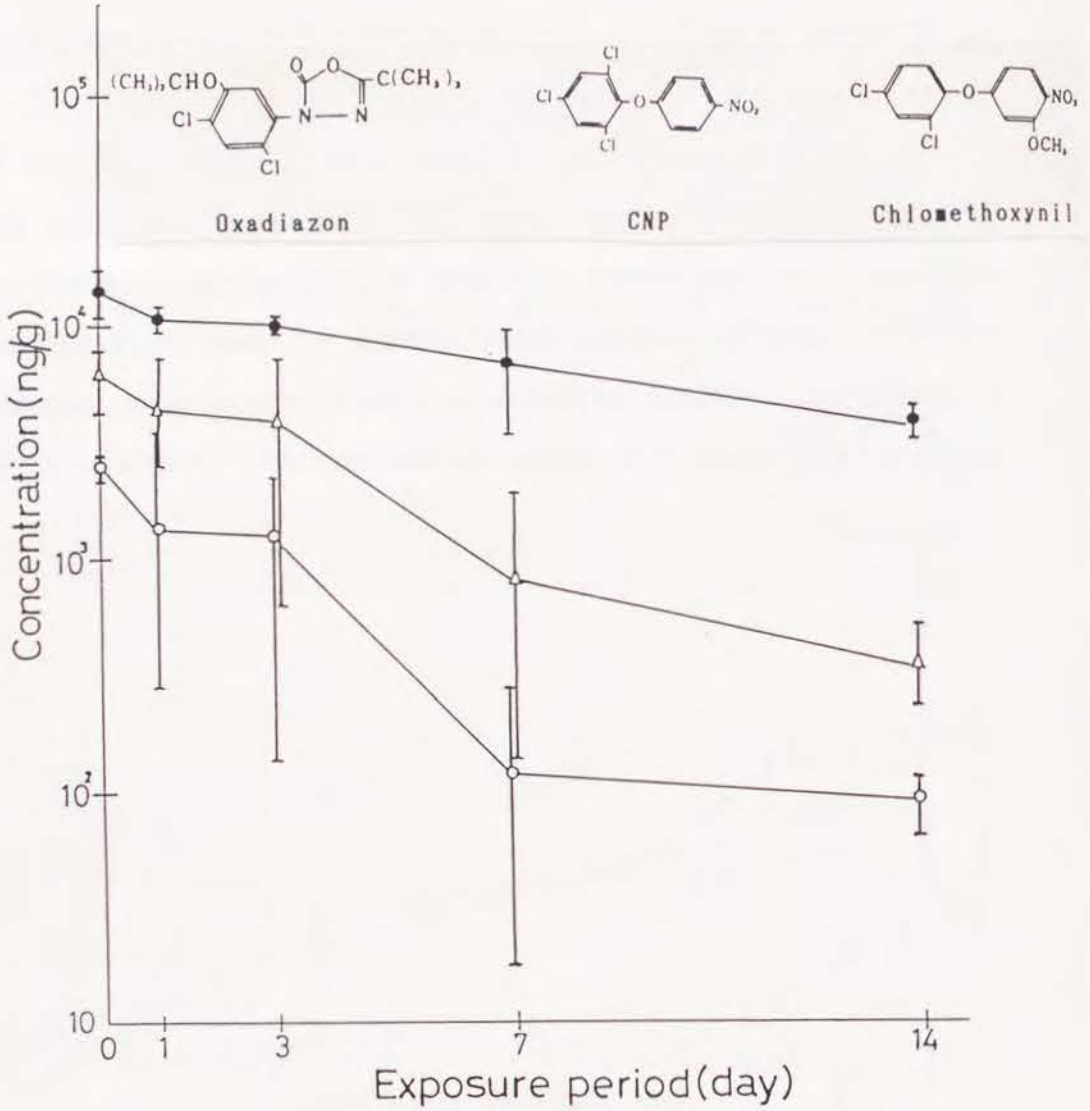

Fig.3 Excretion of oxadiazon, CNP and chlomethoxynil from willow shiner.

○ Oxadiazon ● CNP △ Chlomethoxynil

The experimental results of diazinon, IBP, fenitrothion and malathion are shown in Fig.2. The concentrations of these chemicals in whole body of the fish rapidly decreased, so could not be measured by GC-FTD after 24 - 168 h for malathion, and after 168 h for diazinon, IBP and fenitrothion. The excretion rate constants of diazinon, fenitrothion and malathion could be calculated by assuming their excretion process to be first-order kinetics [Eq.(1)] from the data of Fig.2. But it was impossible for IBP, so its rate constant was calculated by assuming the process to be second-order kinetics [Eq.(2)].

Table 1. Excretion rate constants and biological half-lives of pesticides by willow shiner.

Pesticides	k (h^{-1})	Half-life (h)
Benthiocarb	0.07	9.9
Simetryne	0.13	5.3
Diazinon	0.07	9.9
IBP	{0.0017(g/ng)h^{-1}}[a]	-
Fenitrothion	0.07	9.9
Malathion	0.49	1.4
Oxadiazon	0.0125	55.5
CNP	0.0042	165.0
Chlomethoxynil	0.0088	78.8

[a] This value was calculated assuming the excretion process to be second-order kinetics.

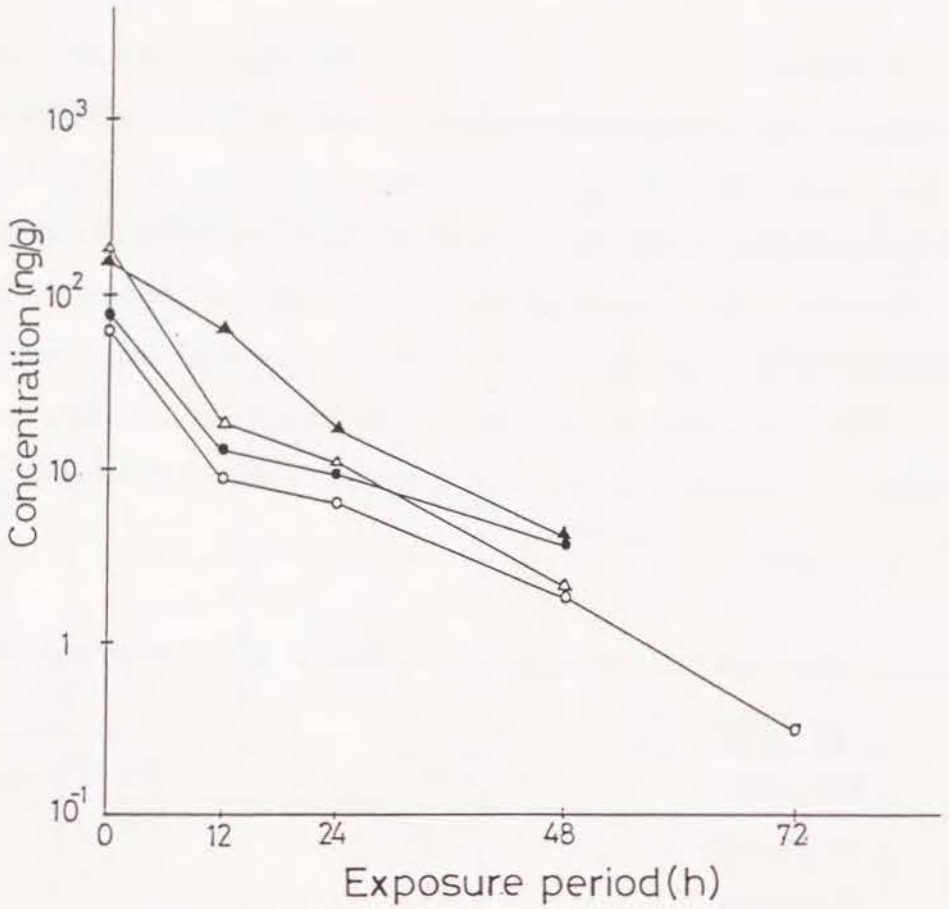

Fig.4 Excretion of benthiocarb from carp.

○ Muscle ● Liver

△ Kidney ▲ Gallbladder

The experimental results of oxadiazon, CNP and chlomethoxynil are shown in Fig.3. The concentrations of these chemicals in whole body of the fish decreased more slowly than those of the chemicals in Fig.1 and Fig.2. The excretion rate constants of oxadiazon, CNP and chlomethoxynil were calculated by assuming their excretion process to be first-order kinetics. The rate constants of all pesticides are summarized in Table 1. Their excretion rate from whole body of willow shiner was considerably rapid

for benthiocarb, simetryne, diazinon, fenitrothion and malathion (biological half-lives: 1.4 - 9.9 h), but a little slower for oxadiazon, CNP and chlomethoxynil (biological half-lives: 55.5 - 165.0 h). The order of these pesticides in their excretion rate was reversed compared with that of their BCF values. That is, the greater was the BCF values, the slower was the excretion rate.

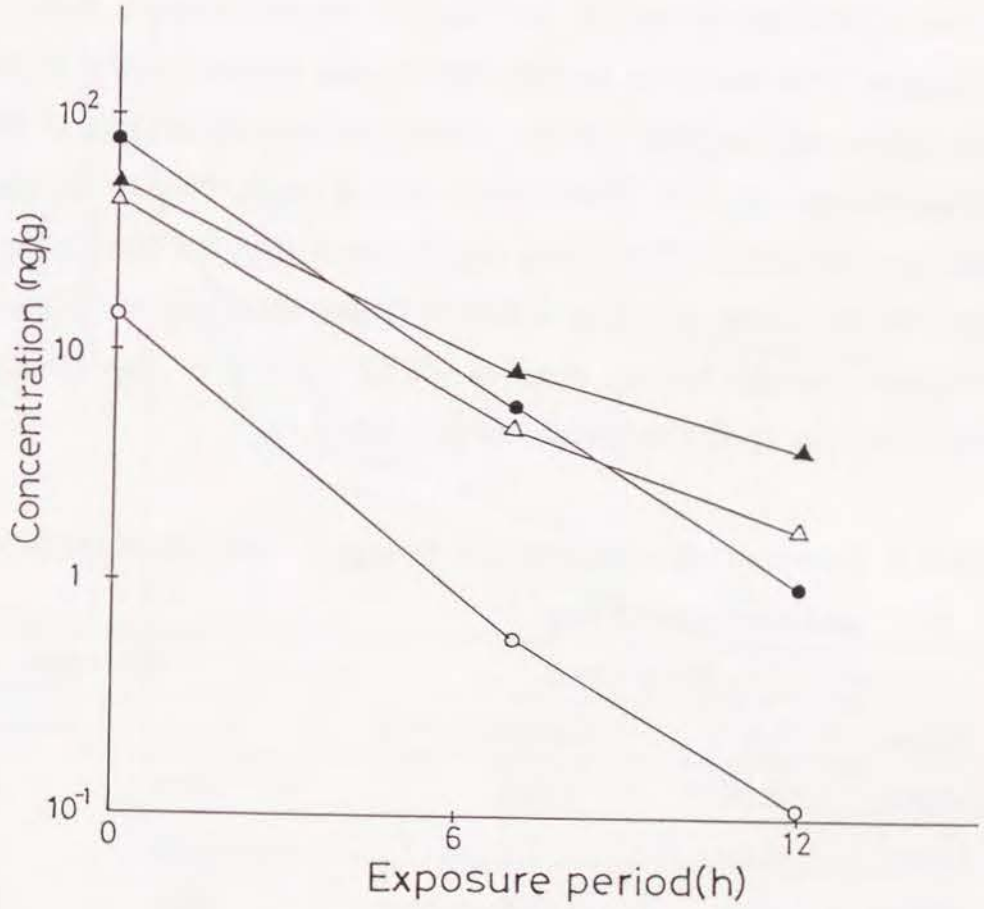

Fig.5 Excretion of simetryne from carp.

○ Muscle ● Liver

△ Kidney ▲ Gallbladder

Excretion of pesticides from carp

Excretion of pesticides from four tissues of carp is shown in Figs.4 - 9. The experimental results of benthiocarb and simetryne are shown in Figs.4 and 5, respectively. The concentrations of both chemicals in he fish rapidly decreased, so could not be measured by GC-FTD after 72 h for benthiocarb in the viscera of the fish and after 24 h for simetryne in all four tissues of the fish. The excretion rate constants (k) and biological half-lives of all four tissues of the fish are shown in Table 2. For benthiocarb, there was no difference in the excretion rate for three tissues (except kidney) of the fish. For simetryne, the rate for the kidney and gallbladder was slower than for the muscle and liver. The excretion rate of benthiocarb for all tissues of the carp ($k = 0.09 - 0.13\ h^{-1}$) was slightly faster than the rate for the whole body of the willow shiner ($k = 0.07\ h^{-1}$) described above. Similarly for simetryne, the rate for the carp ($k = 0.23 - 0.44\ h^{-1}$) was considerably faster than that for the willow shiner ($k = 0.13\ h^{-1}$).

Table 2. Excretion rate constants and biological half-life of benthiocarb and simetryne by carp.

	Benthiocarb		Simetryne	
Organs	$k(h^{-1})$	Half-life(h)	$k(h^{-1})$	Half-life(h)
Muscle	0.10	6.9	0.44	1.6
Liver	0.09	7.7	0.37	1.9
Kidney	0.13	5.3	0.29	2.4
Gallbladder	0.09	7.7	0.23	3.0

The experimental results of diazinon, IBP, malathion and fenitrothion are shown in Figs.6 - 9, respectively. The concentrations of malathion in the muscle and liver of the fish rapidly decreased, so could not be measured by

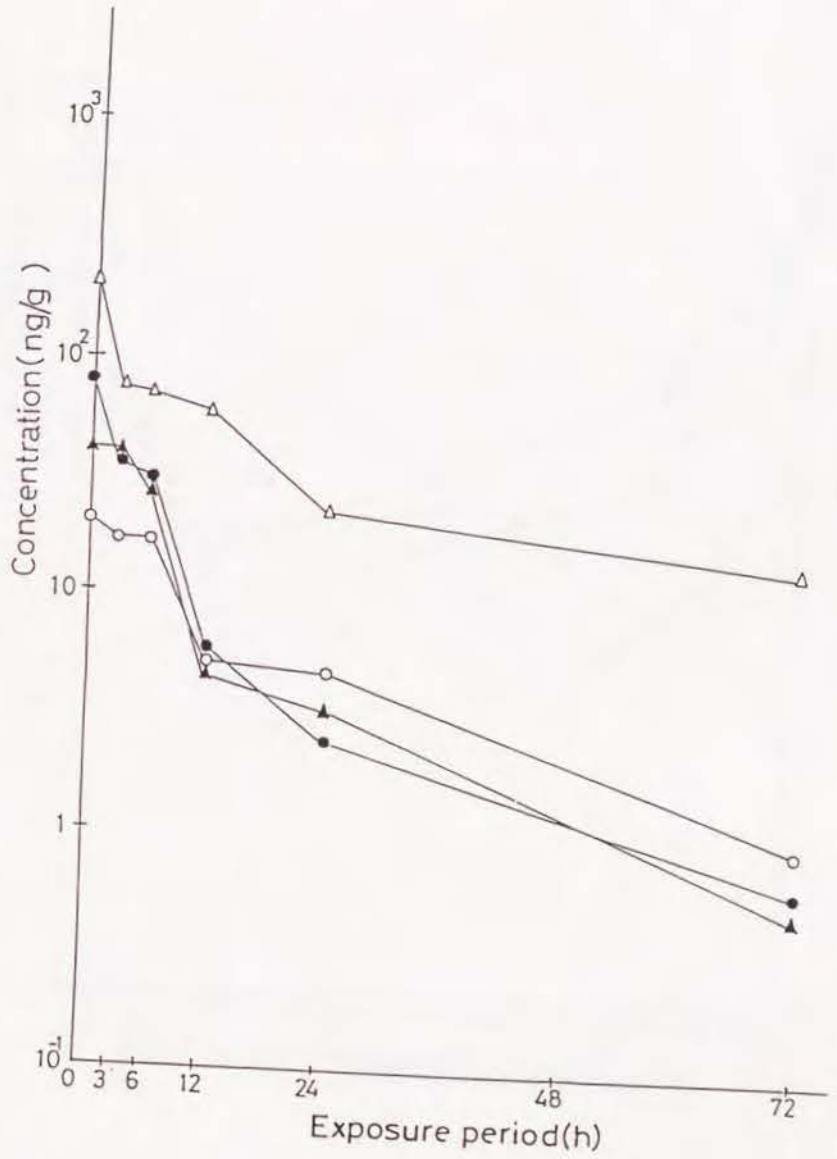

Fig.6 Excretion of diazinon from carp.
○ Muscle ● Liver
△ Kidney ▲ Gallbladder

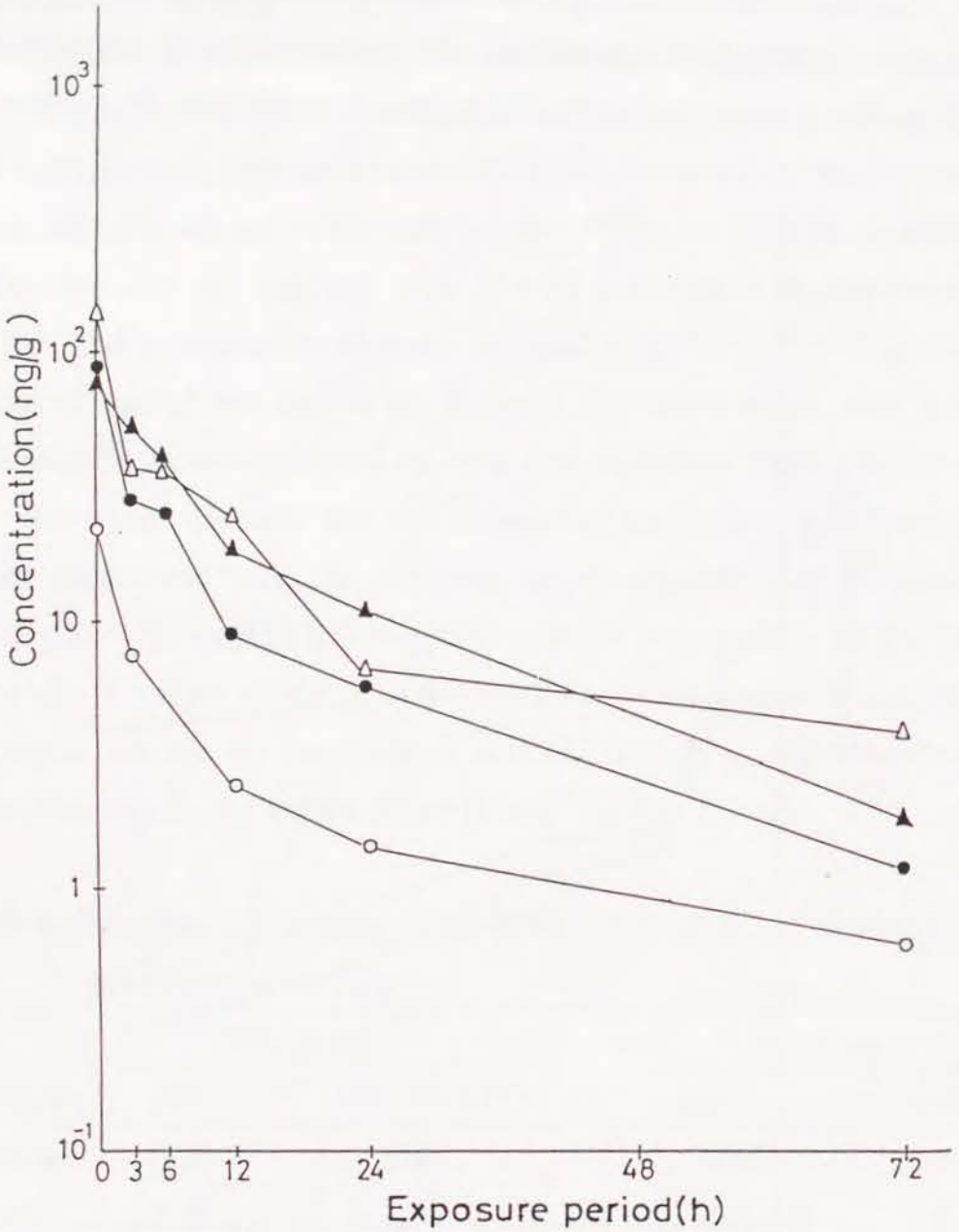

Fig.7 Excretion of IBP from carp.

○ Muscle ● Liver

△ Kidney ▲ Gallbladder

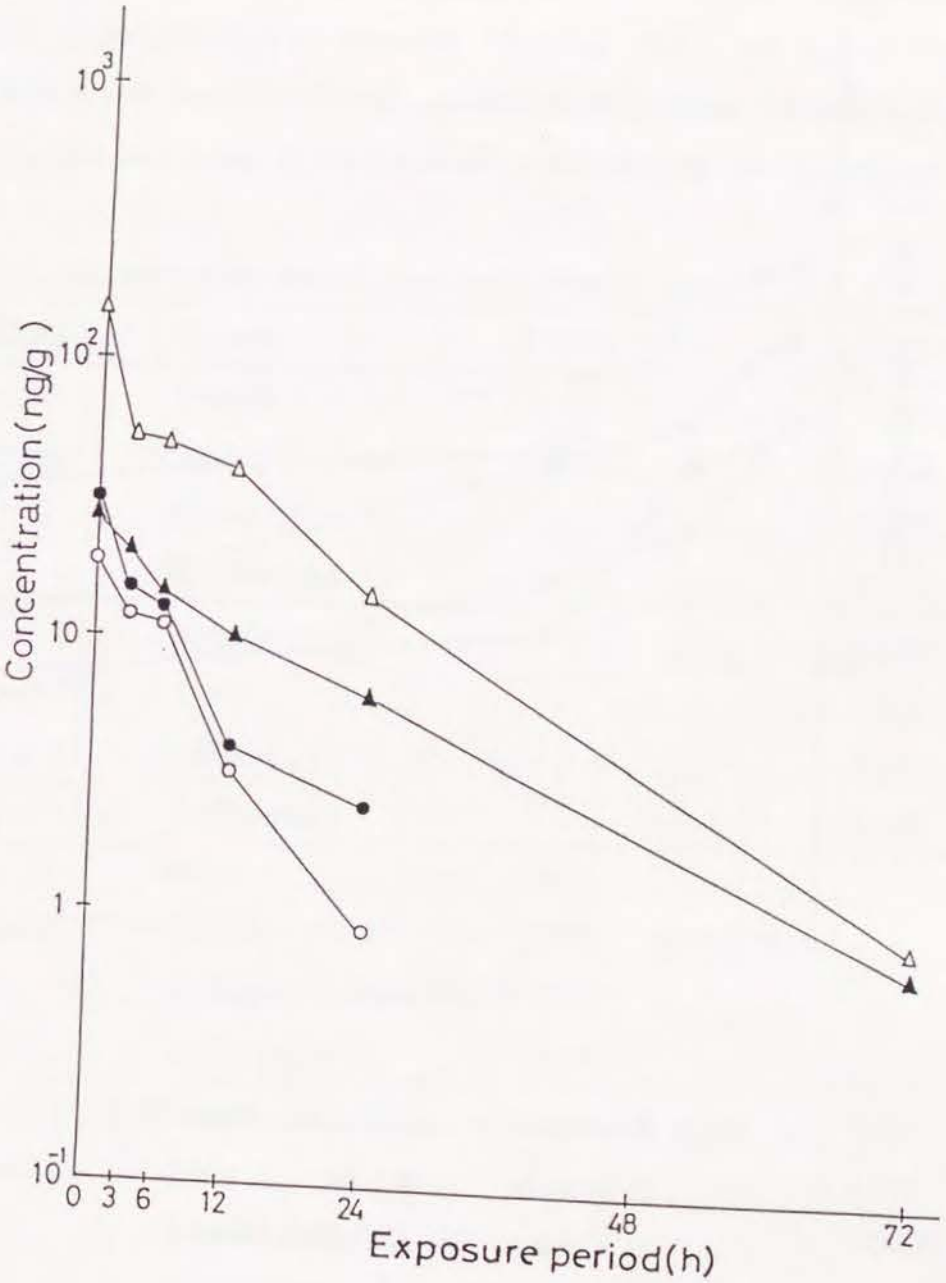

Fig.8 Excretion of malathion from carp.

○ Muscle ● Liver

△ Kidney ▲ Gallbladder

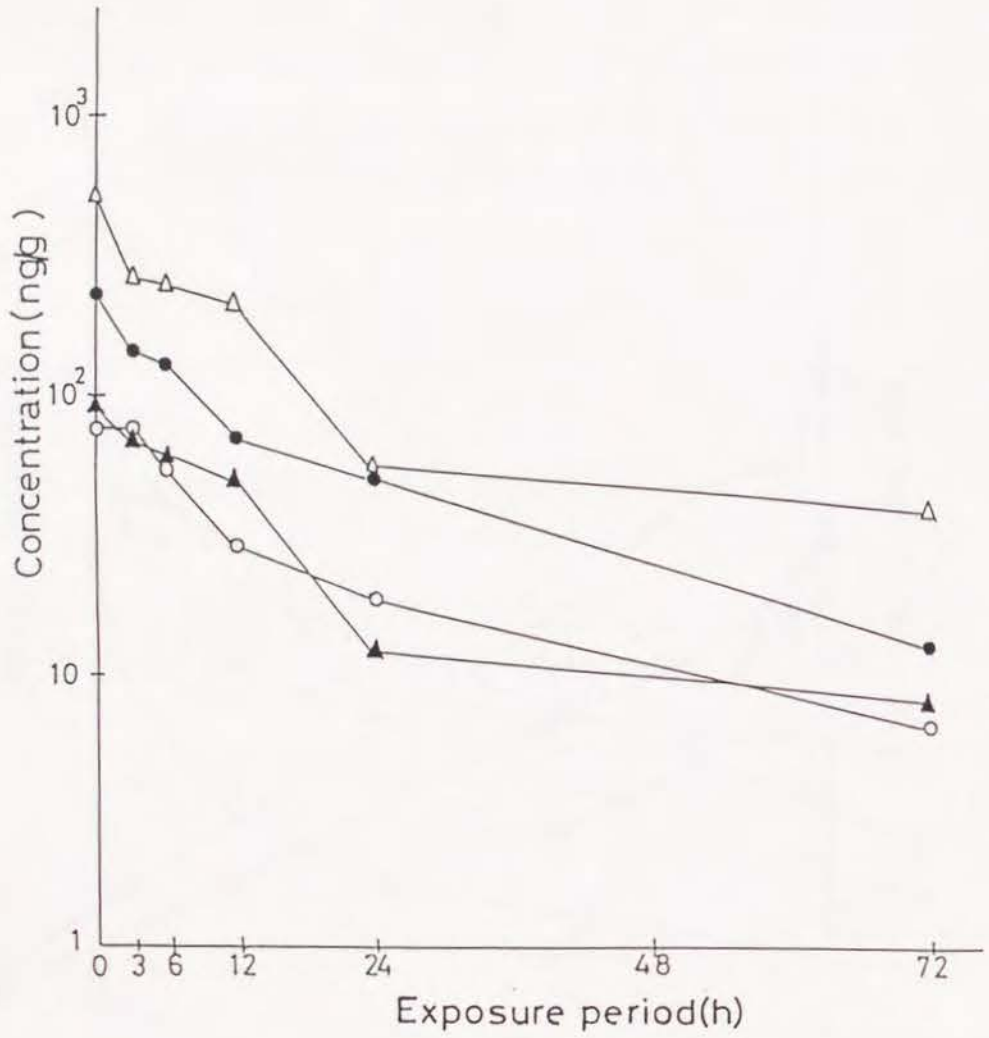

Fig.9 Excretion of fenitrothion from carp.

○ Muscle ● Liver

△ Kidney ▲ Gallbladder

gas chromatography after 72 h. The excretion rate constants (k) of all four tissues of the fish are shown in Table 3. The rate constants of malathion could be calculated by assuming the excretion process to be first-order kinetics [Eq.(1)] from the data of Fig.8. But it was impossible for diazinon,

IBP and fenitrothion, so their rate constants were calculated by assuming the process to be second-order kinetics [Eq.(2)] from the data of Figs.6, 7 and 9. It is not understood why the excretion process follows first-order kinetics for malathion and second-order kinetics for the other pesticides.

Table 3. Excretion rate constants of pesticides by carp.

Pesticides	Organs	k $(h^{-1})^a$	k $\{(g/ng)h^{-1}\}^b$
	Muscle	-	0.013
Diazinon	Liver	-	0.020
	Kidney	-	0.001
	Gallbladder	-	0.023
	Muscle	-	0.024
IBP	Liver	-	0.010
	Kidney	-	0.004
	Gallbladder	-	0.006
	Muscle	0.13	-
Malathion	Liver	0.12	-
	Kidney	0.08	-
	Gallbladder	0.06	-
	Muscle	-	0.002
Fenitrothion	Liver	-	0.001
	Kidney	-	0.0004
	Gallbladder	-	0.002

a First-order kinetics

b Second-order kinetics

In the same experiment on willow shiner, the process follows fist-order kinetics for diazinon, malathion and fenitrothion and second-order kinetics for only IBP.

The orders of the excretion rate from the four tissues of the carp were muscle ≥ liver > kidney ≥ gallbladder for malathion, gallbladder ≥ liver > muscle > kidney for diazinon, muscle > liver > gallbladder ≥ kidney for IBP and muscle ≥ gallbladder ≥ liver > kidney for fenitrothion. These pesticides accumulated in the fish had a tendency to excrete rapidly from muscle and slowly from kidney. The slow excretion rate from kidney is probably because these pesticides in all tissues of the fish are finally transported into kidney and excreted.

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CHAPTER 10

Accumulation and Metabolism of 2,4,6-Trichlorophenyl-4'-nitrophenyl Ether and 2,4,6-Trichlorophenyl-4'-aminophenyl Ether in Carp

Introduction

In recent years, a large amount of pesticides such as herbicides or insecticides have been produced and discharged into the environment. CNP has been one of the most popular herbicides widely used in paddy fields during the rice planting season in Japan. It is well known that 2,4,6-trichlorophenyl-4'-nitrophenyl ether (CNP) in the environment (river, lake and sea) tends to highly accumulate in many species of fish and shellfish from the field data[1-5] and further from the experimental data on the accumulation of CNP in fish[6] and shellfish.[7]

However, few reports[8] have been published regarding the contamination of fish in the environment by the metabolites of CNP.

Therefore, metabolism of CNP was studied for carp simultaneously with the CNP accumulation experiment to predict their contamination of fish in the environment.[9]

Further, the same study was performed for 2,4,6-trichlorophenyl-4'-aminophenyl ether (CNP-amino), highly produced by CNP in paddy fields, using carp.[10]

Experimental Section

Chemicals

2,4,6-Trichlorophenyl-4'-nitrophenyl ether (CNP), purity 99.0%, was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). 2,4,6-Trichlorophenyl-4'-aminophenyl ether (CNP-amino), 2,4,6-trichlorophenyl-4'-actamide phenyl ether (CNP-acetamide) and 2,4,6-trichlorophenyl-4'-formamide phenyl ether (CNP-formamide) were synthesized by the method of Suzuki et al.[8] and these structures were confirmed by gas chromatography-mass spectrometry (GC-MS).

Test fish

Carp (*Cyprinus carpio* L.) were purchased from Nango Suisan Center (Shiga Prefecture, Japan). The body length and weight of the fish were 9.5 - 11.0 cm and 26.2 - 34.3 g, respectively. CNP, CNP-amino, CNP-acetamide and CNP-formamide were not detected in carp before exposure to CNP and CNP-amino. Commercial assorted feed was given 0.5 g/fish once a day throughout the experiment periods.

Test system

The study was carried out without aeration under the continuous flow-through system. The experimental arrangement was the same as in chapter 2.

Aqueous stock solutions of CNP (750 μg/*l*) and CNP-amino (2000 μg/*l*) were diluted continuously 150 and 100 times, respectively, with dechlorinated city water and supplied to each aquaria (about 50 *l*) containing 18 and 15 fish. During the tests, the flow rate and temperature of the test water were maintained 18 *l*/h and 22±1°C for CNP and 12 *l*/h and 23±1°C for CNP-amino, respectively. The concentrations of CNP and CNP-amino in

each test tank water were mean±SD (range) of 0.9±0.1 (0.8 - 1.1) μg/*l* and 3.9±0.3 (3.7 - 4.4) μg/*l*, respectively. Measurements were carried out at 1, 3, 7, 10, 14 and 20 days for CNP and1, 3, 7, 10 and 14 days for CNP-amino. Three fish were taken and immediately analyzed after rinsing with distilled water at each day. The low concentrations of CNP and CNP-amino compared with the fixed concentrations (CNP 5 μg/*l* and CNP-amino 20 μg/*l*) in each test tank water are probably because they are adsorbed to the tubes of the experimental arrangement or to the walls of the aquaria.

Under these conditions, none of the fish showed signs of tiredness or agitation during the experiments.

Analysis

CNP in water

The concentration of CNP in water was determined by the following procedure. A measured volume (100 m*l*) of water was shaken with 50 m*l* of hexane after addition of 5 g of NaCl. The organic layer was analyzed by gas chromatograph equipped with electron capture detector (GC-ECD). Average recoveries (n=3) were 99% at 5 μg/*l* spiked level. The GC (Shimadzu GC-9A) operating conditions were as follows:

GC column: a 1.6 m x 3 mmϕ glass column packed with 5% DC200 on 80/100 mesh Chromosorb W AW DMCS Carrier: N_2 50 m*l*/min
Temperatures: injection and detector 280°C; column 225°C

CNP-amino in water

The concentration of CNP-amino in water was determined by the following procedure. A measured volume (100 m*l*) of water was shaken with

50 m*l* of hexane after addition of 5 m*l* of 8 N KOH. The organic layer was analyzed after rotary-vacuum evaporation to 10 m*l* by GC-ECD. Average recoveries (n=3) were 89 % at 5 μg/*l* spiked level. The GC (Shimadzu GC-9A) operating conditions were as follows:

GC column: a 2.5 m x 3 mmϕ glass column packed with 5 % OV17 on 80/100 mesh Chromosorb W AW DMCS Carrier: N_2 50 m*l*/min
Temperatures: injection and detector 290°C; column 260°C

CNP and its metabolites in fish samples

Analyses were carried out as three separate samples for muscle and as a mixture of three samples for liver, kidney and gallbladder.

(1) CNP Determination of CNP in fish samples was performed according to the following method.[11] A fish sample (0.2 - 5 g) was homogenized with 50 m*l* of acetonitrile by high-speed homogenizer (Ultra-Turrax) after addition of 5 g of anhydrous Na_2SO_4 and the organic layer was filtrated. The residue was again homogenized and filtrated in the same manner. The combined filtrate was rotary-vacuum evaporated just to dryness at 40°C and the residue was dissolved in 20 m*l* of hexane (solution A). A measured volume (5 m*l*) of solution A was diluted with 5 m*l* of hexane, shaken with 20 m*l* of acetonitrile saturated with hexane and the acetonitrile layer was retained. The hexane layer was again shaken with 20 m*l* of acetonitrile saturated with hexane. The combined acetonitrile layer was rotary-vacuum evaporated just to dryness at 40°C and the residue was dissolved with 5 m*l* of hexane. The hexane solution was passed through an activated Florisil column (2.5 g, 1 cmϕ x 6.5 cm) and CNP was eluted with 50 m*l* of ether-hexane (2+8) after washing with 20 m*l* of hexane. The CNP concentration of the

eluate was measured by GC-ECD after rotary-vacuum evaporation to 5 - 20 m*l*. Average recoveries (muscle, n=3) were 91% at 100 ng/g spiked level. Detection limits were 5 ng/g in muscle and 10 ng/g in viscera. The GC operating conditions were the same as in water.

(2) CNP-amino Determination of CNP-amino in fish samples was performed according to the following method.[11] A measured volume (10 m*l*) of solution A was shaken twice with 20 m*l* of 0.2 N HCl. The combined aqueous layer was shaken twice with 20 m*l* of hexane after addition of 2 m*l* of 8 N KOH. The combined hexane layer was dehydrated with anhydrous Na_2SO_4 and rotary-vacuum evaporated to 0.5 - 2 m*l*. The CNP-amino concentration of the solution was measured by GC-ECD. Average recoveries (muscle, n=3) were 87% at 100 ng/g spiked level. Detection limits were 1 ng/g in muscle and 2 ng/g in viscera. The GC operating conditions were as follows:

GC column: a 1.6 m x 3 mmϕ glass column packed with 2% OV17 on 80/100 mesh Chromosorb W AW DMCS Carrier: N_2 50 m*l*/min
Temperatures: injection and detector 280°C; column 250°C

(3) CNP-acetamide and CNP-formamide Determination of CNP-acetamide and CNP-formamide in fish samples was performed according to the method of Suzuki et al.[8] modified as follows. A remaining volume (5 m*l*) of solution A was passed through an activated silica gel column (Kieselgel 60 1.5 g + anhydrous Na_2SO_4 1.5 g, 1 cmϕ x 7 cm) and these metabolites eluted with 60 ml of ethyl acetate-benzene (1.5+8.5) after washing with 20 m*l* of ethyl acetate-benzene (1+9). The metabolites concentrations of the eluate were measured by GC-ECD after rotary-vacuum evaporation to 2 - 5 m*l*. Average

recoveries (muscle, n=3) were 90% for CNP-acetamide and 81% for CNP-formamide at 100 ng/g spiked levels. Detection limits were 2 ng/g in muscle and 10 ng/g in viscera for both metabolites.

(4) GC-MS determination of CNP metabolites GC-MS determination of CNP metabolites was tried by selected ion monitoring (SIM) with ions at m/z=287 for CNP-amino, m/z=329 for CNP-acetamide and m/z=315 for CNP-formamide. The GC-MS (JEOL JMS-DX302) operating conditions were as follows:

GC column: a 1 m x 2.6 mmϕ glass column packed with 2% OV17 on 80/100 mesh Chromosorb W AW DMCS Carrier: He 20 m*l*/min
Ion mode: EI Ionization voltage: 70 eV Ionization current: 300 μA
Operating temperatures: injection 300°C; column 260°C
separator 300°C

CNP-amino and its metabolites in fish samples

Analyses were carried out as three separate samples for muscle and as a mixture of three samples for liver, kidney and gallbladder.

(1) Free CNP-amino Determination of free CNP-amino in fish samples was performed according to the following method.[11] A fish sample (0.2 - 5 g) was homogenized with 50 m*l* of acetonitrile by high-speed homogenizer (Ultra-Turrax) and the organic layer was filtrated. The residue was again homogenized and filtrated in the same manner. The combined filtrate was rotary-vacuum evaporated just to dryness at 40°C after dehydration with anhydrous Na_2SO_4 and the residue was dissolved in 10 m*l* of hexane (solution A). A 5 m*l* of solution A was diluted with 5 m*l* of hexane and shaken twice with 20 m*l* of 0.2 N HCl. The combined aqueous layer was

shaken twice with 20 m*l* of hexane after addition of 2 m*l* of 8 N KOH. The combined hexane layer was dehydrated with anhydrous Na_2SO_4 and rotary-vacuum evaporated to 5 - 20 m*l*. The CNP-amino concentration of the solution was measured by GC-ECD under the same GC operating conditions as above. Average recoveries (muscle,n=3) were 87 % at 100 ng/g spiked level.

(2) Bound CNP-amino Determination of bound CNP-amino in fish samples was performed according to the method of Watanabe et al.[12] The residue of fish samples after acetonitrile extraction described above was saponified with 25 m*l* of 4 N NaOH for 4 h at 80°C. The reaction mixtures were cooled and shaken with 50 m*l* of hexane after addition of 25 m*l* of acetone. The hexane layer was shaken with 50 m*l* of 0.4 N HCl. The aqueous layer was again shaken with 50 m*l* of hexane after addition of 5 m*l* of 10 N NaOH. The hexane layer was dehydrated with anhydrous Na_2SO_4 and rotary-vacuum evaporated to 10 - 50 m*l*. The CNP-amino concentration of the solution was measured by GC-ECD under the same GC operating conditions as above. Average recoveries (muscle, n=3) were 80 % at 100 ng/g spiked level.

(3) CNP metabolites Determination of CNP-acetamide and CNP-formamide in fish samples was performed according to the method of Suzuki et al.[8] modified as follows. A remaining volume (5 m*l*) of solution A was passed through an activated silica gel column (Kieselgel 60 1.5 g + anhydrous Na_2SO_4 1.5 g, 1 cmϕ x 7 cm) and these metabolites were eluted with 60 m*l* of ethyl acetate-benzene (1.5+8.5) after washing with 20 ml of benzene. The metabolites concentrations of the eluate were measured by GC-ECD after rotary-vacuum evaporation to 10 - 20 m*l*. Average recoveries (muscle, n=3)

were 99 % for CNP-acetamide and 96 % for CNP-formamide at 100 ng/g spiked levels. The GC operating conditions were as follows:

GC column: J &W DB 1701 (30 m x 0.53 mmϕ) Carrier: N_2 30m*l*/min
Temperature: injection and detector 290°C; column 260°C

(4) GC-MS confirmation of CNP metabolites GC-MS confirmation of CNP metabolites was tried for the fish samples. The GC-MS operating conditions were as follows:

GC column: J &W DB 1701 (30 m x 0.32 mmϕ) Carrier: He 0.8 m*l*/min
Ion mode: EI Ionization voltage: 70 eV Ionization current: 300 μA
Operating temperature: injection 290°C; column 60°C (2 min) 32°C/min to 270°C

Calculation of bioconcentration factor (BCF)

BCF was calculated by the following equation.

$$BCF = \frac{\text{chemical concentration in each tissue of fish}}{\text{chemical concentration in water}}$$

The chemical concentration in water at each sampling time was used for the calculation of BCF.

Results and Discussion

Accumulation of CNP

Fig.1 shows BCF of CNP in muscle, liver, kidney and gallbladder of carp through the 20 days exposure period. The CNP concentration in muscle

nearly reached plateaus after 7 days, and probably after 10 - 14 days for viscera. These periods were slightly longer than the period 7 days in whole

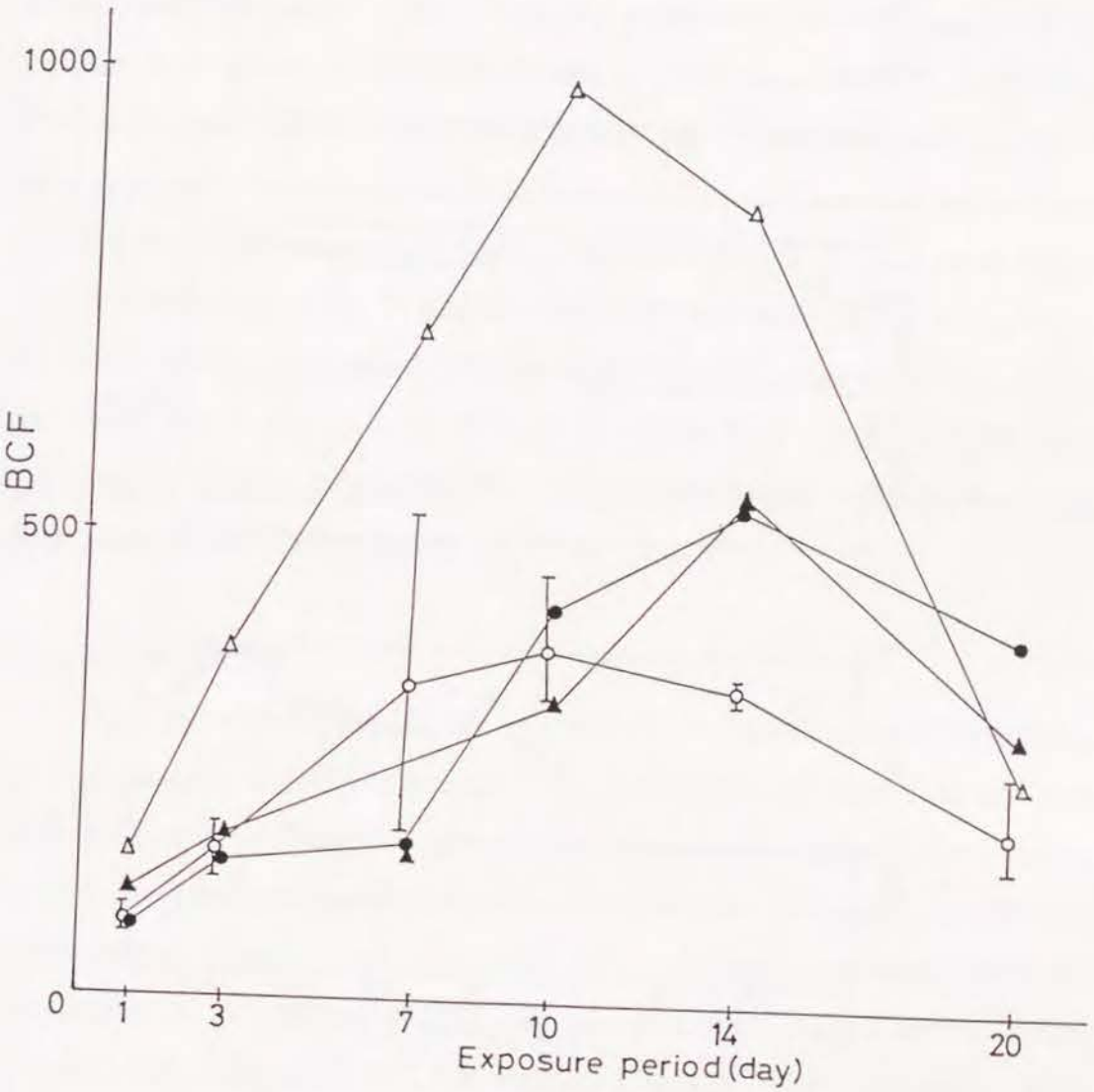

Fig.1 Accumulation of CNP in carp.

○ Muscle ● Liver
△ Kidney ▲ Gallbladder

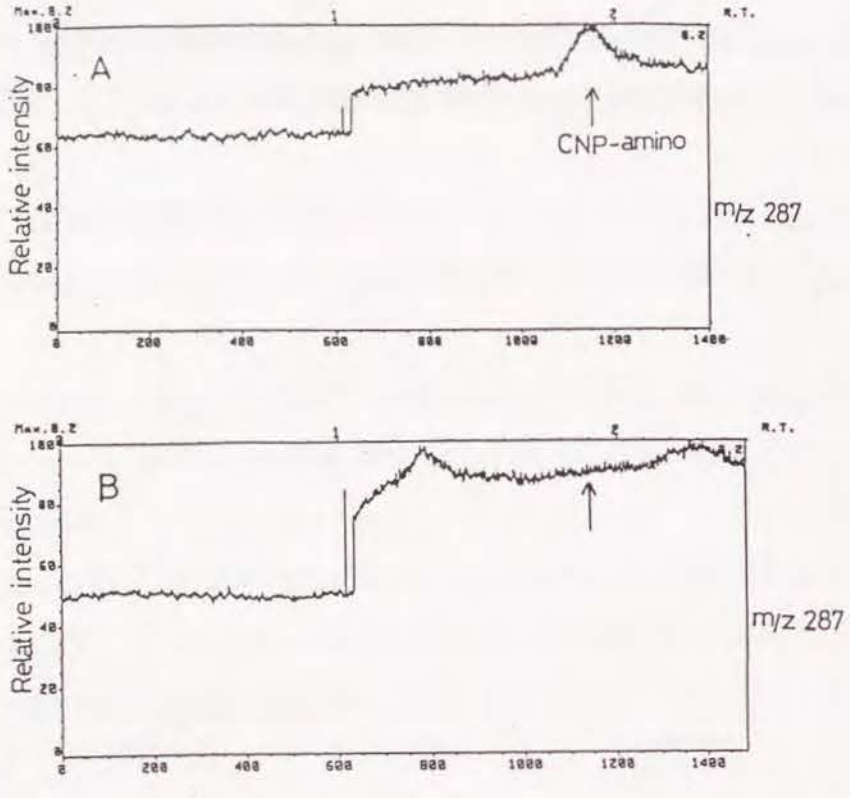

Fig.2 Typical GC-MS fragmentgrams of CNP-amino in muscle of carp.

A: Standard CNP-amino 0.2 ng

B: Muscle of carp (after 7 days exposure)

C: Muscle of carp (after 14 days exposure)

body of fresh-water fish, topmouth gudgeon[13] or the period 5 - 9 days in various tissue of mussel.[7] After 20 days exposure, the CNP concentrations in the four kinds of tissue of carp had a tendency to decrease. This decrease tendency is presumed to be temporary compared with the result of the similar experiment.[13] The BCF values of CNP were 334±13 (mean±SD, n=3) in muscle, 542 in liver, 862 in kidney and 547 in gallbladder after 14 days exposure. The order of BCF in the four kinds of tissue was not constant through the 20 days exposure period, but the values in viscera were higher than that in muscle after 14 and 20 days CNP exposure. These values were slightly lower than the value 1109 in whole body of topmouth gudgeon[6] and the value 950 in whole body of goldfish.[14] The BCF values of CNP were presumed to change by the difference of the fish species or the environment (temperature, concentration, etc.).

Metabolism of CNP

The CNP metabolites in fish or shellfish are known to be CNP-amino, CNP-acetamide and CNP-formamide by the studies of Suzuki et al.[8] and Watanabe et al.[12] Therefore, these metabolites were analyzed for muscle, liver, kidney and gallbladder of carp in the above accumulation experiment. CNP-amino in muscle of carp after 10 - 20 days exposure could be determined by GC-MS using SIM with ions at m/z=287. Fig.2 shows typical GC-MS fragmentgrams of CNP-amino in muscle of carp together with standard CNP-amino. The results of GC-MS determination agreed with those by GC-ECD. For CNP-acetamide and CNP-formamide, GC-MS determination was impossible for all samples of carp because of low concentrations of these metabolites. The concentrations of CNP in muscle nearly reached plateaus after 7 days exposure, and those of the metabolites probably after 10

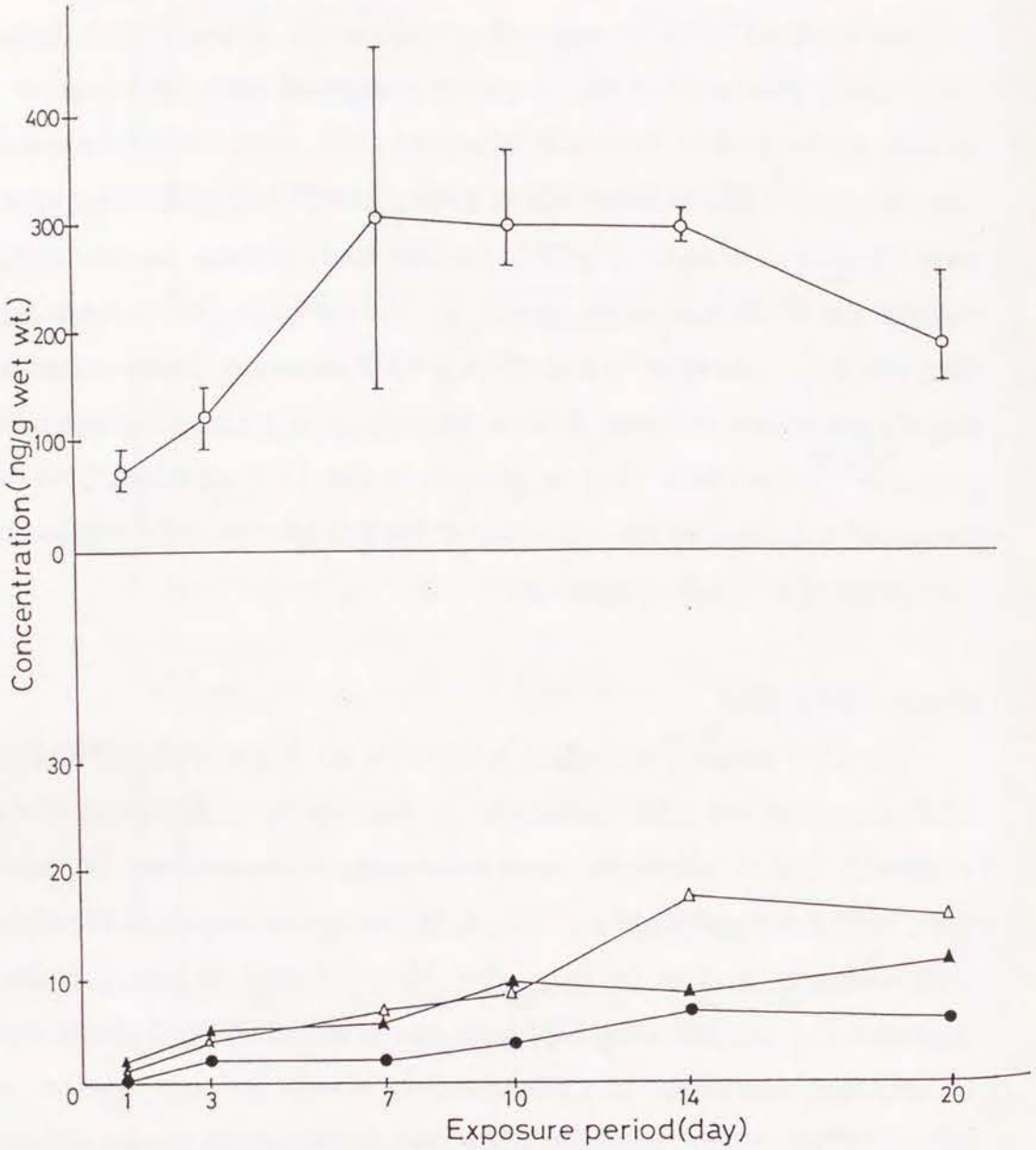

Fig.3 Concentrations of CNP metabolites in muscle of carp.

○ CNP ● CNP-amino △ CNP-acetamide ▲ CNP-formamide

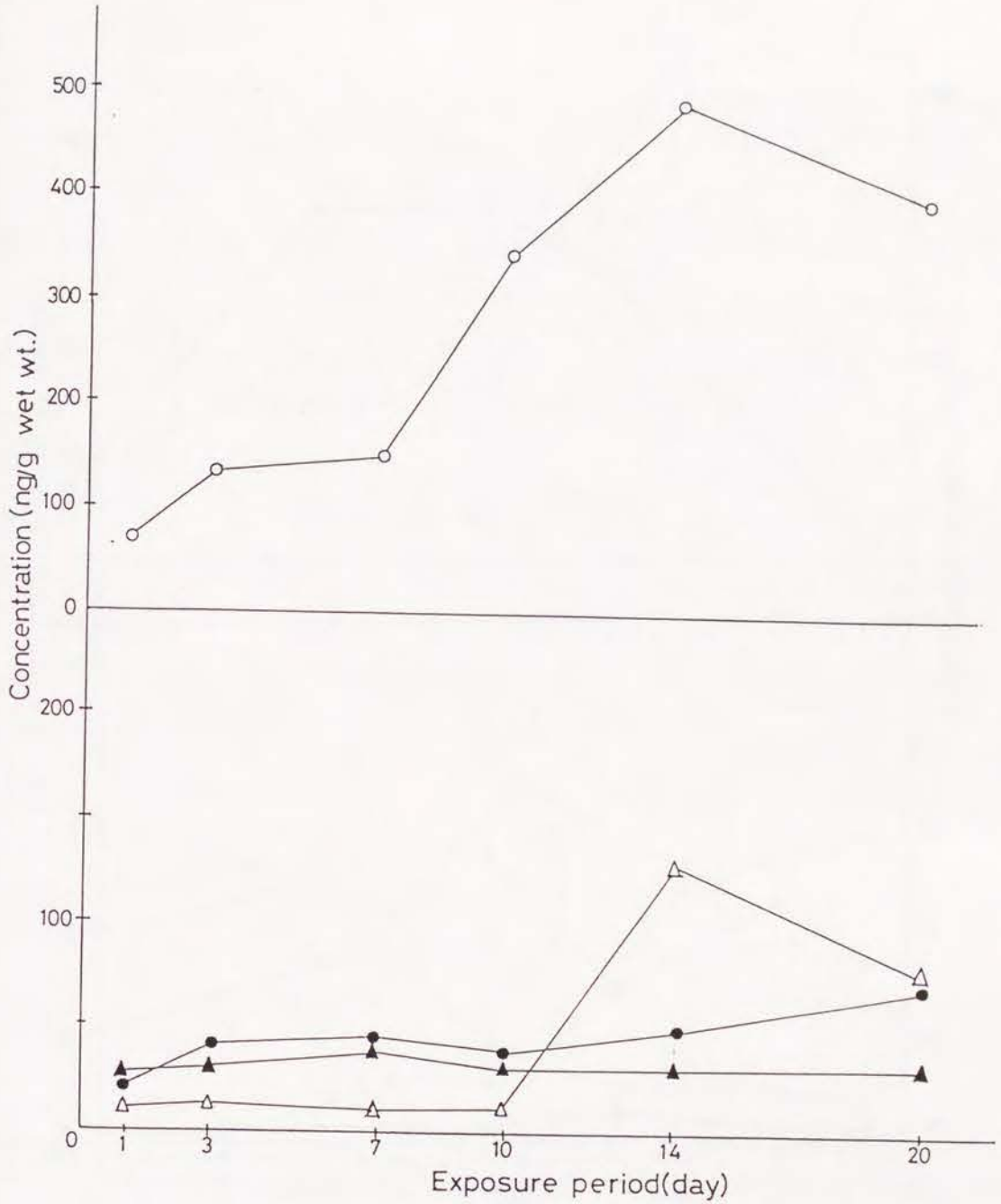

Fig.4 Concentrations of CNP metabolites in liver of carp.

○ CNP ● CNP-amino △ CNP-acetamide ▲ CNP-formamide

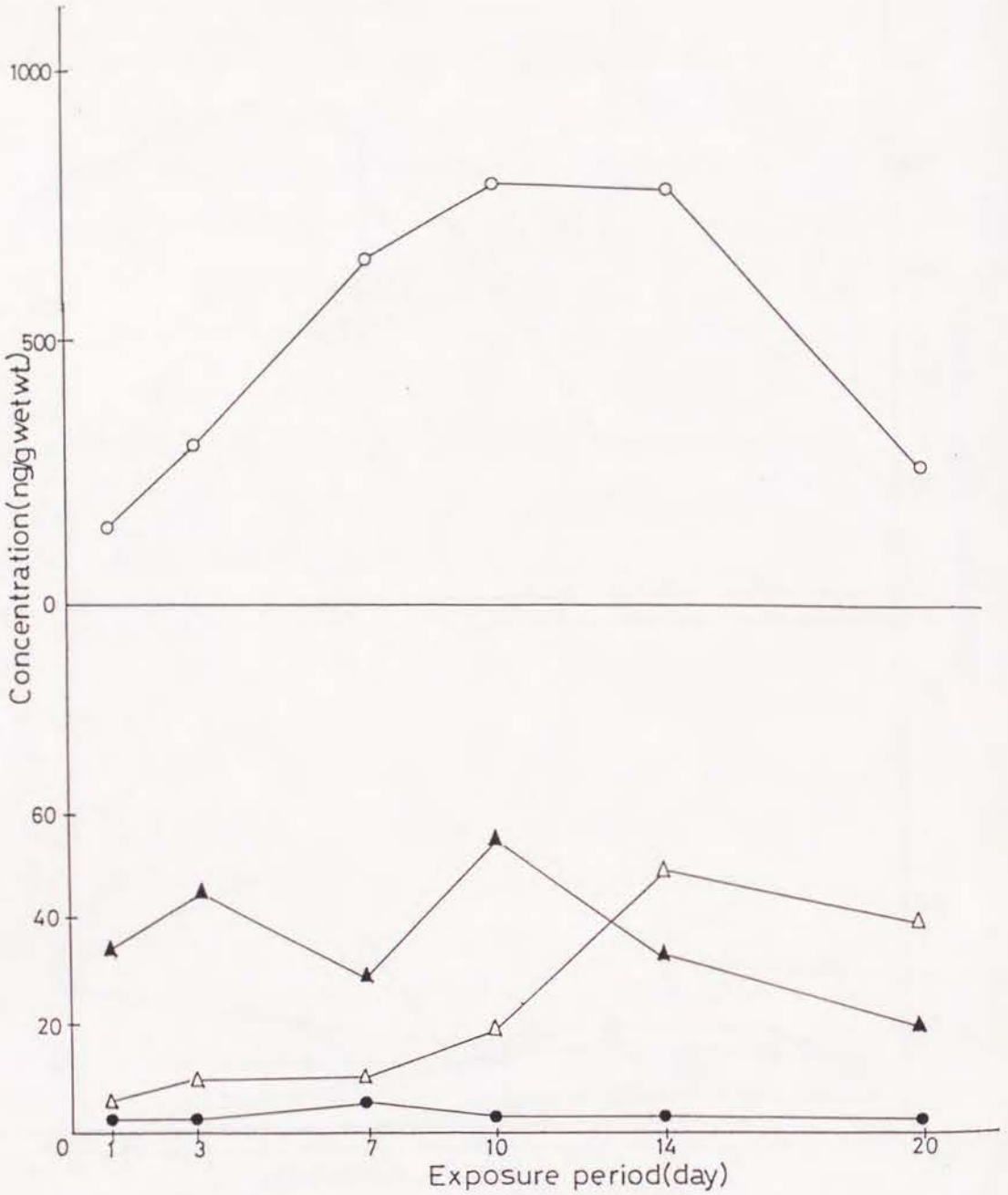

Fig.5 Concentrations of CNP metabolites in kidney of carp.

○ CNP ● CNP-amino △ CNP-acetamide ▲ CNP-formamide

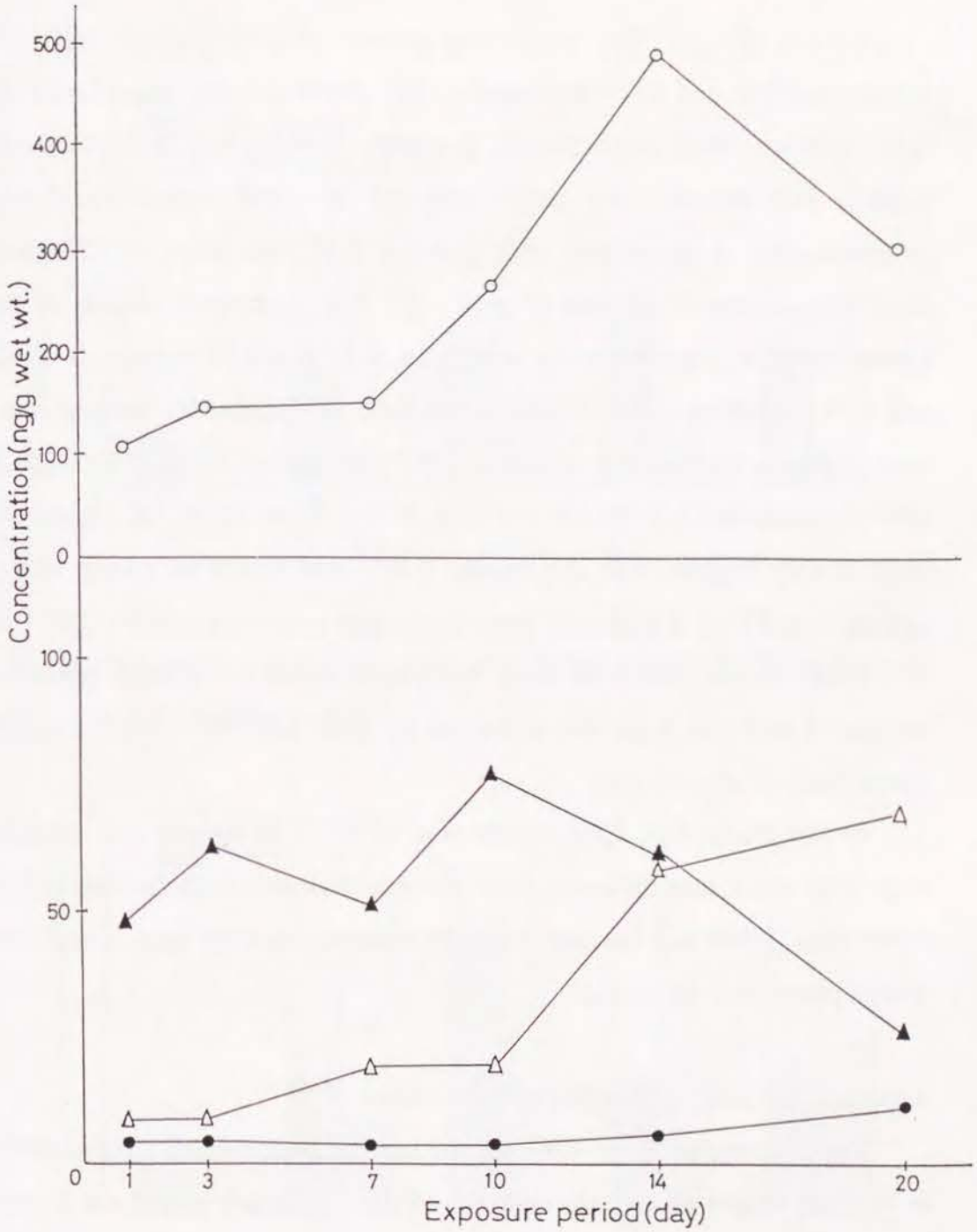

Fig.6 Concentrations of CNP metabolites in gallbladder of carp.

○ CNP ● CNP-amino △ CNP-acetamide ▲ CNP-formamide

- 14 days (Fig.3). The maximum values of CNP-amino/CNP, CNP-acetamide/CNP and CNP-formamide/CNP (each concentration ratio) were 0.03, 0.08 and 0.06, respectively, in muscle through the 20 days exposure period. For viscera, the concentrations of CNP-acetamide changed approximately in agreement with those of CNP, but those of CNP-amino and CNP-formamide did not (Figs.4 - 6). The maximum values of these concentration ratios for viscera were 0.18, 0.27 and 0.08 in liver, 0.01, 0.16 and 0.08 in kidney and 0.03, 0.23 and 0.43 in gallbladder through the 20 days exposure period. The values of CNP-acetamide/CNP (0.08 - 0.23) and CNP-formamide/CNP (0.06 - 0.43) in the four kinds of tissue were considerably higher than the values 0.002 and 0.005 in whole body of crucian carp.[8] This is probably because the low concentrations in CNP (300 - 800 ng/g) in the tissue of carp are metabolized on a high percentage compared with the high concentration of CNP (25,000 - 30,000 ng/g) in whole body of crucian carp.

In this study, free CNP-amino was detected in muscle and viscera of carp. This result was different from the results in the same studies by fresh-water clam, loach and crucian carp,[8] by mussel,[12] and by carp, crucian carp, tenaga prawn and corbicula.[5]

Accumulation and metabolism of CNP-amino

The concentrations of CNP-amino and the metabolized CNP-acetamide in the four tissues of carp through the 14 days exposure period are shown in Figs.7 - 10. CNP-amino in carp could be divided into CNP-amino(**I**) in a free condition and CNP-amino(**II**) in a bound condition. CNP-amino in muscle of carp comprised about 90 % of CNP-amino(**I**) and about 10 % of CNP-amino(**II**) over the 14 days exposure period. But the percentage 20 -

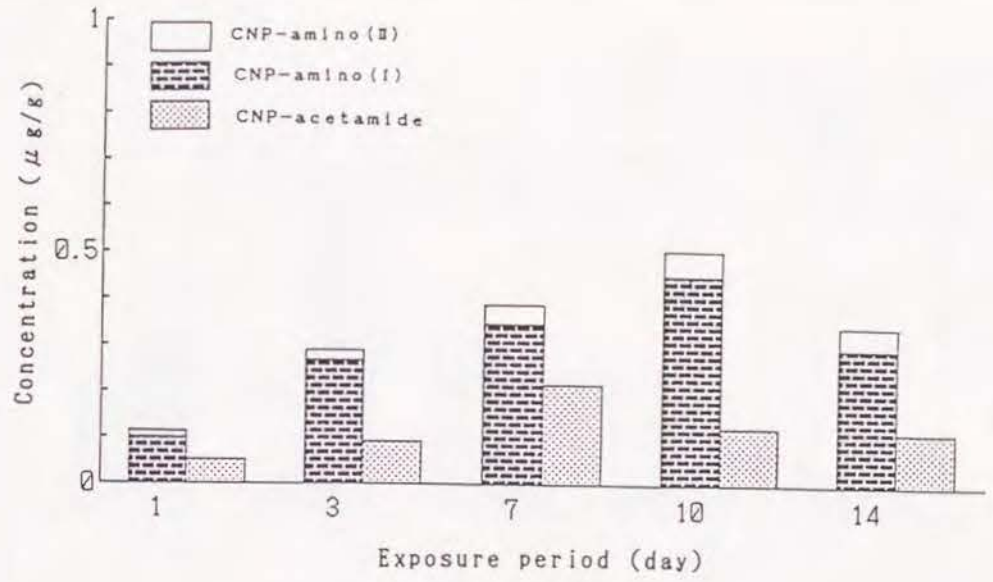

Fig.7 Concentrations of CNP-amino and the metabolized CNP-acetamide in muscle of carp.

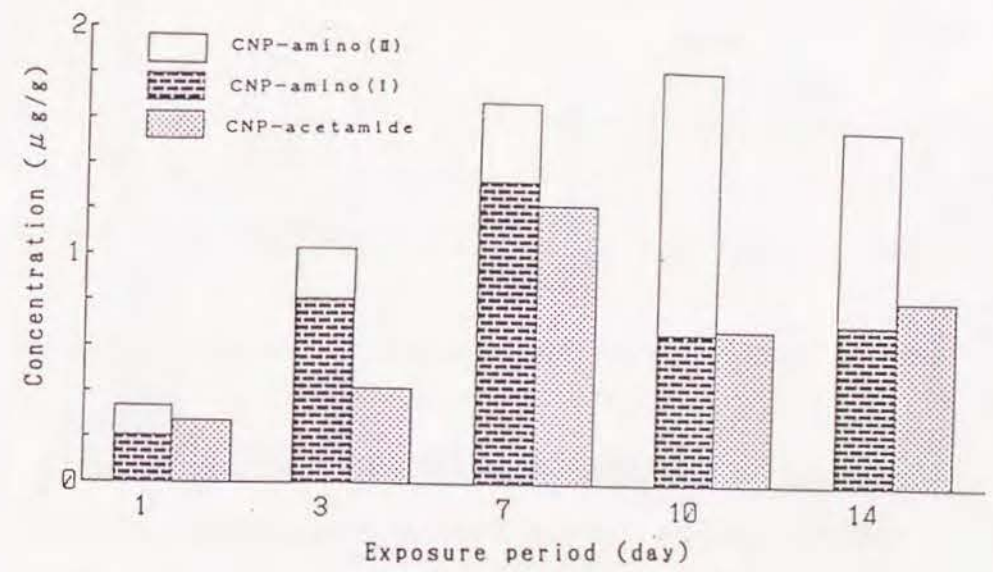

Fig.8 Concentrations of CNP-amino and the metabolized CNP-acetamide in liver of carp.

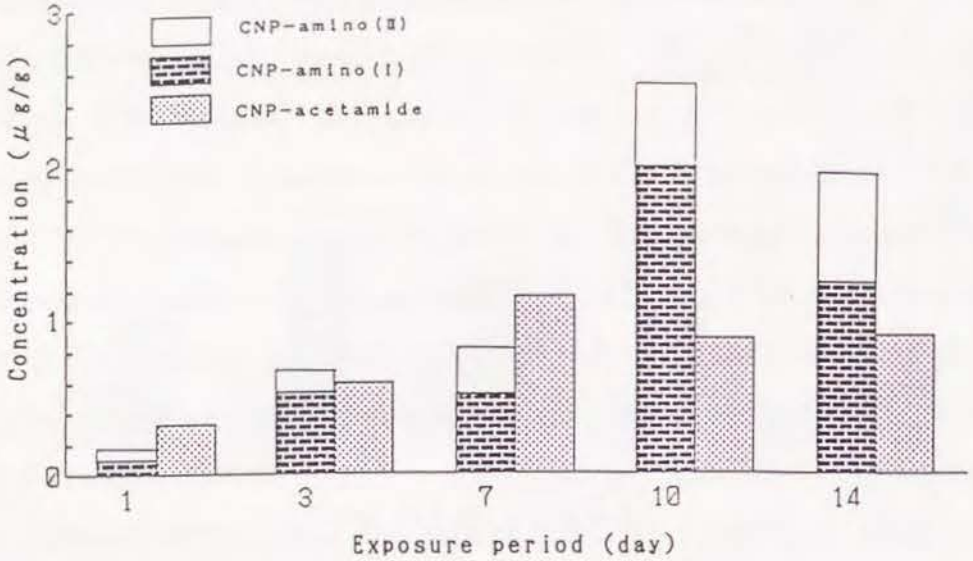

Fig.9 Concentrations of CNP-amino and the metabolized CNP-acetamide in kidney of carp.

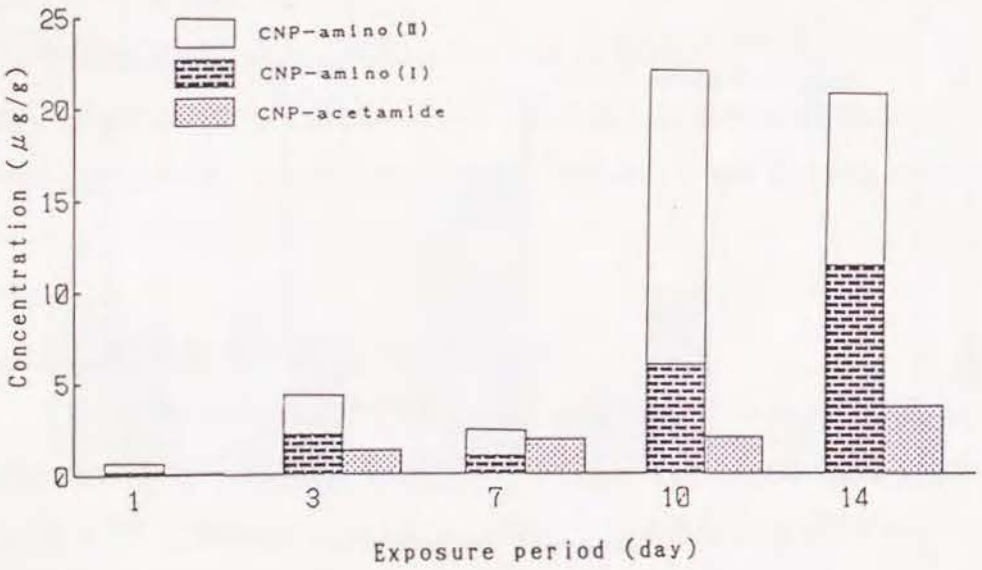

Fig.10 Concentrations of CNP-amino and the metabolized CNP-acetamide in gallbladder of carp.

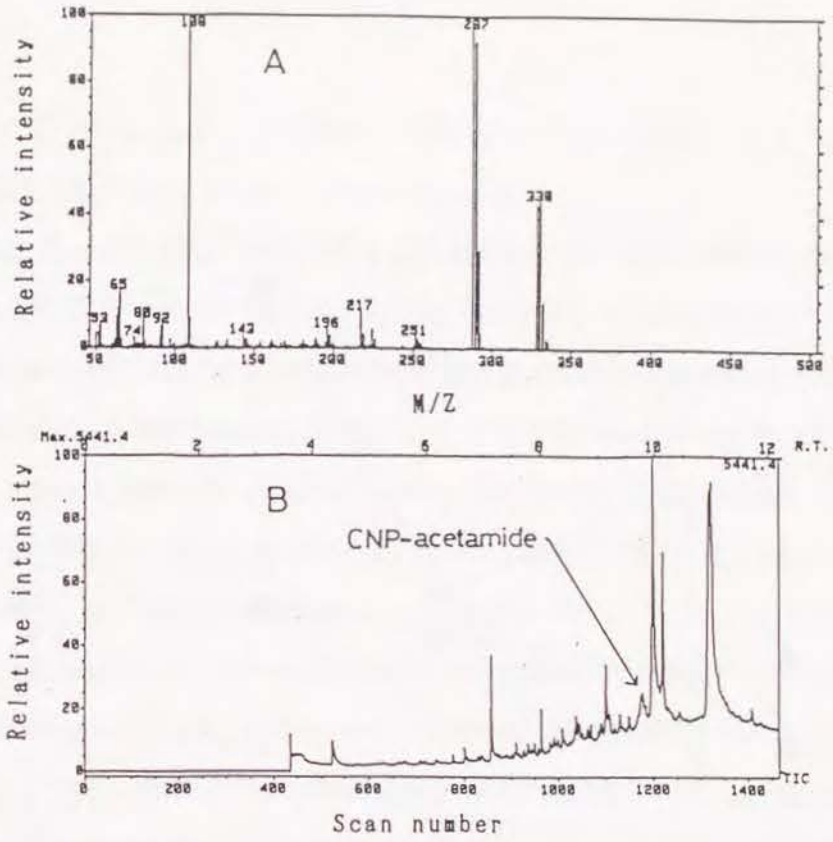

Fig.11 Identification of CNP-acetamide in gallbladder of carp.

A: A mass spectrum of standard CNP-acetamide (200 ng)

B: A total ion chromatogram of the sample solution obtained from the gallbladder of carp after 14 days exposure

C: A mass spectrum corresponding to CNP-acetamide in the gallbladder

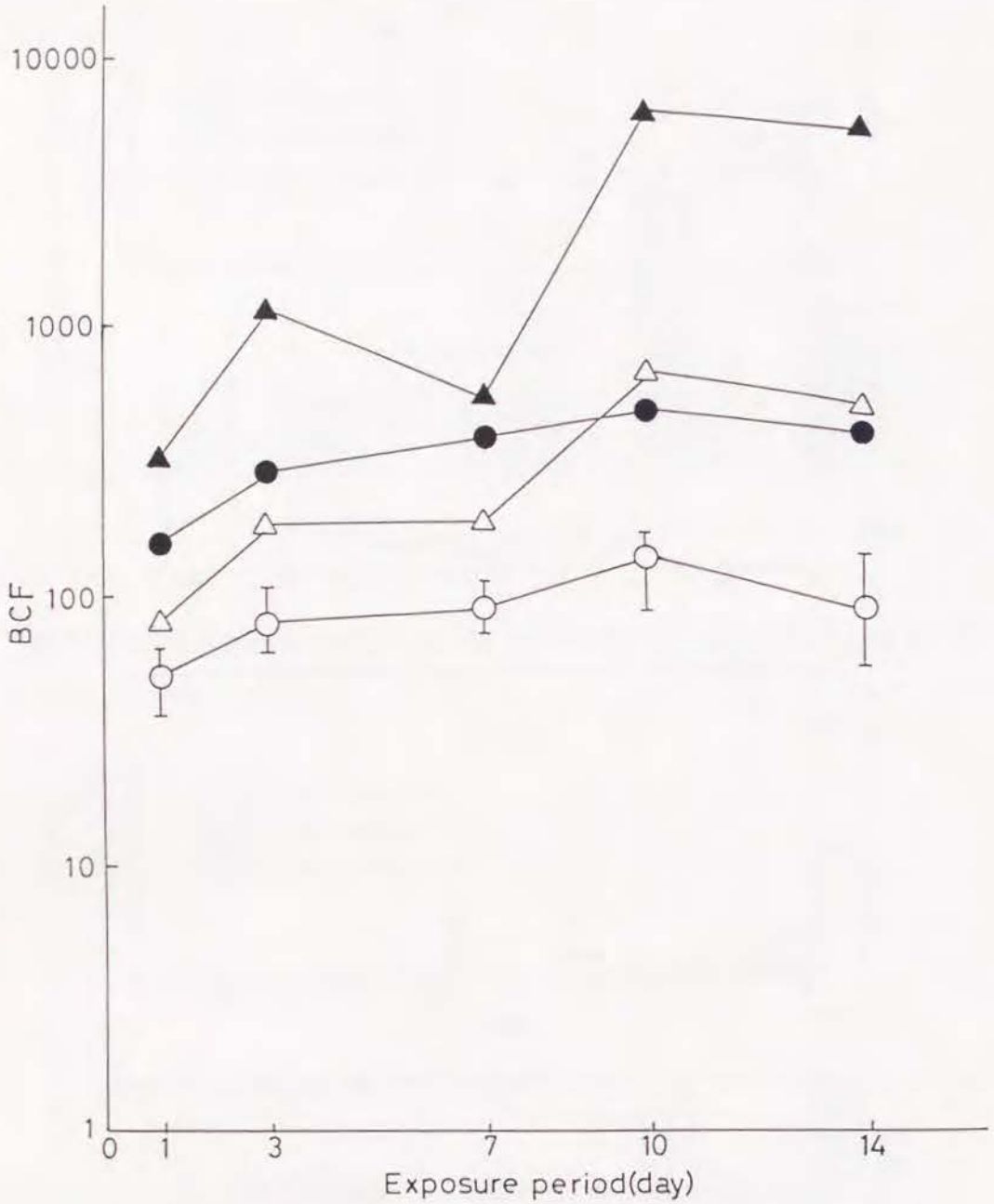

Fig.12 Accumulation of CNP-amino(I+II) in carp.

○ Muscle ● Liver △ Kidney ▲ Gallbladder

70 % of CNP-amino(II) in liver, kidney and particularly gallbladder was higher than the value about 10 % in muscle.

It is known that both of CNP-acetamide and CNP-formamide are detected as CNP-metabolites in fish by the study of Suzuki et al.[8] But CNP-formamide could not be detected from the accumulation experiment of CNP-amino in carp. CNP-acetamide in carp could be confirmed by GC-MS, so a typical mass spectrum of CNP-acetamide in the gallbladder of carp was shown in Fig.11. Ions at m/z 329, 287 and 108 were chosen for the confirmation of CNP-acetamide according to the study by Suzuki et al.[8] The patterns of ions in the mass spectrum corresponding to CNP-acetamide in the fish sample agreed very closely with those of the standard CNP-acetamide.

The rate of CNP-amino metabolization was presumed to be considerably rapid from the data of Figs.7 - 10. That is, the concentration ratios of CNP-acetamide/CNP-amino (I+II) in the four tissues of carp were considerably high even in the initial days of the exposure period. For example, the values after one day exposure were 0.50 in muscle, 0.82 in liver, 1.91 in kidney and 0.26 in gallbladder.

BCF of CNP-amino(I+II) in the four tissues of carp through the 14 days exposure period are shown in Fig.12. The BCF values in the all four tissues of carp reached plateaus after 10 days. Those were 90±38 (mean±SD, n=3) in muscle, 402 in liver, 501 in kidney and 5368 in gallbladder after 14 days exposure. The value 5368 in gallbladder was considerably higher than the values in the other tissues of carp. The order of BCF after reaching plateaus was gallbladder > kidney ≥ liver > muscle. This order was slightly different from that of CNP (kidney > gallbladder ≥ liver > muscle). There were large differences among the BCF values of CNP-amino in the four

tissues of carp (90 - 5368), but small differences among those of CNP (334 - 862).

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CHAPTER 11

Accumulation and Excretion of Pesticides Used in Golf Courses by Freshwater-Fishes

Introduction

In recent years, a large number of pesticides have been produced and discharged into the environment. Quite recently, water pollution by pesticides used in golf courses has become a serious problem in Japan. At present, a tentative plan on the concentrations of 21 pesticides in waste water from golf courses and in city water is provided by Environment Agency and Welfare Ministry in Japan.

In chapters 8 and 9, the accumulation and excretion of herbicides (benthiocarb, simetryne, oxadiazon, CNP and chlomethoxynil), insecticides (diazinon, malathion and fenitrothion) and fungicides (IBP) using freshwater fishes were already described. Diazinon and fenitrothion are included in the 21 pesticides.

In this study,[1] the same accumulation and excretion experiments using willow shiner and carp were performed for other 9 pesticides used in golf courses, that is, herbicides (simazine), insecticides (chlorpyriphos and isoxathion) and fungicides (tolclofos-methyl, flutolanil, isoprothiolane, chlorothalonil, captan and iprodione).

Experimental Section

Chemicals

Simazine [2-chloro-4,6-(ethylamino)-1,3,5-triazine], tolclofos-methyl (O-2,6-dichloro-p-tolyl O,O-dimethyl phosphorothioate), chlorpyriphos (diethyl 3,5,6-trichloro-2-pyridyl phosphorothionate), isoxathion (diethyl 5-phenyl-3-isoxazolyl phosphorothionate), flutolanil (α,α,α-trifluoro-3-isopropoxy-O-toluanilide), isoprothiolane (diisopropyl 1,3-dithiolane-2-ylidene malonate), chlorothalonil (tetrachloroisophthalonitrile), captan [N-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide] and iprodione [N-isopropyl-3-(3,5-dichlorophenyl)-2,4-dioxoimidazolidine-carboxamide] were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). These chemicals, each reagent grade (more than 98.0%), were used without further purification.

Test fish

Carp (*Cyprinus carpio* L.) were purchased from Nango Suisan Center (Shiga Prefecture, Japan) and willow shiner (*Gnathopogon caerulescens*) were obtained from Shiga Prefecture Fisheries Experimental Station. The body length and body weight of the fishes were 7.5 - 9.5 cm and 14.0 - 21.9 g for carp and 3.8 - 4.3 cm and 0.93 - 1.43 g for willow shiner. All these pesticides were not detected in the fishes before exposure to them. Commercial assorted feed was given at 0.3 g/fish for carp and 20 mg/fish for willow shiner once a day throughout the experiments.

Test system

The study was carried out without aeration under the continuous flow-through system. The experimental arrangement was the same as in chapter

2. For the accumulation experiment on willow shiner, a stock solution (simazine 800 μg/*l*, chlorothalonil 400 μg/*l* and captan 800 μg/*l*,10 *l*) was diluted continuously 100 times with dechlorinated city water and supplied to an aquaria containing 30 fish. During the test, the flow rate and temperature of test water were maintained 12 *l*/h and 20±1°C, respectively. The concentrations of these chemicals in the test water were mean±SD (n=6) of 7.7±0.9 μg/*l* for simazine, 1.1±0.2 μg/*l* for chlorothalonil and 0.16±0.01 μg/*l* for captan. Measurements were carried out at 0, 1, 3, 7, 10 and 14 days. Three fish were taken at 1, 3, 7, 10 and 14 days.

For the accumulation experiment on carp, stock solution A (tolclofos-methyl, chlorpyriphos, flutolanil and isoprothiolane, each 500 μg/*l*, 10 *l*) and B (chlorothalonil, captan, isoxathion and iprodione, each 500μg/*l*,10 *l*) were respectively diluted continuous 150 times with dechlorinated city water and supplied to each of the two aquaria containing 30 fish. During the test, the flow rate and temperature of each test water were maintained 18 *l*/h and 25±1°C, respectively. The concentrations of these chemicals in each test water were mean±SD (n=6) of 0.54±0.13 μg/*l* for tolclofos-methyl, 0.49±0.11 μg/*l* for chlorpyriphos, 3.1±0.5 μg/*l* for flutolanil and 3.4±0.4μg/*l* for isoprothiolane (experiment 1), and 1.4±0.3 μg/*l* for chlorothalonil, 1.1±0.2 μg/*l* for captan, 0.59±0.24 μg/*l* for isoxathion and 0.76±0.11 μg/*l* for iprodione (experiment 2). Measurements were carried out at 0, 1, 3, 7, 10 and 14 days. Three fish were taken at 1, 3, 7, 10 and 14 days.

The concentrations of these pesticides in each test water were fixed by reference to the data of 48 h LC_{50} to carp.[2] The low concentrations of these chemicals are probably because they are adsorbed to the tubes of the experimental arrangement or the walls of the aquaria. The pH, the

concentration of dissolved oxygen and the hardness in each test water were 6.7 - 6.9, more than 7 mg/*l* and 36 - 38 mg/*l* as $CaCO_3$, respectively. Measurements were carried out every day for pH and once after 3 days for dissolved oxygen and hardness.

For the excretion experiment on willow shiner, 15 fish remaining in the aquaria were transferred into another aquaria and dechlorinated city water was supplied to it. During the test, the flow rate and temperature of the test water were maintained 60 *l*/h and 21±1°C, respectively. Three fish were taken at 3, 6, 12, 24 and 72 h.

For the excretion experiment on carp, 15 fish remaining in each aquaria were were separately transferred into each of the other two aquaria and dechlorinated city water was supplied to each of them. During the tests, the flow rate and temperature of each test water were maintained 60 *l*/h and 23±1°C, respectively. Three fish were taken at 12, 24, 72 and 168 h.

The pH and the concentrations of dissolved oxygen in each test water were 6.7 - 6.8 and more than 7 mg/*l*, respectively. Measurements were carried out every day for pH and once after 3 days for dissolved oxygen.

Under these conditions, none of the fishes showed signs of tiredness and agitation during the accumulation and excretion experiments.

Analysis

Each concentration of the pesticides in water samples was determined by the following procedure. A measured volume (200 m*l*) of water was shaken with 100 m*l* of dichloromethane after addition of 10 g of NaCl. The organic layer was rotary-vacuum evaporated just to dryness at 40°C. The residue was dissolved with 4 m*l* hexane and analyzed by gas chromatograph equipped with flame thermionic detector (GC-FTD) for simazine and by gas

chromatograph equipped with electron capture detector (GC-ECD) for 8 pesticides except simazine. Average recoveries (n=3) were 100% for simazine, 94% for tolclofos-methyl, 93% for chlorpyriphos, 94% for flutolanil, 92% for isoprothiolane, 100% for chlorothalonil, 100% for captan, 100% for isoxathion and 94% for iprodione at 2 μg/*l* spiked levels.

Determination of each pesticide in fish samples was performed as follows. A fish sample was homogenized with 30 m*l* of acetonitrile by high-speed homogenizer (Ultra-Turrax, Germany) after addition of 5 g of anhydrous Na_2SO_4 and the organic layer was filtrated. The residue was again homogenized and filtrated in the same manner. The combined filtrate was rotary-vacuum evaporated just to dryness at 40°C and the residue was dissolved in 10 m*l* of hexane. The hexane solution was shaken with 30 m*l* of acetonitrile saturated with hexane and the acetonitrile layer was retained. This operation was repeated and the combined acetonitrile layer was rotary-vacuum evaporated just to dryness at 40°C. The residue was dissolved with 5 ml of hexane and the hexane solution was passed through a SEP-PAK florisil cartridge (Waters Associates, USA). Each pesticide was eluted with 10 m*l* of acetone-hexane (10+90) after washing with 10 m*l* of hexane. Each pesticide concentration of the eluate was measured by GC-FTD or GC-ECD. Average recoveries (whole body of carp 5 g, n=3) were 84% for simazine, 89% for tolclofos-methyl, 85% for chlorpyriphos, 81% for flutolanil, 95% for isoprothiolane, 70% for chlorothalonil, 80% for captan, 89% for isoxathion and 75% for iprodione at 100 ng/g spiked levels. The GC (Shimadzu GC-9A) operating conditions were as follows:

GC-FTD

GC column: J&W DB 1701 (30 m x 0.53 mmϕ). Carrier: He 20 m*l*/min.
Temperatures: injection and detector 250°C; column 200°C.

H_2: 4 m*l*/min. Air: 150 m*l*/min.

GC-ECD

(1) GC column: a 2.1 m x 3 mmϕ glass column packed with 5% 0V 17 on 80/100 mesh Chromosorb WHP. Carrier: N_2 50 m*l*/min.
Temperatures: injection and detector 260°C; column 230°C.

(2) GC column: J&W DB 1701 (30 m x 0.53 mmϕ). Carrier: N_2 20 m*l*/min.
Temperatures: injection and detector 250°C; column 200°C.

Analysis was carried out as a sample for whole body of each fish at each sampling time of the accumulation and excretion experiments.

Calculation of bioconcentration factor (BCF) and excretion rate constant (k)

BCF was calculated by the following equation.

$$BCF = \frac{\text{chemical concentration in whole body of fish}}{\text{chemical concentration in water}}$$

The chemical concentration in the water at each sampling time was used for the calculation of BCF. The excretion rate constant (k) of the chemicals from whole body of the fishes was calculated assuming the excretion process to be first-order kinetics.

Results and Discussion

Accumulation of pesticides

The experimental results are shown in Table 1 and 2. The concentrations of 3 pesticides in whole body of willow shiner reached plateaus in 1 day exposure. The average BCF values in whole body were 3.9 for simazine, 18 for chlorothalonil and 350 for captan after 1 - 14 days

Table 1. BCF of pesticides in willow shiner.

	BCF [a] in whole body of fish				
Pesticides	1 day	3 days	7 days	10 days	14 days
Simazine	3.9±0.4	3.8±0.4	3.6±0.9	3.4±0.5	4.6±0.5
Chlorothalonil	24±6	20±6	13±1	14±1	19±5
Captan	200±80	270±60	510±10	250±30	500±70

[a] Mean value±SD (n=3)

Table 2. BCF of pesticides in carp.

	BCF [a] in whole body of fish				
Pesticides	1 day	3 days	7 days	10 days	14 days
Tolclofos-methyl	230±10	250±20	180±60	250±70	210±60
Chlorpyriphos	310±70	540±300	340±50	550±60	410±100
Flutolanil	21±1	16±5	13±3	31±4	21±4
Isoprothiolane	28±7	26±7	29±5	29±3	22±5
Chlorothalonil	27±9	30±2	41±17	14±7	13±4
Captan	95±13	92±7	80±9	160±24	74±15
Isoxathion	730±60	450±80	340±50	490±10	200±70
Iprodione	110±50	560±240	300±10	330±70	230±10

[a] Mean value±SD (n=3)

exposure. The concentrations of 8 pesticides in whole body of carp reached plateaus in 1 day exposure for tolclofos-methyl, flutolanil, isoprothiolane, chlorothalonil, captan, isoxathione, and in 3 days exposure for chlorpyriphos and iprodione. The average BCF values in whole body were 220 for tolclofos-methyl, 20 for flutolanil, 27 for isoprothiolane, 25 for chlorothalonil, 100 for captan and 440 for isoxathion after 1 - 14 days

exposure, and 460 for chlorpyriphos and 360 for iprodione after 3 -14 days exposure. There was no difference in the BCF values of chlorothalonil between willow shiner and carp, but the BCF value of captan in willow shiner was higher than that in carp.

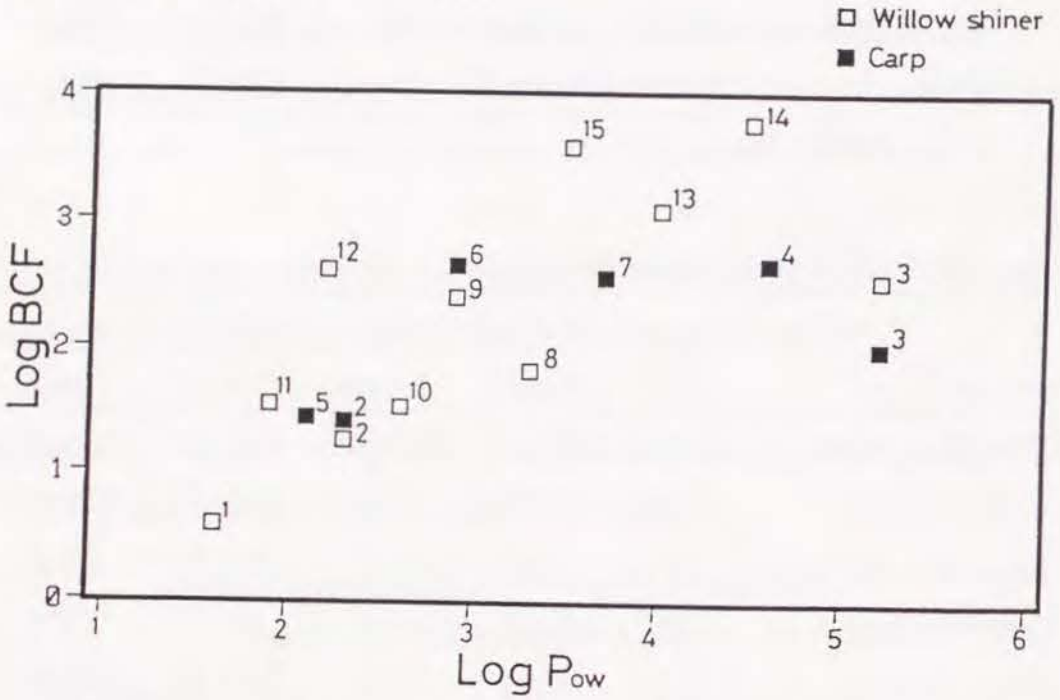

Fig.1 Relationship between $logP_{ow}$ and logBCF in fish.

1 Simazine	6 Isoxathion	11 Malathion
2 Chlorothalonil	7 Iprodione	12 Fenitrothion
3 Captan	8 Benthiocarb	13 Oxadiazon
4 Chlorpyriphos	9 Diazinon	14 CNP
5 Isoprothiolane	10 IBP	15 Chlomethoxynil

It is generally known that there is a linearity between $logP_{ow}$ and logBCF in fish, and estimation of BCF by P_{ow} is a very useful method as screening test. For the pesticides studied here and already described in

chapter 8, the correlations between $logP_{ow}$ and logBCF were investigated. The data of $logP_{ow}$ were cited from the study by Okumura and Imamura.[3] These correlations for willow shiner and carp are shown in Fig.1. The r values were 0.6336 (n=11) for willow shiner and 0.4666 (n=17) for both fishes, which did not show high correlations. However, the r values except captan were higher, that is, 0.9391 (n=10) for willow shiner and 0.7747 (n=15) for both fishes. The BCF values of captan in both fishes were considerably lower than those estimated from the P_{ow} value.

Excretion of pesticides

The experimental results are shown in Fig.2 for willow shiner and in Fig.3 and 4 for carp. From these data, the k values and biological half-lives of the fishes were calculated by assuming their excretion process to be first-order kinetics and these are shown in Table 3 for willow shiner and in Table 4 for carp. The excretion rate of captan from willow shiner was two times as rapid as that from carp. The excretion process of iprodione from carp could not be assumed to be first-order kinetics or the concentration of chlorothalonil in carp could not be satisfactorily measured, so calculation of these excretion rate was impossible. The excretion rate of these pesticides was simazine > chlorothalonil > captan for willow shiner and isoprothiolane > flutolanil > tolclofos-methyl > isoxathion > chlorpyriphos > captan for carp. As shown in Table 1 and 2, the orders of their BCF values were captan > chlorothalonil > simazine for willow shiner and chlorpyriphos ≥ isoxathion > tolclofos-methyl > captan > isoprothiolane ≥ flutolanil for carp. The order of their excretion rate was reversed compared with that of their BCF values for willow shiner, and similarly for carp except captan. That is,

the greater the BCF value, the slower the excretion rate. This tendency was the same as in chapter 9.

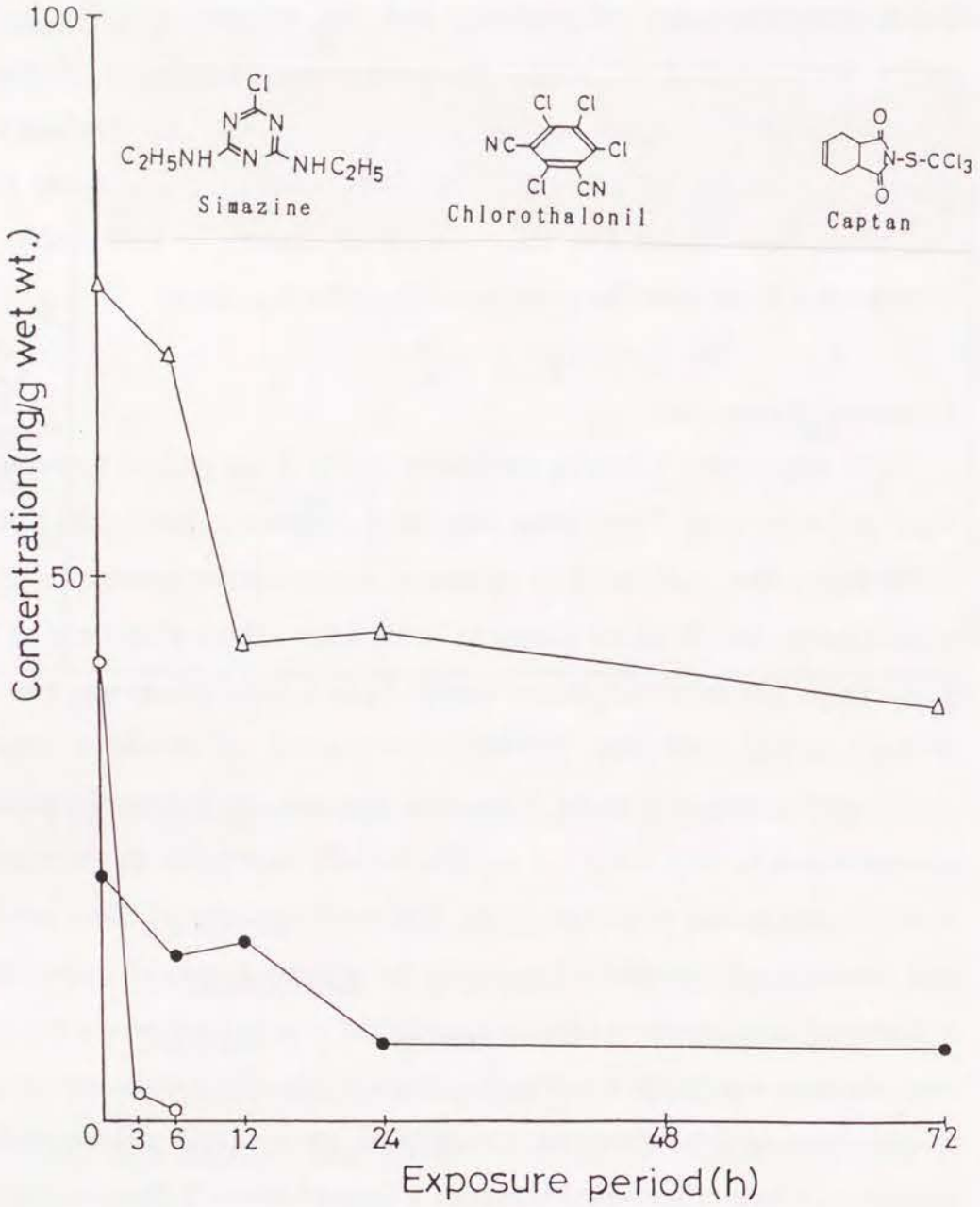

Fig.2 Excretion of pesticides from willow shiner.

○ Simazine ● Chlorothalonil △ Captan

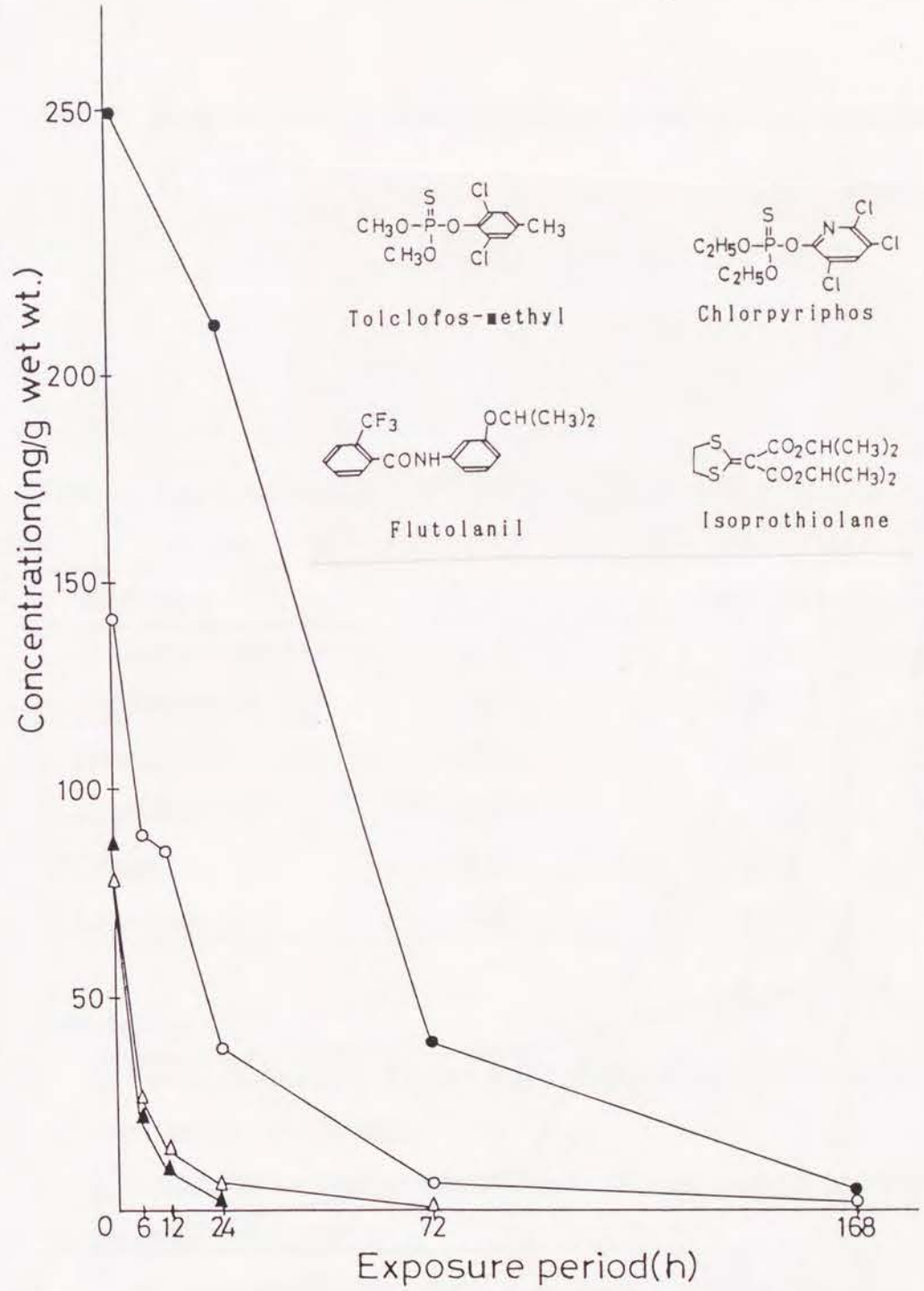

Fig.3 Excretion of pesticides(1) from carp.

○ Tolclofos-methyl ● Chlorpyriphos

△ Flutolanil ▲ Isoprothiolane

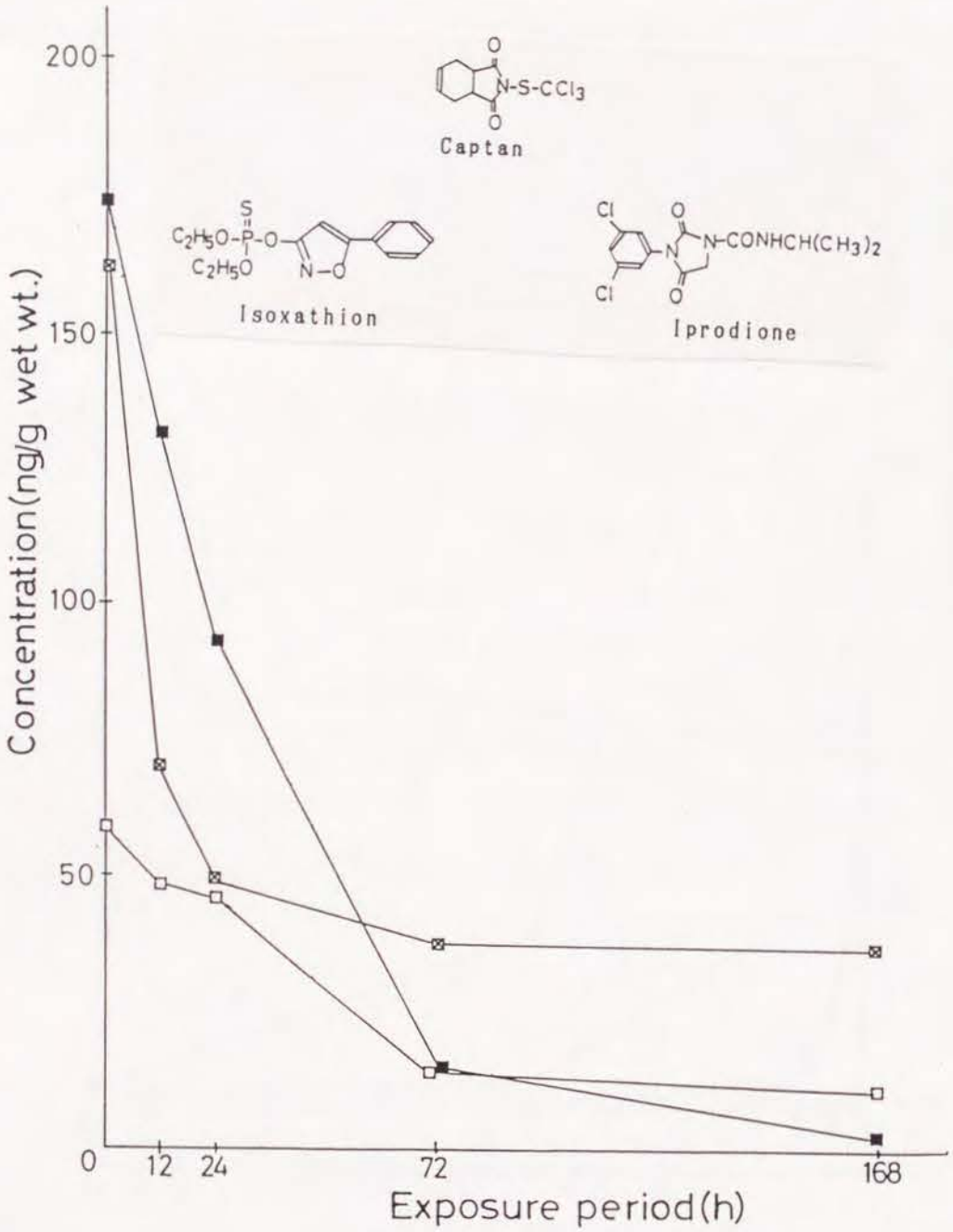

Fig.4 Excretion of pesticides(2) from carp.

□ Captan ■ Isoxathion ⊠ Iprodione

Table 3. Excretion rate constants and biological half-lives of pesticides by willow shiner.

Pesticides	k (h^{-1})	Half-lives (h)
Simazine	0.77	0.9
Chlorothalonil	0.04	17.3
Captan	0.02	34.7

Table 4. Excretion rate constants and biological half-lives of pesticides by carp.

Pesticides	k (h^{-1})	Half-lives (h)
Tolclofos-methyl	0.04	17.3
Chlorpyriphos	0.02	34.7
Flutolanil	0.12	5.8
Isoprothiolane	0.19	3.6
Captan	0.01	69.3
Isoxathion	0.03	23.1

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CHAPTER 12

Comparison of Experimental Data with Field Data

Introduction

In recent years, a large number of industrial chemical substances have been produced and discharged into the environment. At present, the environmental contamination by organotin compounds and pesticides is a serious problem in Japan. To precisely evaluate the contamination of fishes in the environment by these compounds, laboratory experiments for their accumulation and excretion in several species of fishes have been performed as described in chapters 2 - 11.

In this chapter, these experimental data are compared with the field data that are the results of surveys in Lake Biwa for butyltin and phenyltin compounds and those in rivers flowing into Lake Biwa for pesticides.

Experimental Section

Sample collection

For butyltin and phenyltin compounds, water and fish (crucian carp) samples were collected from seven harbors of Lake Biwa in June and July (1986) and from two harbors of Lake Biwa in June and July (1989).

For pesticides, water and fish (pale chub and ayu sweetfish) samples were collected in seven rivers flowing into Lake Biwa once or twice every month in 1988 and 1989. Water samples were immediately analyzed and fish sample were frozen and preserved for analysis.

Analysis

Butyltin and phenyltin compounds were determined as their hydrides by GC-ECD or GC-FPD according to the method described in chapter 2.

Pesticides were determined by GC-FTD for molinate, diazinon, IBP, benthiocarb and simetryne, and by GC-ECD for CNP, oxadiazon and chlomethoxynil according to the method described in chapter 8. Further, determination of these compounds in water and fish samples were performed using selected ion monitoring (SIM) by gas chromatography-mass spectrometory (GC-MS). The GC-MS (JEOL JMS-DX302) operating conditions were as follows:

For Bu_3SnH and Ph_3SnH

GC column: J&W DB1 (0.32 mmϕ x 30 m)

Carrier: He 1.0 m*l*/min

Operating temperatures: injection 280°C;
column 60°C (2 min)
32°C/min to 160°C (0 min)
16°C/min to 260°C (5 min)

For pecticides

GC column: J&W DB WAX (0.53 mmϕ x 30 m)

Operating temperatures: injection 300°C;
column 210°C (0 min) 4°C/min to 230°C

Carrier: He 30 m*l*/min

Ion mode: EI Ionization voltage: 70 eV
Ionization current: 300 μA

Table 1. Concentrations of butyltin compounds in water and fish muscle from harbors of Lake Biwa.

	Water [a]	Crucian carp [a]				
L [c]	Bu_3Sn^+ (ng/m*l*)	Body length (cm)	Weight (g)	Bu_3Sn^+ (ng/g)[b]	Bu_2Sn^{2+} (ng/g)[b]	$BuSn^{3+}$ (ng/g)[b]
		11.5	51.9	7.7	0.8	< 4.0
	0.007	10.5	38.5	1.8	< 0.4	< 4.0
A		9.0	27.4	1.8	0.4	< 4.0
		10.0	29.1			
	< 0.004			3.7	0.4	< 4.0
		9.0	20.5			
		7.5	17.0			
B	0.01	8.0	17.7	15.3	< 0.4	< 4.0
		8.0	17.7			
		8.0	20.4			
C	< 0.004	8.0	20.3	4.0	0.8	< 4.0
		8.0	19.5			
D	0.007	20.0	250.3	2.9	0.4	< 4.0
E	< 0.007	13.0	93.7	5.8	0.8	< 4.0
F	< 0.004	10.5	47.2	3.3	< 0.4	< 4.0
		11.0	50.5	23.0	1.6	< 4.0
G	0.015					
		12.0	59.6	10.2	< 0.4	< 4.0

[a] Collected in June and July (1986)

[b] Expressed as Sn(ng/g) in wet weight

[c] Location

Results and Discussion

Surveys of butyltin and phenyltin compounds in harbors of Lake Biwa

The concentrations of butyltin compounds in water and fish samples from harbors of Lake Biwa are presented in Table 1. For water, the concentrations of Bu_3Sn^+ were mostly < 0.007 ng/ml. For fish (crucian carp), those of butyltin compounds were 1.8 - 23.0 ng/g for Bu_3Sn^+, < 0.4 - 1.6 ng/g for Bu_2Sn^{2+} and < 4.0 ng/g for $BuSn^{3+}$.

Table 2. Bioconcentration factors (BCF) and concentration ratios (Bu_2Sn^{2+}/Bu_3Sn^+ and $BuSn^{3+}/Bu_3Sn^+$) in muscle of crucian carp from Lake Biwa.

Location	BCF [a]	Bu_2Sn^{2+}/Bu_3Sn^+ [b]	$BuSn^{3+}/Bu_3Sn^+$ [c]
	1050	0.10	< 0.5
A	250	< 0.22	< 2.2
	>1000	0.11	< 1.1
B	1400	< 0.02	< 0.3
C	>1100	0.20	< 1.0
D	400	0.14	< 1.4
E	> 800	0.14	< 0.7
F	> 900	< 0.12	< 1.2
G	1580	0.07	< 0.2
	700	< 0.04	< 0.4

a Concentration of Bu_3Sn^+ in fish / concentration of Bu_3Sn^+ in water

b Concentration of Bu_2Sn^{2+} in fish / concentration of Bu_3Sn^+ in fish

c Concentration of $BuSn^{3+}$ in fish / concentration of Bu_3Sn^+ in fish

Table 3. Concentrations of tri-n-butyltin and triphenyltin compounds in crucian carp from harbors of Lake Biwa.

Fish [a]	Location	Body length (cm)	Weight (g)	Tissues	Bu_3Sn^+ (ng/g)[b]	Ph_3Sn^+ (ng/g)[b]
				muscle	4.0	12.6
				liver	14.2	16.0
	A	22.0	307.7			
				kidney	16.0	18.5
				gallbladder	7.7	51.4
				muscle	6.9	29.3
				liver	19.0	14.2
RCC [c]	A	18.5	205.2			
				kidney	22.6	45.3
				gallbladder	9.5	64.1
	B	15.0	103.9	muscle	16.8	45.5
	A	22.5	370.5	muscle	6.6	21.3
	B	13.5	82.7	muscle	6.2	1.5
				muscle	9.5	4.0
DCC [d]				liver	101.1	64.1
	B	13.5	121.2			
				kidney	67.2	28.6
				gallbladder	30.3	< 10.0

a Collected in June and July (1989).

b Expressed as Sn (ng/g) in wet weight.

c Round crucian carp

d Deep crucian carp

Bioconcentration factors (BCF) and concentration ratios (Bu_2Sn^{2+}/Bu_3Sn^+ and $BuSn^{3+}$/Bu_3Sn^+) in crucian carp were calculated from the field data (Table 1) and are presented in Table 2. The BCF values of tri-n-butyltin compounds in the muscle of crucian carp (800±500, n=7) were slightly higher than those of $(Bu_3Sn)_2O$ in the muscle of round crucian carp (590) and carp (500) calculated from the experimental data in chapter 2.

The ratios (Bu_2Sn^{2+}/Bu_3Sn^+ and $BuSn^{3+}$/Bu_3Sn^+) were < 0.02 - 0.22 (n=11) and < 0.2 - < 2.2 (n=11), respectively, in the muscle of crucian carp obtained from harbors of Lake Biwa. The ratio (Bu_2Sn^{2+}/Bu_3Sn^+) was higher than that (0.02) in the metabolism experiment of $(Bu_3Sn)_2O$ in carp (chapter 5). For the ratio ($BuSn^{3+}$/Bu_3Sn^+), the same comparison was impossible because the accurate field data were not obtained.

The concentrations of tri-n-butyltin and triphenyltin compounds in crucian carp obtained from harbors of Lake Biwa are presented in Table 3.

In the field data, the orders of BCF in round crucian carp were kidney ≥ liver > gallbladder > muscle for tri-n-butyltin compounds and gallbladder > kidney > liver > muscle for triphenyltin compounds. For the experimental data in chapter 2, the orders in carp were kidney > gallbladder > liver > muscle for $(Bu_3Sn)_2O$ and kidney > liver > muscle ≥ gallbladder for Ph_3SnCl. The orders were slightly different between the experimental and field data, but the BCF values were higher in the viscera than in the muscle for both data.

The concentrations (Ph_3Sn^+ > Bu_3Sn^+) in round crucian carp obtained from a harbor of Lake Biwa (Table 3) were presumed to be accumulated mainly by oral intake when considered with the results of the via gill intake

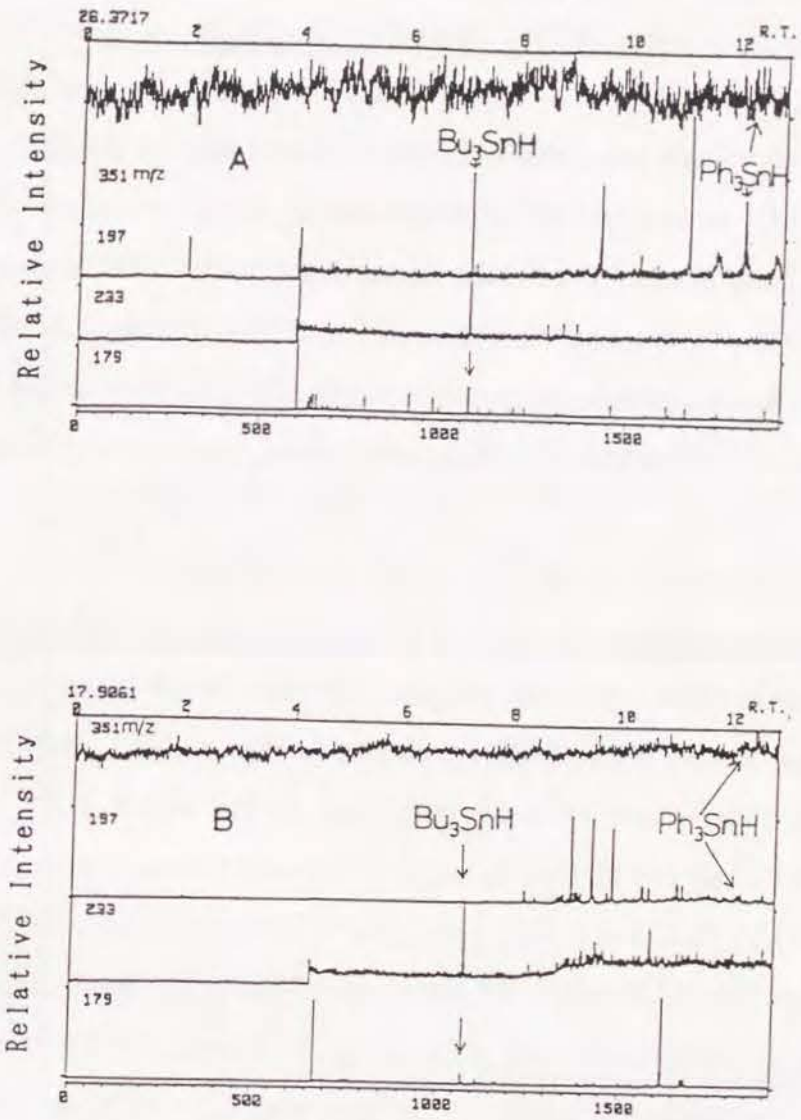


Fig.1 Typical GC-MS SIM chromatograms of Bu_3SnH and Ph_3SnH.

A: Std.Bu_3SnH and Ph_3SnH each 1 ng

B: Muscle of round crucian carp obtained from Harbor B

experiment (BCFg: Bu_3SnCl > Ph_3SnCl) on the same fish (chapter 2) and the oral intake experiment (BCF_o: Ph_3SnCl > Bu_3SnCl) on goldfish (chapter 7). However, those (Bu_3Sn^+ > Ph_3Sn^+) in deep crucian carp obtained from the same location were presumed to be accumulated mainly via gill intake.

GC-MS determination of organotin hydrides in round crucian carp obtained from Harbor B of Lake Biwa was tried by SIM with ions at m/z= 179 and 233 for Bu_3SnH and m/z=197 and 351 for Ph_3SnH. As shown in Fig.1, the determination was possible for Bu_3SnH but impossible for Ph_3SnH. The result of Bu_3SnH by GC-MS agreed closely with that by GC-FPD.

Surveys of pesticides in rivers flowing into Lake Biwa

The concentration changes of molinate, diazinon, IBP, benthiocarb and simetryne in water and fish samples obtained from River A and River B throughout the survey in 1988 are shown in Figs.2 and 3, respectively.

The BCF values of these pesticides in the whole body of pale chub calculated from the field data were 108 (n=1) for molinate, 135 (n=2) for diazinon, 17 (n=5) for IBP, 204 (n=3) for benthiocarb and 4 (n=3) for simetryne. The BCF values of these pesticides in the whole body of willow shiner from the experimental data (chapter 8) were 248 for diazinon, 33 for IBP, 65 for benthiocarb and 2 for simetryne. The BCF values of IBP and simetryne in the experimental data were nearly equal to those in the field data.

The concentration changes of oxadiazon, CNP and chlomethoxynil in water and fish samples obtained from River A and River B throughout the survey in 1989 are shown in Figs.4 and 5, respectively.

The average BCF values of these pesticides in the whole body of pale chub calculated from the field data were 1180 (n=9) for oxadiazon and 3140

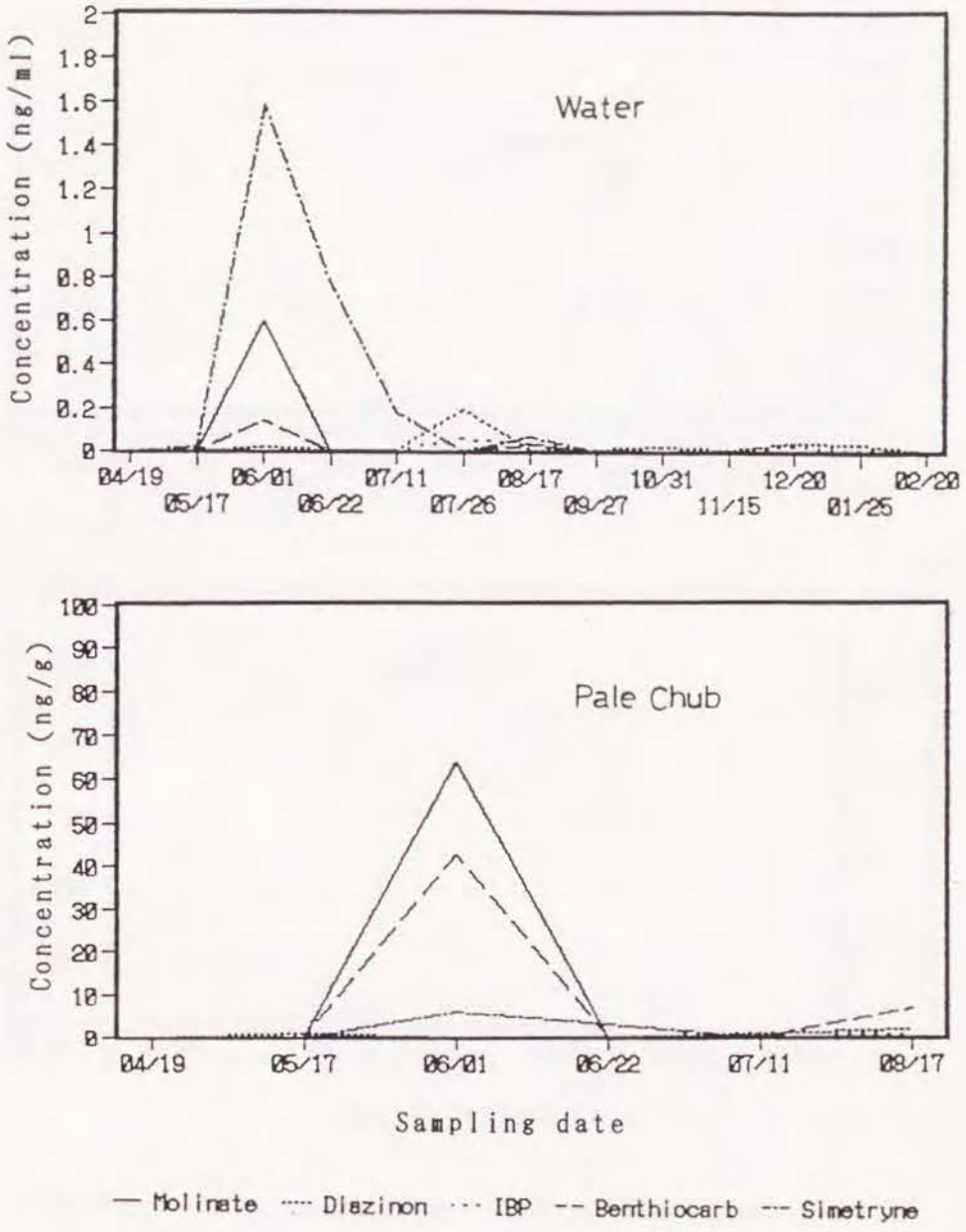


Fig.2 Survey on pesticides in water and fish samples (1988) obtained from River A.

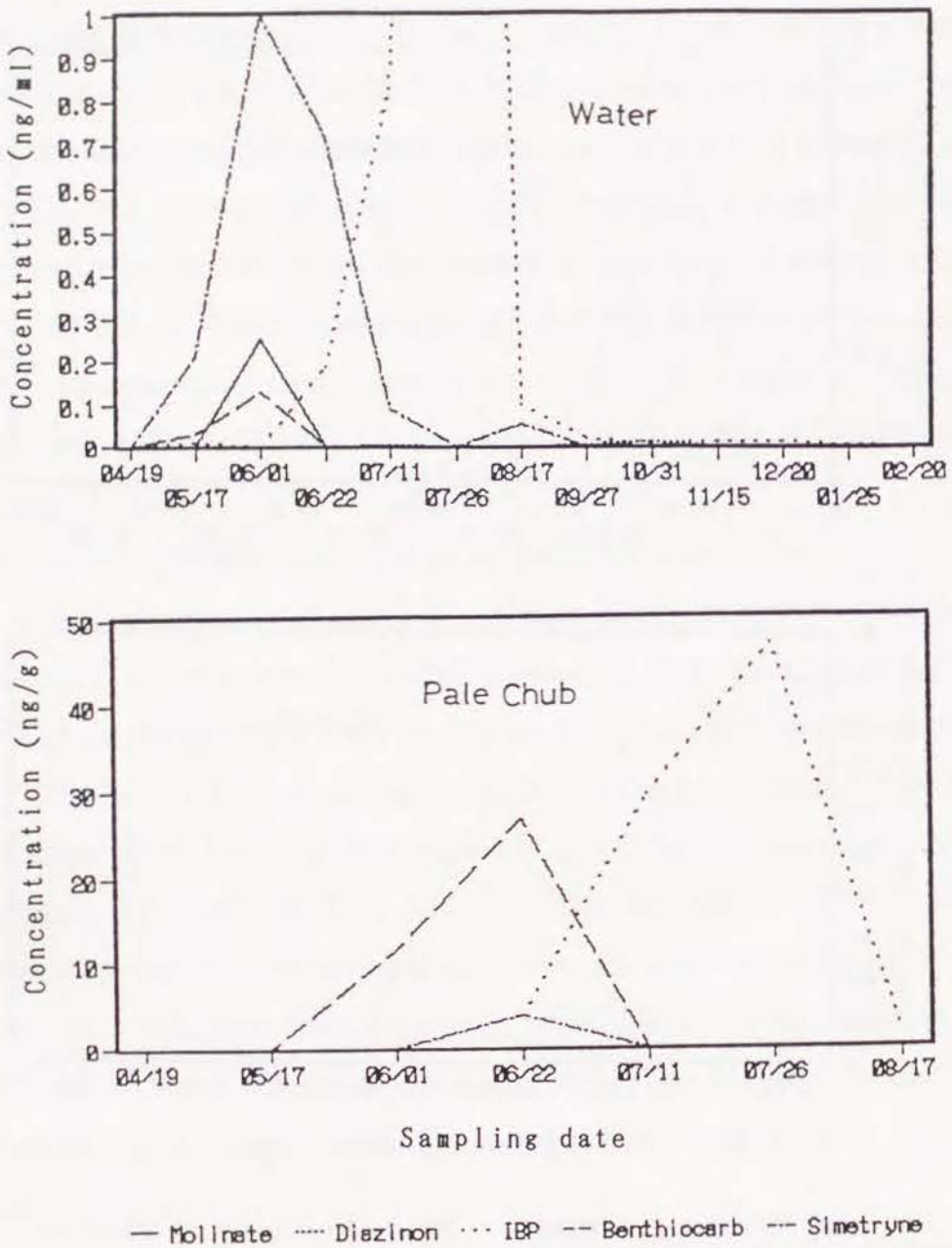


Fig.3 Survey on pesticides in water and fish samples (1988) obtained from River B.

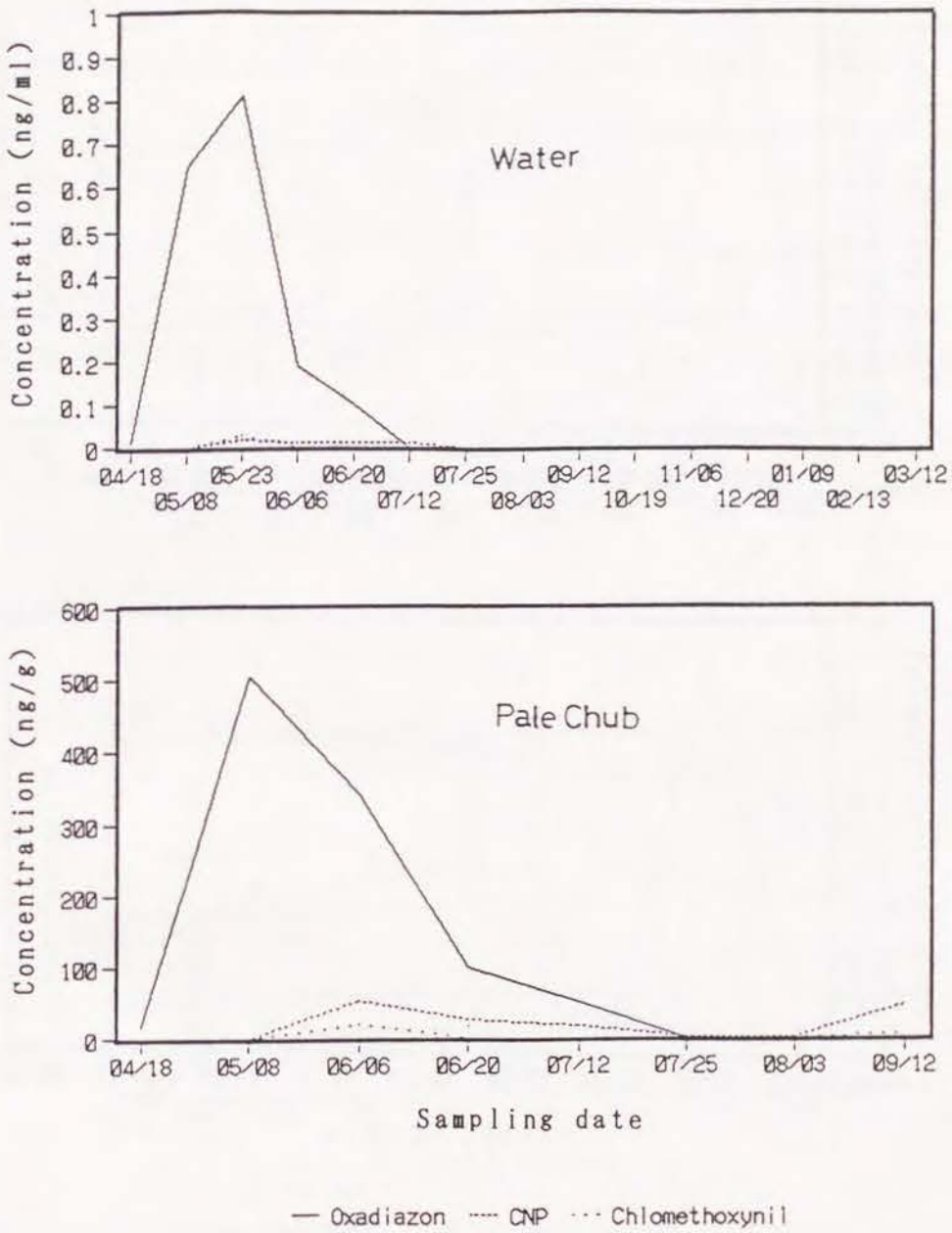


Fig.4 Survey on pesticides in water and fish samples (1989) obtained from River A.

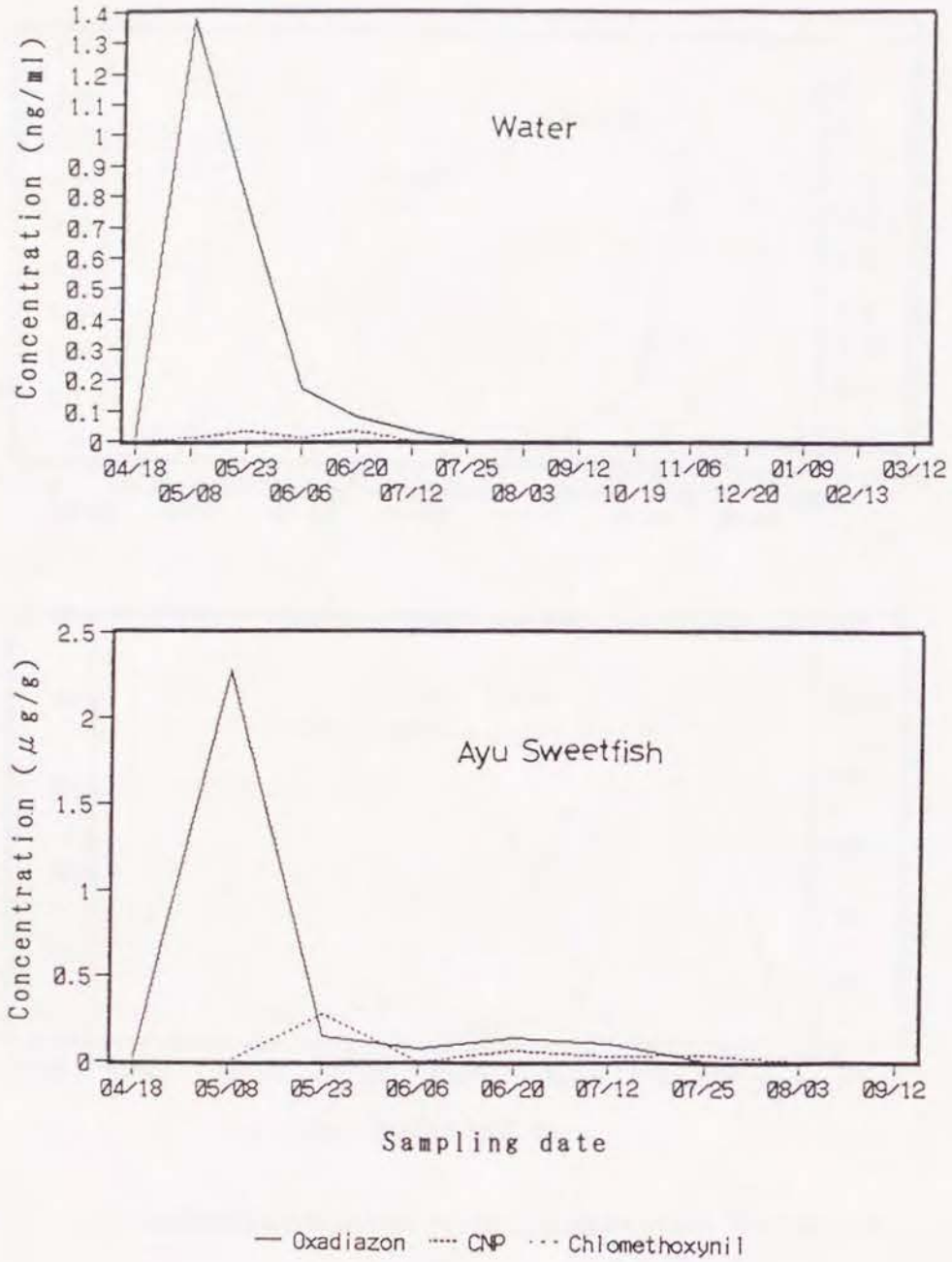
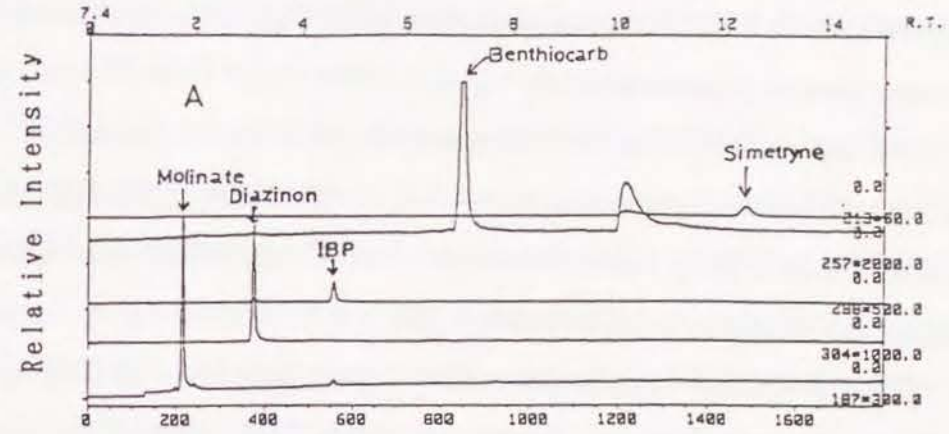


Fig.5 Survey on pesticides in water and fish samples (1989) obtained from River B.



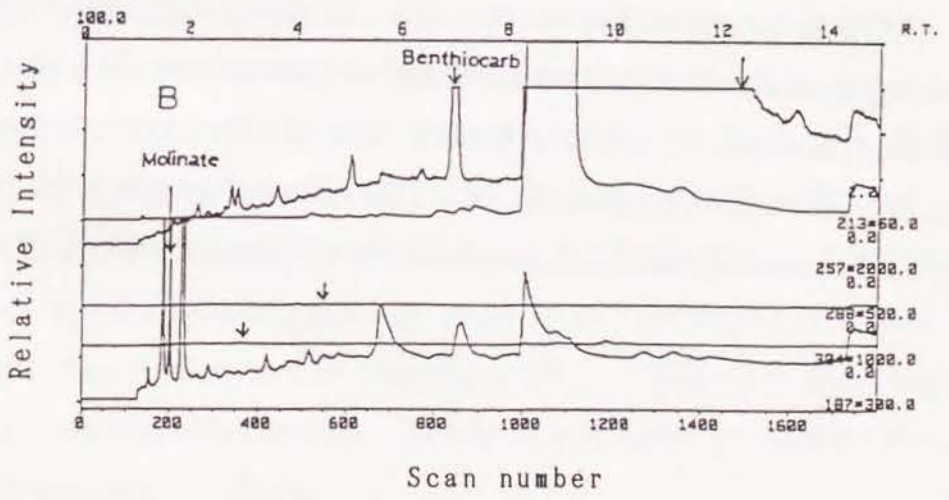


Fig.6 Typical GC-MS SIM chromatograms of pesticides.
A: Std.Pesticides each 1.25 ng
B: Whole body of ayu sweetfish obtained from River C

(n=7) for CNP. Similarly, those in the whole body of ayu sweetfish were 1500 (n=5) for oxadiazon and 3000 (n=4) for CNP. The BCF values of these pesticides in the whole body of willow shiner from the experimental data (chapter 8) were 1226 for oxadiazon and 5689 for CNP. The BCF values in the experimental data were nearly equal to those in the field data.

The order of BCF in the fishes calculated from the field data (CNP > oxadiazon > benthiocarb > diazinon > IBP > simetryne) were approximately equal to that in willow shiner calculated from the experimental data (CNP > oxadiazon > diazinon > benthiocarb > IBP > simetryne).

The low residue of these pesticides in pale chub and ayu sweetfish after stopping their use has also become apparent from the field data. These results are consistent with the experimental data that their excretion rate from willow shiner is rapid (chapter 9).

GC-MS determination of pesticides in fishes obtained from rivers flowing into Lake Biwa was tried by SIM with ions at m/z=187 for molinate, m/z=304 for diazinon, m/z=288 for IBP, m/z=257 for benthiocarb and m/z=213 for simetryne. Typical GC-MS SIM chromatograms were shown in Fig.6. The results by GC-MS agreed closely with those by GC-FTD.

General Conclusion

This thesis has dealt with studies on biological transport phenomena of environmental chemical pollutants. The conclusion of each chapter is as follows:

In chapter 1, recent developments in analytical methods of butyltin and phenyltin compounds in biological samples were reviewed and our proposed method was described. We have developed a sensitive and rapid gas chromatographic method for the determination of butyltin and phenyltin compounds in biological samples. The method could be satisfactorily applied to the fish samples used in the accumulation and excretion experiments and obtained from harbors of Lake Biwa and the rivers.

In chapter 2, the accumulation of butyltin and phenyltin compounds in freshwater-fishes was described. The bioconcentration factor (BCF) in carp, crucian carp and goldfish was measured for butyltin and phenyltin compounds. The orders of BCF were $(Bu_3Sn)_2O > BuSnCl_3 > Bu_2SnCl_2$ and $Ph_3SnCl > Ph_2SnCl_2 > PhSnCl_3$ or $Ph_3SnCl > PhSnCl_3 > Ph_2SnCl_2$ in the muscle and the viscera (liver, kidney and gallbladder) of carp. The BCF values of carp and crucian carp were higher in the viscera than in the muscle. The BCF values of $(Bu_3Sn)_2O$ (500 - 3200) were about two - five times as high as those of Ph_3SnCl (260 - 2100) in the four tissues of carp and the whole body of goldfish.

In chapter 3, the influence of pH on the accumulation of Bu_3SnCl and Ph_3SnCl in carp was studied to evaluate more precisely their accumulation in the fishes which are living in the contaminated lake or sea. The order of BCF at three pH values was pH 7.8 > pH 6.8 > pH 6.0 for both Bu_3SnCl and Ph_3SnCl. It is presumed that the chemical forms of Bu_3SnCl

and Ph_3SnCl change from the dissociated (Bu_3Sn^+ and Ph_3Sn^+) to the undissociated forms (Bu_3SnOH and Ph_3SnOH) with an increase in pH.

In chapter 4, the excretion of tri-n-butyltin and triphenyltin compounds from freshwater-fishes was described. The excretion rate constants of these compounds were calculated by assuming the excretion process to be first-order kinetics. The biological half-lives were 1.7 days for $(Bu_3Sn)_2O$ and 1.4 days for Ph_3SnCl in the muscle of carp. For goldfish, the biological half-lives were 8.7 days for $(Bu_3Sn)_2O$ and 17.3 days for Ph_3SnCl in the whole body of goldfish.

In chapter 5, the metabolism of $(Bu_3Sn)_2O$ and Ph_3SnCl in carp and goldfish was described. Bu_2Sn^{2+} and $BuSn^{3+}$ or Ph_2Sn^{2+} and $PhSn^{3+}$, metabolites of $(Bu_3Sn)_2O$ or Ph_3SnCl were detected in both fishes. The ratio of Ph_3SnCl: Ph_2Sn^{2+}: $PhSn^{3+}$ concentrations in the metabolism of carp corresponded to that of the BCF of carp for the same phenyltin chlorides. The accumulation potential (BCF) of Ph_2SnCl_2 and $PhSnCl_3$ could be used as an explanation for the concentrations of metabolized Ph_2Sn^{2+} and $PhSn^{3+}$ in carp. But the same potential of Bu_2SnCl_2 and $BuSnCl_3$ could not be applied to the metabolism of $(Bu_3Sn)_2O$.

In chapter 6, the differences between freshwater and seawater-acclimated guppies in the accumulation and excretion of Bu_3SnCl and Ph_3SnCl were described. The BCF values of both Bu_3SnCl and Ph_3SnCl (460 and 1100) in the freshwater-fish were about twice as high as those (240 and 530) in the seawater-acclimated fish. The excretion rate of Ph_3SnCl from the seawater-acclimated fish (0.30 day^{-1}) was about twice as rapid as that from the freshwater-fish (0.13 day^{-1}), but there was no difference between the fishes in the rate for Bu_3SnCl (0.35 and 0.32 day^{-1}). These differences between the two types of fishes in the accumulation and excretion of these

chemicals are probably due to the differences in their chemical forms or properties as well as the physiological mechanisms operating for regulation of osmotic pressure in the fishes. In chapters 2, 3 and 4, the accumulation of butyltin and phenyltin compounds only via gill intake of fishes was studied. To explain the contamination of fishes in lake or sea by these compounds, the data on their accumulation by oral intake as well as via gill intake were absolutely necessary.

In chapter 7, the accumulation of Bu_3SnCl and Ph_3SnCl via gill and by oral intakes of goldfish was described. The average BCF values of Bu_3SnCl and Ph_3SnCl (n=2) via gill intake were 1980 and 1380, respectively, after 21 - 28 days of exposure. Similarly, their BCF values by oral intake were 0.04 and 0.10, respectively, after 35 days of exposure. The accumulation potential of Bu_3SnCl and Ph_3SnCl via gill intake was considerably higher than that by oral intake. The order of these compounds (Ph_3SnCl > Bu_3SnCl) in the potential by oral intake was opposite to that (Bu_3SnCl > Ph_3SnCl) via gill intake.

In chapter 8, the accumulation of pesticides in freshwater-fishes was described. The BCF in willow shiner and carp was measured for herbicides, insecticides and fungicides. The accumulation potential of these pesticides in whole body of willow shiner was low for simetryne, IBP, malathion and benthiocarb (BCF: 2 - 65), middle for diazinon and fenitrothion (BCF: 248 and 399) and high for oxadiazon, chlomethoxynil and CNP (BCF: 1226 - 5689). The order of BCF in the four tissues of carp was kidney > liver ≥ gallbladder > muscle for benthiocarb, diazinon, IBP and fenitrothion.

In chapter 9, the excretion of pesticides from freshwater-fishes was described. The excretion rate constant was calculated by assuming the

excretion process to be first-order or second-order kinetics. Their excretion rate from whole body of willow shiner was considerably rapid for simetryne, malathion, benthiocarb, diazinon and fenitrothion (biological half-lives: 1.4 - 9.9 h), but a little slower for oxadiazon, chlomethoxynil and CNP (biological half-lives: 55.5 - 165.0 h). These pesticides accumulated in carp had a tendency to excrete rapidly from muscle and slowly from kidney. The slow excretion rate from kidney is probably because these pesticides in all tissues of the fish are finally transported into kidney and excreted.

In chapter 10, the accumulation and metabolism of CNP and CNP-amino in carp were described. The BCF values of CNP were 334 in muscle, 542 in liver, 862 in kidney and 547 in gallbladder. Those of CNP-amino were 90 in muscle, 402 in liver, 501 in kidney and 5368 in gallbladder. The orders of BCF were kidney > gallbladder ≥ liver > muscle for CNP and gallbladder > kidney ≥ liver > muscle for CNP-amino. The order of CNP was slightly different from that of CNP-amino. CNP-amino, CNP-acetamide and CNP-formamide were detected as metabolites of CNP in carp. But CNP-formamide could not be detected from the metabolism experiment of CNP-amino in carp.

In chapter 11, the accumulation and excretion of pesticides used in golf courses by carp and willow shiner were described. The BCF values in whole body of willow shiner were 3.9 for simazine, 18 for chlorothalonil and 350 for captan. Those in whole body of carp were 220 for tolclofos-methyl, 460 for chlorpyriphos, 20 for flutolanil, 27 for isoprothiolane, 25 for chlorothalonil, 100 for captan, 440 for isoxathion and 360 for iprodione. Their excretion rate from whole body of willow shiner was considerably rapid for simazine (biological half-life: 0.9 h), but slower for chlorothalonil

and captan (biological half-lives: 17.3 and 34.7 h). The rate from whole body of carp was rapid for flutolanil and isoprothiolane (biological half-lives: 5.8 and 3.6 h), but slower for tolclofos-methyl, chlorpyriphos, captan and isoxathion (biological half-lives: 17.3 - 69.3 h). The correlations between P_{ow} and BCF in willow shiner and carp were investigated for the pesticides studied in this thesis. Correlation factors (r) except captan were 0.9391 (n=10) for willow shiner and 0.7747 (n=15) for both fishes.

In chapter 12, the comparison of these experimental data with the field data was described. The field data were the results of surveys in Lake Biwa for butyltin and phenyltin compounds and those in rivers flowing into Lake Biwa for pesticides. The BCF values of tri-n-butyltin compounds in muscle of crucian carp (800 ±500, n=7) calculated from the field data in the harbors of Lake Biwa were slightly higher than those of $(Bu_3Sn)_2O$ in muscle of round crucian carp (590) and carp (500) calculated from the experimental data. The concentration ratio (Bu_2Sn^{2+}/Bu_3Sn^+) was < 0.02 - 0.22 (n=11) in the muscle of crucian carp obtained from the same locations. The ratio was higher than that (0.02) in the metabolism experiment of $(Bu_3Sn)_2O$ in carp. The concentrations ($Ph_3Sn^+ > Bu_3Sn^+$) in round crucian carp obtained from a harbor of Lake Biwa were presumed to be accumulated mainly by oral intake when considered with the results of the via gill intake experiment (BCF_g: $Bu_3SnCl > Ph_3SnCl$) on the same fish and the oral intake experiment (BCF_o: $Ph_3SnCl > Bu_3SnCl$) on goldfish. However, those ($Bu_3Sn^+ > Ph_3Sn^+$) in deep crucian carp obtained from the same location were presumed to be accumulated mainly via gill intake.

The survey on pesticides was carried out in seven rivers flowing into Lake Biwa once or twice every month in 1988 and 1989. Here, two rivers were selected and the field data on two species of fishes (pale chub and ayu

sweetfish) were compared with the experimental data. The order of BCF in the fishes calculated from the field data (CNP > oxadiazon > benthiocarb > diazinon > IBP > simetryne) were approximately equal to that in willow shiner calculated from the experimental data (CNP > oxadiazon > diazinon > benthiocarb > IBP > simetryne). The low residue of these pesticides in the fishes after stopping their use has become apparent from the field data. These results are consistent with the experimental data that their excretion rate from willow shiner is rapid.

The results from these studies will be important fundamental data for the evaluation of safety of these environmental chemical pollutants to man and fish as well as for the evaluation of contamination of freshwater and marine fishes by these compounds.

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T. Tsuda, H. Nakanishi, T. Morita and J. Takebayashi, Simultaneous gas chromatographic determination of dibutyltin and tributyltin compounds in biological and sediment samples. *J. Assoc. Off. Anal. Chem.*, **69**, 981 - 984 (1986).

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