Accumulation, Transformation, and Elimination of Bis(tri-*n*-butyltin) Oxide in Red Sea Bream, *Pagrus major*, under Laboratory Conditions

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Red sea bream (*Pagrus major*) were exposed to bis(tri-*n*-butyltin) oxide (TBTO), and accumulation, transformation, and excretion of TBTO in the muscle, liver, gills, and digestive tract were observed. Only two organotin compounds, tri-*n*-butyltin chloride (TBTC) and di-*n*-butyltin chloride (DBTC), were detected in the muscle, and TBTC accounted for more than 96% of the organotin compounds at 8 weeks after TBTO treatment. In addition to these compounds, hydroxylated, carboxylated, and oxygenated compounds of TBTC or DBTC were also found in the liver, gills, and digestive tract. The metabolic pattern in the red sea bream liver was significantly different from that in the liver of mammalian but similar to that in the gills and digestive tract and in general agreement with that found in the natural marine environment, which was surveyed in an earlier work.

Keywords: *TBTO; bis(tri-n-butyltin) oxide; red sea bream; Pagrus major; fish; uptake; accumulation; metabolism; depuration; elimination; viscera; gas chromatography/helium atmospheric pressure microwave-induced plasma/atomic emission detection system; GC/MIP/AED*

INTRODUCTION

Organotin compounds, such as trialkyltin and triaryltin compounds, have been increasingly used since the early 1970s as biocidal agents for boat paints or fishing nets to prevent algae and shellfish from becoming encrusted on ship bottoms and fishing nets. However, reports have shown that the use of these organotin compounds had resulted in pollution of the aquatic environment and marine products on a global scale (Becker et al., 1992; Huggett et al., 1992). Due to growing concern regarding the impact on the environment, several countries have imposed restrictions on the use of organotin compounds (U.S. EPA, 1988; Alzieu, 1991).

Severe pollution of marine products due to the use of such chemicals has also been reported in Japan (Sasaki et al., 1988a,b; Ishizaka et al., 1989a; Hashimoto et al., 1991; Higashiyama et al., 1991). This led to the passing of bills in Japan in December 1989, banning the use of bis(tri-*n*-butyltin)oxide (TBTO) for such purposes, including restrictions on the use of other organotin compounds.

Suzuki et al. (1992) investigated the molecular species of organotin compounds in marine products of natural origin, such as hairtail, yellowtail, and dab, which were obtained in retail stores in the Tokyo metropolitan area. They found high concentrations of di-*n*-butyltin dichloride (DBTC), tri-*n*-butyltin chloride (TBTC), and triphenyltin chloride, as well as lower concentrations of ten other organotin compounds including dealkylated, hydroxylated, carboxylated, and oxo compounds in the muscle, liver, and eggs. However, there have been few attempts to systematically conduct laboratory experiments on the accumulation, metabolism, and elimination of these organotin compounds in fish or other marine organisms.

In this study, therefore, red sea bream (*Pagrus major*), which is cultured in Japanese marine farms and is commercially available, was experimentally exposed to TBTO in the laboratory, and the bioaccumulation, metabolism, and elimination of TBTO in the muscle and other tissues were observed over a 16-week period.

MATERIALS AND METHODS

Gas Chromatography/Helium Atmospheric Pressure Microwave-Induced Plasma/Atomic Emission Detection System (GC/MIP/AED). An HP model 5890 Series II gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a split/splitless injection port interfaced to an HP model 5921A atomic emission detector equipped with a turbo makeup gas valve was used. Injections were made with an HP model 7673A automatic sampler. Four capillary columns were used: a cross-linked methyl silicone [HP-1; Hewlett-Packard; 0.32 mm (i.d.) × 25 m × 0.17 μm (film thickness)], a cross-linked 5% phenyl methyl silicone [DB-5; J&W Scientific, Folsom, CA; 0.25 mm (i.d.) × 30 m × 0.25 μm (film thickness)], a cross-linked 14% cyanopropylphenyl methyl silicone [DB-1701; J&W Scientific; 0.25 mm (i.d.) × 30 m × 0.25 μm (film thickness)], and a cross-linked 50% cyanopropylphenyl methyl silicone [DB-225; J&W Scientific; 0.25 mm (i.d.) × 30 m × 0.25 μm (film thickness)]. Operating conditions were as follows for the HP-1: column oven, programmed from 35 °C (hold 2 min) at the rate of 30 °C/min to 200 °C (hold 0 min), followed by the rate of 15 °C/min to 280 °C (hold 6 min); injection port (splitless), 250 °C; and AED solvent vent off time, 3 min. The conditions for AED were established essentially as described by Łobiński et al. (1992); namely, AED cavity temperature, 280 °C; AED cavity pressure, 1.5 psi; AED cavity scavenger gases, 3.5 kg/cm^2 (H_2) and 1.4 kg/cm^2 (O_2); AED spectrometer purge flow (N_2), 2 L/min; and wavelengths for measurement at 303.319 nm. Operating conditions were as follows for the DB-5: column oven, programmed from 35 °C (hold 2 min) at

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the rate of 30 °C/min to 200 °C (hold O min), followed by the rate of 15 °C/min to 280 °C (hold 4 min); AED solvent vent-off time, 4 min; and other conditions were the same with those for HP-1. All operating conditions of the DB-1701 were exactly the same as those for the DB-5. Operating conditions were as follows for the DB-225: column oven, programmed from 35 °C (hold 2 min) at the rate of 30 °C/min to 220 °C (hold 15 min); AED solvent vent off time, 3.6 min; AED cavity temperature, 220 °C; and other conditions were the same with those for HP-1.

Treatment of Red Sea Bream. Red sea bream, *Pagrus major*, purchased from Kanagawa Prefectural Fisheries Research Station were acclimated in the laboratory to 21 ± 1 °C for about 1 week before being used for experiments, and those with a body weight of 17.7 ± 2.1 g (mean ± SD) were used. They were separated into three groups of 33 fish in each: a control group, a low-concentration TBTO exposure group (low-TBTO group), and a high-concentration TBTO exposure group (high-TBTO group). The fish were reared in continuous flow-through rearing glass aquaria (60 cm × 30 cm × 36 cm, approximately 45 L). The rearing aquaria were supplied with activated charcoal-processed seawater at a rate of 500 mL/min and also with air. The fish were fed with commercially available formula feeds (Kyowa Hakko, type C-3000; TBTC concentration < 10 ppb) at a rate of 30 mg/g body weight/day, twice a day from Monday to Saturday. TBTO was dissolved in a mixture of acetone−dimethyl sulfoxide (1:9, v/v). An appropriate amount of the TBTO solution was diluted with 20 L of well-aerated tap water and then supplied to the aquaria at a rate of 5 mL/min by a micro glass pump. The control group was supplied only with the mixture of acetone−dimethyl sulfoxide without TBTO. The concentrations of TBTO in the rearing aquaria seawater were measured before red sea bream were exposed to TBTO and at 1, 2, 4, 6, and 8 weeks after TBTO exposure. Measured TBTO values were <2 ng/L in the control group (as TBTC), 163 ± 38 ng/L (n = 6, mean ± SD, as TBTC) in the low-TBTO group and 326 ± 59 ng/L (n = 6, mean ± SD, as TBTC) in the high-TBTO group during the course of the experiments.

The fish were reared for 8 weeks in seawater containing TBTO and then transferred to the TBTO-free seawater systems, where they were reared for a further 8 weeks for depuration. Three fish were collected for each analysis at 10 different points in the time course: before TBTO treatment; 1, 2, 4, 6, and 8 weeks after the start of TBTO treatment; and 1, 2, 4, and 8 weeks after termination of TBTO treatment, respectively. After each fish was weighed, the muscle, liver, gills, and digestive tract were dissected and frozen in screw-capped vials at −40 °C until extraction.

Authentic Standards. TBTO (95%) and TBTC (95%) were obtained from Wako Pure Chemical Industries, Ltd. (Tokyo). DBTC (>97%) and tri-*n*-propyltin chloride (Pr_3SnCl) were purchased from Tokyo Kasei Kogyo Company, Ltd. (Tokyo) and Kanto Chemicals Company, Inc. (Tokyo), respectively. *n*-Butyltin trichloride (MBTC, 95%) was purchased from Aldrich Chemical Company (Milwaukee, WI).

Di-n-butyl(3-hydroxybutyl)tin Chloride (T3OH), Di-n-butyl-(3-oxobutyl)tin Chloride (T3CO), and Di-n-butyl(4-hydroxybutyl)tin Chloride (T4OH). The materials were synthesized as described by Fish et al. (1976) and then purified by the method reported by Ishizaka et al. (1989b).

n-Butyl(3-hydroxybutyl)tin Dichloride (D3OH), n-Butyl(3-oxobutyl)tin Dichloride (D3CO), and n-Butyl(4-hydroxybutyl)-tin Dichloride (D4OH). The materials were synthesized according to the method described by Ishizaka et al. (1989b).

Di-n-butyl(3-carboxypropyl)tin Chloride (TCOOH) and n-Butyl(3-carboxypropyl)tin Dichloride (DCOOH). The materials were synthesized by the method of Suzuki et al. (1992). The chemical names and their abbreviations used throughout are shown in Table 1.

Preparation of Matrix Solution. In order to equalize, as far as possible, the effects of matrices in sample solutions with those in the standard solutions in GC/MIP/AED measurement, a matrix solution was added to both the sample solutions and the standard solutions. The matrix solution was prepared as follows. To 200 g of rainbow trout muscle was added 3% sodium chloride (NaCl) solution (400 mL), and then the mixture was homogenized and extracted with *n*-hexane (300 mL). The *n*-hexane layer was separated by centrifugation at 300 rpm. The aqueous layer was extracted with diethyl ether (Et_2O, 300 mL) and centrifuged again. The combined mixture of *n*-hexane and Et_2O extracts was filtered through a filter paper if necessary and then concentrated to 40 mL under reduced pressure.

Table 1. Standards and Abbreviations

standard	abbrev
bis(tri-*n*-butyltin) oxide	TBTO
n-butyltin trichloride	MBTC
di-*n*-butyltin dichloride	DBTC
tri-*n*-butyltin chloride	TBTC
n-butyl(3-hydroxybutyl)tin dichloride	D3OH
n-butyl(3-oxobutyl)tin dichloride	D3CO
n-butyl(4-hydroxybutyl)tin dichloride	D4OH
n-butyl(3-carboxypropyl)tin dichloride	DCOOH
di-*n*-butyl(3-hydroxybutyl)tin chloride	T3OH
di-*n*-butyl(3-oxobutyl)tin chloride	T3CO
di-*n*-butyl(4-hydroxybutyl)tin chloride	T4OH
di-*n*-butyl(3-carboxypropyl)tin chloride	TCOOH
tri-*n*-propyl ethyltin	Pr_3EtSn

Preparation of Sample Solution. After all adhering tissues were scraped off, fish digestive tract was put on a filter paper, held with tweezers on one end, stripped off using the dull portion of another tweezers to remove contents of the digestive tract, and then weighted accurately. Other tissues were weighted as usual. The extraction method is essentially that of Suzuki et al. (1992). After addition of 0.9% NaCl solution (10 mL/0.2−2.0 g of tissue) to samples in a 50-mL centrifuge tube with a screw-cap, the mixture was homogenized with a Biotrona 6403 (Kinematica AG, Littau, Switzerland). Hydrochloric acid (HCl) (36%, 12 mL) was added to the homogenate, and the mixture was shaken vigorously and allowed to stand for 10 min. After the addition of Et_2O (20 mL) and NaCl (2 g), the mixture was shaken vigorously for 10 min on a KM shaker (Iwaki Company, Ltd.) and then centrifuged at 3000 rpm for 10 min. This extraction procedure was repeated twice. The supernatant extract was dried over sodium sulfate (Na_2SO_4) and evaporated under reduced pressure, and then the residue was dissolved in *n*-hexane (20 mL) saturated with acetonitrile (MeCN). The solution was transferred to a 50-mL centrifuge tube with a screw-cap on it, to which MeCN (20 mL) saturated with *n*-hexane was added, and the mixture was shaken vigorously and centrifuged. The under layer (MeCN layer) was removed by pipetting with a Pasteur pipet. This extraction procedure was repeated once more. The remaining *n*-hexane layer was concentrated nearly to dryness under reduced pressure below 35 °C, and the residue was dissolved in a small volume of Et_2O and then applied to a column (1 cm i.d.) prepared from a slurry of Florisil (Floridin Company, Hancock, WV; 3 g) and Et_2O (50 mL). The column was washed with Et_2O (40 mL) and then eluted with a mixture of acetic acid (AcOH)−Et_2O (1:99 v/v; 40 mL), which was evaporated under reduced pressure at 35 °C. Complete removal of AcOH is facilitated by the addition of a small amount of *n*-hexane to the residue, followed by evaporation nearly to dryness. This residue was combined with the MeCN extract described above, the mixture was concentrated nearly to dryness under reduced pressure, and the final volume of solvent was removed under an N_2 atmosphere. The residue was dissolved in a small volume of *n*-hexane−ethyl acetate (EtOAc) (2:1, v/v), and the resulting solution was transferred to a 1-cm i.d. chromatographic column containing 5 g of HCl-treated silica gel (Wakogel C-100) (Hattori et al., 1984), prepared with *n*-hexane, with 1 cm of Na_2SO_4 on the top. The column was eluted with a mixture of *n*-hexane-EtOAc (2:1 v/v; 50 mL). The eluate was evaporated under reduced pressure, and the residue was dissolved in Et_2O (5 mL) and transferred into a screw-capped centrifuge tube (50 mL). Methyl magnesium bromide [MeMgBr, Tokyo Kasei Kogyo Company, Ltd. (Tokyo), ca. 3 M in Et_2O] (4 mL) was added carefully to the solution described above [*Caution*! MeMgBr/Et_2O and ethylmagnesium bromide (EtMgBr)/Et_2O are corrosive, form a

flammable combination, and react violently with water!], and the mixture was mixed gently, screw-capped, and then allowed to stand for 1 h in a water bath at 40 °C. A 2-mL portion of Et_2O and 10 mL of water were then added drop by drop to the solution in an ice bath until violent bubbling ceased after addition of water (1−2 mL). After gentle mixing, anhydrous sodium sulfite (Na_2SO_3, 0.2 g) and saturated ammonium chloride (NH_4Cl) solution (10 mL) were added to the solution, and then it was shaken vigorously. This reaction mixture was extracted twice with *n*-hexane (10 mL), and the combined *n*-hexane extract was dried over Na_2SO_4, concentrated exactly to 1 mL, and kept at −40 °C after addition of Na_2SO_3 until analysis (Sasaki et al., 1988b).

Standard solutions for calibration, with the exception of tri-*n*-propyl ethyltin (Pr_3SnEt), were prepared by methylation of organotin salts as described earlier (Suzuki et al., 1992). Briefly, diluted solutions of TCOOH and DCOOH in acetone (20 μg/2 mL, each), diluted standard solutions of MBTC and DBTC in *n*-hexane (5 μg/0.5 mL, each), TBTC in *n*-hexane (10 μg/1 mL), and others in *n*-hexane (20 μg/2 mL, each) were put into a flask (100 mL) containing 4 drops of 4 N HCl. Then the solvent was distilled off under reduced pressure nearly to dryness below 35 °C and then diluted with Et_2O to exactly 20 mL. This solution (0.5, 1, 2, and 4 mL each) was transferred to a 50-mL centrifuge tube, made up to a volume of about 20 mL with Et_2O, and then methylated as described above. On the other hand, an internal standard, Pr_3SnEt (100 μg/mL based on Pr_3SnCl), was prepared with EtMgBr [Tokyo Kasei Kogyo Company, Ltd., ca. 3 M in Et_2O] in place of MeMgBr as just described, and the resulting stock solution of Pr_3SnEt was diluted daily with *n*-hexane to afford a concentration of 0.5 μg/mL. The obtained mixtures of methylated organotin compounds were made up the volumes exactly to 2.0 mL with *n*-hexane after addition of the internal standard Pr_3SnEt (200 μL) and the matrix solution (200 μL).

To an aliquot of the sample solution in a small test tube was added Pr_3SnEt (0.5 μg/mL, 50 μL), the matrix solution (50 μL), and *n*-hexane exactly to make the volume 500 μL, and then the sample solutions and working standards were transferred to 100-μL autosampler vials for analysis. The organotin concentrations reported here are usually mean values from two fish.

All results in the low- and high-TBTO groups were not corrected for the control group.

Detection limits in the muscle, which were calculated as three times the standard deviation of the noise levels of peak height, were 0.6 (MBTC, DB-5), 0.2 (DBTC, DB-5), 0.6 (D3OH, DB-5), 0.6 (D3CO, DB-5), 0.3 (TBTC, DB-5), 0.5 (D4OH, DB-1701), 1.4 (DCOOH, DB-5), 0.7 (T3OH, DB-5), 0.5 (T3CO, DB-5), 0.5 (T4OH, DB-5), and 1.5 (TCOOH, HP-1) ng/g. Detection limits in the liver, gills, and digestive tract were 1 (MBTC), 0.4 (DBTC), 1 (D3OH), 1 (D3CO), 0.5 (TBTC), 0.8 (D4OH), 3 (DCOOH), 1 (T3OH), 0.8 (T3CO), 0.8 (T4OH), and 3 (TCOOH) ng/g (the columns used are the same as those in the muscle).

Analysis of Organotin Compounds in Seawater. Extraction of organotin compounds were essentially performed following the method proposed by Dirkx et al. (1989) with a slight modification of extraction solvent (*n*-hexane in place of *n*-pentane) and equipment used. To 800 mL of seawater sample in a 1-L separatory funnel was added 200 mL of a citric acid/phosphate buffer solution at pH 5.0. If necessary, 1 N sodium hydroxide was added in a dropwise manner to adjust the pH to 5.0, which was monitored by putting a drop of the sample solution on an indicator paper. Next, sodium diethyldithiocarbamate solution (1 M, 2 mL) and *n*-hexane (40 mL) were added to the seawater and the mixture was shaken for 2 min. This extraction procedure was repeated twice. The combined *n*-hexane extracts were evaporated under reduced pressure nearly to dryness at 35 °C, transferred by pipetting to a Teflon-capped 50-mL centrifuge tube with the aid of Et_2O (20 mL), and then methylated with MeMgBr (2 mL). The centrifuge tube was tightly screw-capped and then warmed to 40 °C for 1 h in a water bath. After addition of 10 mL of water (added drop by drop until violent bubbling ceased), Pr_3SnEt (0.5 μg/mL, 100 μL), anhydrous Na_2SO_3 (ca. 0.2 g), saturated NH_4Cl (5 mL), and *n*-hexane (10 mL) were added, and then the solution was shaken vigorously using a mechanical shaker. After centrifugation, the organic layer was collected and the same extraction procedure was repeated again. The combined extracts were dried over Na_2SO_4, decanted into a flask, and then concentrated to 0.5 mL under reduced pressure below 30 °C. The data are reported as the means of duplicate trials.

Calculation of Bioconcentration Factor (BCF). BCF was calculated by the following equation (Laughlin et al., 1986):

BCF =
(concentration of TBTC in tissues of red sea bream)/
(concentration of TBTC in seawater)

1, 2, 4, 6, and 8 weeks after the start of exposure, the concentrations of TBTC in tissues of red sea bream were determined and used for the calculation of BCF.

Calculation of Half-Life. The biological half-life, $t_{1/2}$, which gives an indication of persistence of a chemical, is calculated according to a definition described in the legend to Table 2.

RESULTS AND DISCUSSION

For the sake of brevity, each of the organotin species is referred to in the paper as if it existed only in chlorides, but this is not meant to imply the exact identities of these species in fish tissues. All organotin concentrations were expressed as the chlorides, in ng/L or ng/g, and analytical results were not corrected for recovery.

Effect of TBTO on Fish. There were no significant differences in relative body weight gains between groups throughout the experimental period (data not shown). However, three of the red sea bream in the high-TBTO group died during the latter half of the accumulation experiment. With the exception of bluegill sunfish, the reported acute toxicities of TBTO to marine and estuarine fish range from 2.3 to 34.8 ng/L (as TBTC) in 96-h LC_{50} (WHO, 1990). The mortality rate in the present study (3/33) is, therefore, considered to be reasonable.

Identification. Figure 1 shows a representative GC/MIP/AED chromatogram (HP-1) of liver extract from the high-TBTO group at 1 week after TBTC treatment. Peaks on the gas chromatogram were assigned to individual organotin compounds, as shown in the figure, on the basis of retention time, i.e., in the order of elution to *n*-butyltin trichloride (MBTC), di-*n*-butyltin dichloride (DBTC), *n*-butyl(3-hydroxybutyl)tin dichloride (D3OH), *n*-butyl(3-oxobutyl)tin dichloride (D3CO), tri-*n*-butyltin chloride (TBTC), *n*-butyl(4-hydroxybutyl)tin dichloride (D4OH), *n*-butyl(3-carboxypropyl)tin dichloride (DCOOH), di-*n*-butyl(3-hydroxybutyl)tin chloride (T3OH), di-*n*-butyl(3-oxobutyl)tin chloride (T3CO), and di-*n*-butyl(4-hydroxybutyl)tin chloride (T4OH). These assignments were also confirmed with the other two columns, DB-5 and DB-225, and an additional DB-1701 when necessary. Further confirmation of alkyltin compounds was carried out by the previously reported tin emission pattern method (Łobiński et al., 1992; Suzuki et al., 1994, 1996), i.e., the peaks observed on the chromatogram were identified as organotin compounds by the presence of the characteristic four lines (300.914, 303.419, 317.505, and 326.234 nm) on the emission spectrum of each peak after subtraction of helium background noise.

Tissue Distribution, Metabolism, and Depuration. *(1) Muscle.* Figure 2 indicates the concentrations of organotin compounds in muscle tissues of the low-TBTO group (A) and the high-TBTO group (B). The first 8 weeks are the accumulation period, and the next 8 weeks are the depuration period following the termi-

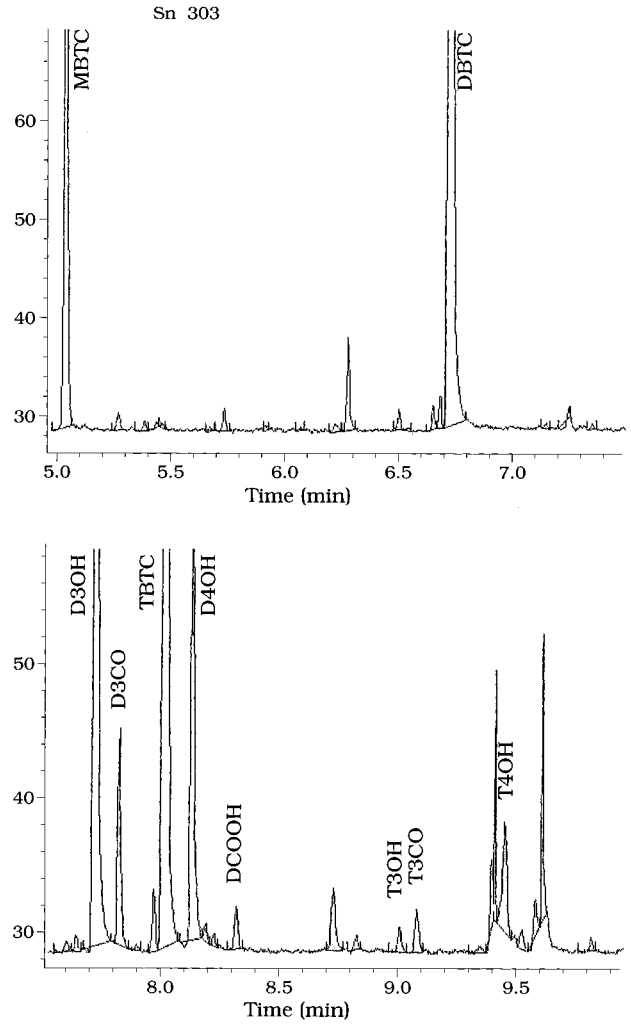

Figure 1. GC/MIP/AED chromatogram of methylated alkylated liver extract obtained from red sea bream, *P. major*, exposed to TBTO (DB-5) (exposure level: 326 ng/L as TBTC).

nation of TBTO treatment. Throughout the 16-week experimental period, the organotin concentration was measured in ten compounds at the ten points in time. The TBTC concentrations in natural seawater are reported to be around 100 ng/L at relatively highly polluted sites, 1−10 ng/L at relatively low polluted sites (as Bu_3Sn^+; WHO, 1990), and sometimes rise above 200 ng/L (Maguire et al., 1986; Suzuki et al., 1996). Thus, the TBTO levels (TBTC levels) used in this study are considered to be somewhat higher than the general concentration levels in natural marine and freshwater environments. As can be seen from the figure, only two organotins, DBTC and TBTC, were detected in the muscles, and a tendency to approach a steady state was not evident at either exposure level. A notable deposition was not found in the control group in contrast to that in the other two groups (data not shown). The TBTC concentration showed a peak at the 8th week after TBTO treatment in both the low-TBTO group (540 ng/g) and the high-TBTO group (940 ng/g). The accumulated concentrations appeared to be almost in proportion to the concentrations to which the fish were exposed, but the accumulated concentrations in the high-TBTO group did not reach twice the low-TBTO group over the course of the experiment. DBTC levels in the muscles in both groups were very low, and the TBTC/DBTC ratio was more than 26 at 8 weeks after TBTO treatment. Suzuki et al. (1992) investigated organotin compounds in the muscle, eggs, and liver of 35 species of marine products, such as hairtail, yellowtail, and dab, which were obtained from several retail stores in the Tokyo metropolitan area. In addition to DBTC and TBTC, they found metabolites that were oxygenated at the 3- or 4-position of the butyl moiety of DBTC and TBTC mainly in the fish liver and in some fish eggs and muscle. In the present study, however, only DBTC and TBTC were found in the muscle of red sea bream, and no other metabolites were detected. Lower TBTC/DBTC ratios were found in the muscles of various species of fish in previous reports: range = 2.4−30, n = 12, mean = 9.4 (Sasaki et al., 1988); n = 5, mean = 2.9 (Martin et al., 1989); and range = 0.9−16.7, n = 28, mean = 5.6 (Suzuki et al., 1992). The large variations in the TBTC/DBTC ratios in the muscle between laboratory and field conditions are attributed to species differences, the elapsed time from

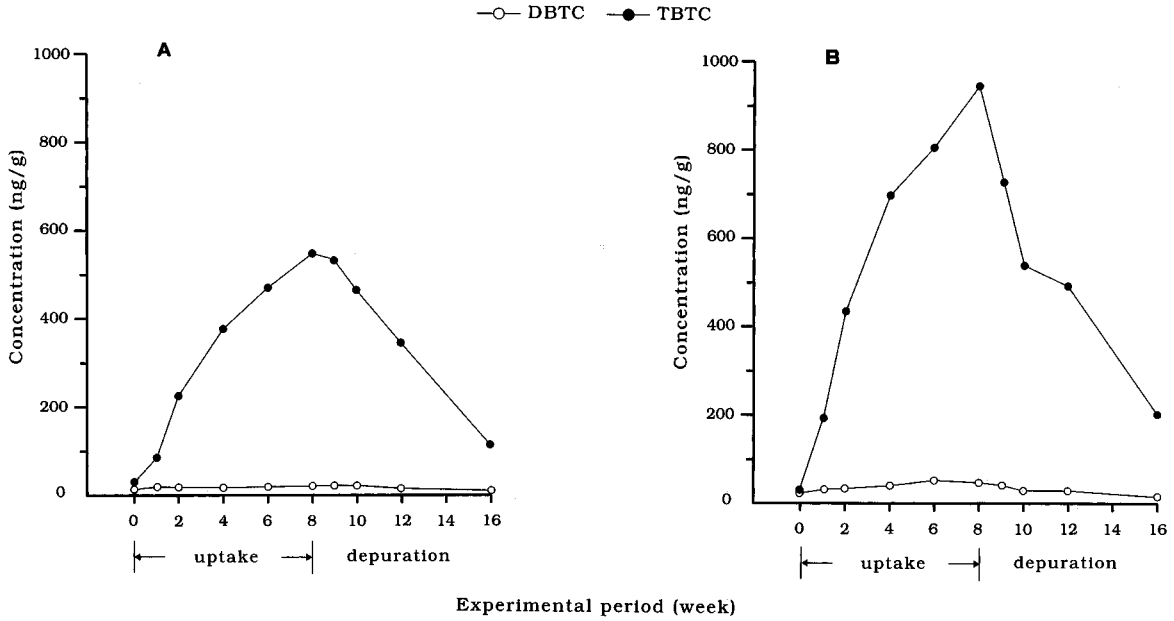

Figure 2. Accumulation and depuration of organotin compounds in the muscle of red sea bream, *P. major*, exposed to TBTO-containing seawater: A, 163 ng/L as TBTC; B, 326 ng/L as TBTC.

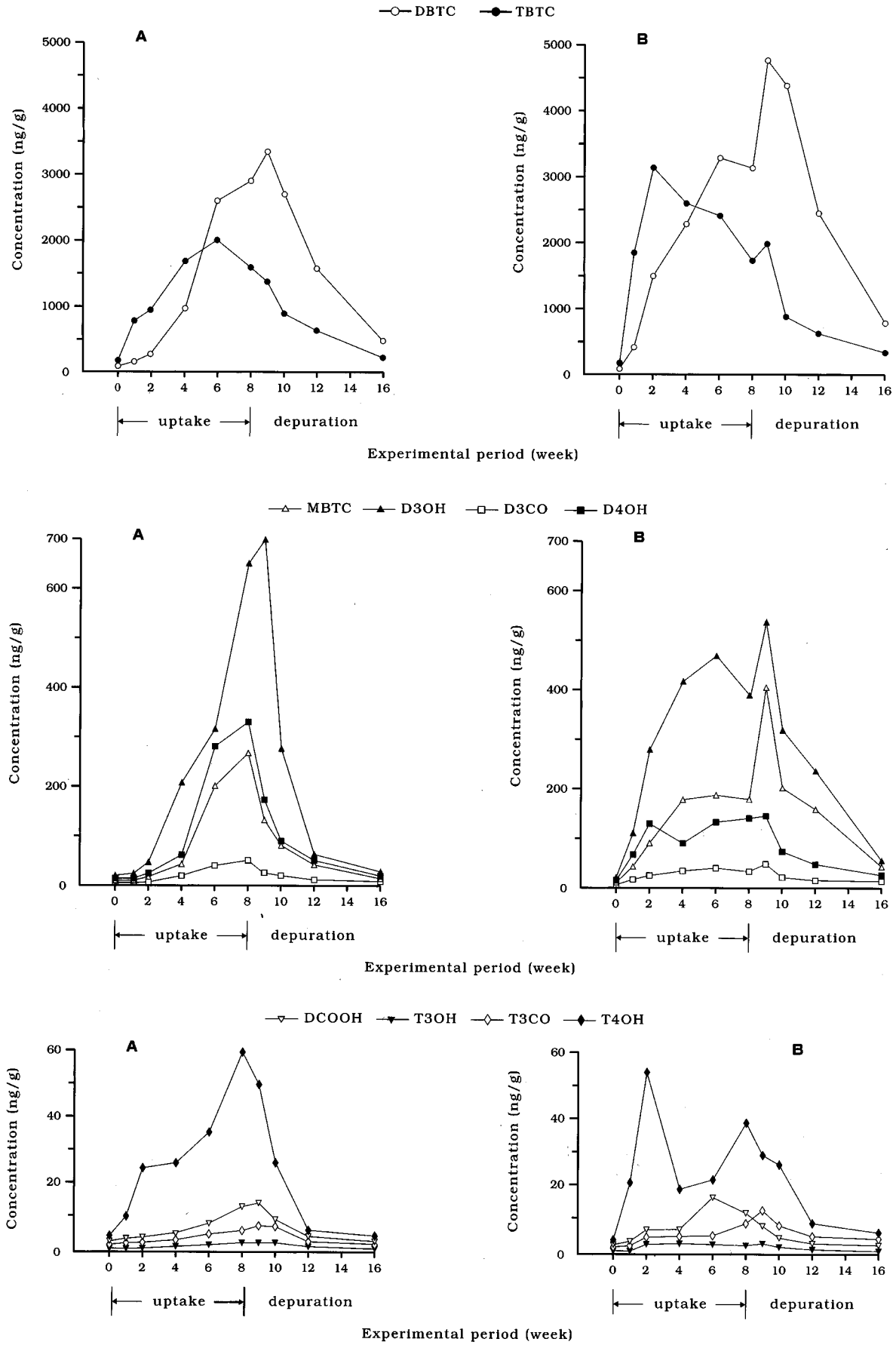

Figure 3. Accumulation and depuration of organotin compounds in the liver of red sea bream, *P. major*, exposed to TBTO-containing seawater: A, 163 ng/L as TBTC; B, 326 ng/L as TBTC.

exposure to TBTO until collection, exposure concentration, and differences in uptake form.

(2) Liver. Figure 3 shows the results for the liver. Ten organotin compounds were detected and identified

in the liver as shown in Figure 1. The results shown in Figure 3 were apparently different from those for the muscle; the concentrations of TBTC or DBTC were much higher than those in the muscle (top), and the concentrations of TBTC were higher than those of DBTC during the first 4 weeks of exposure but reversed after the 6th week in both the low-TBTO group and the high-TBTO group. The TBTC level in the low-TBTO group, unlike that in the muscle, rose to a peak at the 6th week, whereas the TBTC level in the high-TBTO group reached a maximum at the 2nd week. Two possible reasons are considered for these phenomena: activation of a drug-metabolizing enzyme system and inhibition of absorption of TBTC. However, the latter possibility is easily denied by the increment of TBTC in muscles for the same accumulation periods as shown in Figure 2 and an increase of DBTC in the liver of fish in the high-TBTO group up to twice that of fish in the low-TBTO group as shown in Figure 3. Accordingly, it would be reasonable to assume that TBTC, after reaching a certain level in the liver, is quickly metabolized and thus does not accumulate in large quantities. The presence of maxima before the 8th week, which was the last week of uptake, indicates that a drug-metabolizing enzyme system was induced in the fish liver, and this system predominated when the TBTC level in the seawater was high. On the other hand, DBTC reached a maximum at the 9th week both in the low-TBTO group and the high-TBTO group after completion of the uptake period.

MBTC, D3OH, D3CO, and D4OH in the low-TBTO group showed steep peaks around the 8th week, while those in the high-TBTO group showed the rapid increases of concentrations from the start of uptake and became relatively constant after the 2nd or 3rd week (middle). The levels of D3OH and D4OH at the 8th week of uptake were lower in the high-TBTO group than in the low-TBTO group. These trends were very similar to that of DBTC. The level of DCOOH, which is a main metabolite in the rat liver (Matsuda et al., 1993), showed maxima (about 15 ng/g) at the 9th week in the low-TBTO group and at the 6th week in the high-TBTO group in red sea bream, though the levels were low. The concentrations of D3OH at the 4th week were 210 ng/g in the low-TBTO group and 420 ng/g in the high-TBTO group. This order was reversed at the 9th week and the low-TBTO group showed about 700 ng/g, whereas the high-TBTO group showed 550 ng/g. These phenomena may be attributed to elevated drug enzyme systems as in the case of TBTC described above. The levels of dibutyltin derivatives in the low-TBTO group were in the order of D3OH > D4OH > D3CO > DCOOH.

The tributyltin derivatives T3OH, T3CO, and T4OH in the low-TBTO group showed peak maxima at the 8th to 9th week, whereas those in the high-TBTO group showed peak maxima at the 8th week or earlier (bottom). For example, a difference in the concentrations of T4OH was observed between the low-TBTO and high-TBTO groups. T4OH gradually increased in the low-TBTO group, reaching a peak at the 8th week, and the other tributyltin derivatives, T3OH and T3CO, also peaked at the 8th week, though the levels were low. On the other hand, in the high-TBTO group, T4OH increased dramatically within 2 weeks to a maximum of 56 ng/g but later decreased. This suggests an increased metabolic activity of fish liver in the high-TBTO group and the rapid degradation of the metabolic intermediates T4OH and D4OH or other oxygenated metabolites.

Fish et al. (1976) and Kimmel et al. (1977) showed that Bu_3Sn^+ is metabolized in vitro by a rat liver microsome enzyme system to give DBTC, T3OH, T3CO, and T4OH. Ishizaka et al. (1989b) reported that DBTC administered intraperitoneally to the rat is metabolized to hydroxylated products at the 3- and 4-positions. Furthermore, Matsuda et al. (1993) administered TBTC orally to rats and reported that DCOOH and D3OH were the main metabolites in the liver and kidney, while TBTC remained in only small amounts and tributyltin derivatives such as T3OH, T3CO, T4OH, and TCOOH were not produced. In the present study, however, both dibutyltin derivatives (D3OH, D3CO, and DCOOH) and tributyltin derivatives (T3CO, T3OH, and T4OH) were detected in the liver of red sea bream. These differences were attributed to the different metabolic activities between in vivo and in vitro studies and to different experimental animals. In the in vivo experiments, rats, which have the strongest metabolic activity among experimental animals, would metabolize intermediates such as T3OH and T4OH to more oxygenated or degraded forms such as DCOOH or MBTC. However, in the in vitro experiments, the rat liver does not have sufficient ability to degrade the intermediates such as T3OH, T4OH, and T3CO, and therefore these metabolites may stay at those steps without further degradations. On the other hand, it is a well-established fact that the metabolic activities of the fish liver microsomes are generally much lower than those of the liver microsomes from terrestrial animals (Adamson, 1967), and therefore it is considered natural that intermediates such as T3OH and T3CO appear in marine vertebrates (Suzuki et al., 1992).

Lee (1985), Seligman et al. (1988), and Suzuki et al. (1996) investigated the decomposition of organotin compounds in the seawater, and they suggested or confirmed that TBTO is decomposed in seawater by various factors to form metabolites such as MBTC, DBTC, and hydroxylated products. Vertebrates and molluscs in the marine environment may partly absorb such degradation products, but their accumulation from seawater would be negligible because the formation rate of such metabolites from TBTO is considered to be very rapid compared with the absorption rate from seawater.

(3) Gills. Figure 4 shows the results for the gills. The same ten organotin compounds that were detected in the liver were also detected in the gills. Generally, their levels were lower than in liver. The patterns of DBTC and TBTC were similar to those in the muscle except for a sharp rise in the TBTC level followed by a constant TBTC level from the 2nd to 8th week in the high-TBTO group. Lower TBTC/DBTC ratios were observed in both groups compared to those in the muscle (top).

MBTC levels were higher than D3OH levels in both groups (middle), and these results were different from those for the liver. All the metabolites except D3CO in the low-TBTO group showed peak maxima at the 8th week, while those in the high-TBTO group showed maxima at earlier times than those in the low-TBTO group.

Tributyltin derivatives in the low-TBTO group showed peak maxima between the 8th and 10th week, whereas those in the high-TBTO group peaked at earlier times of uptake and retained the levels or otherwise decreased gradually after that (bottom).

(4) Digestive Tract. Figure 5 shows the results for the digestive tract, which includes both the stomach and intestine. The TBTC levels in the digestive tract were lower than those in the gills in both the low- and high-

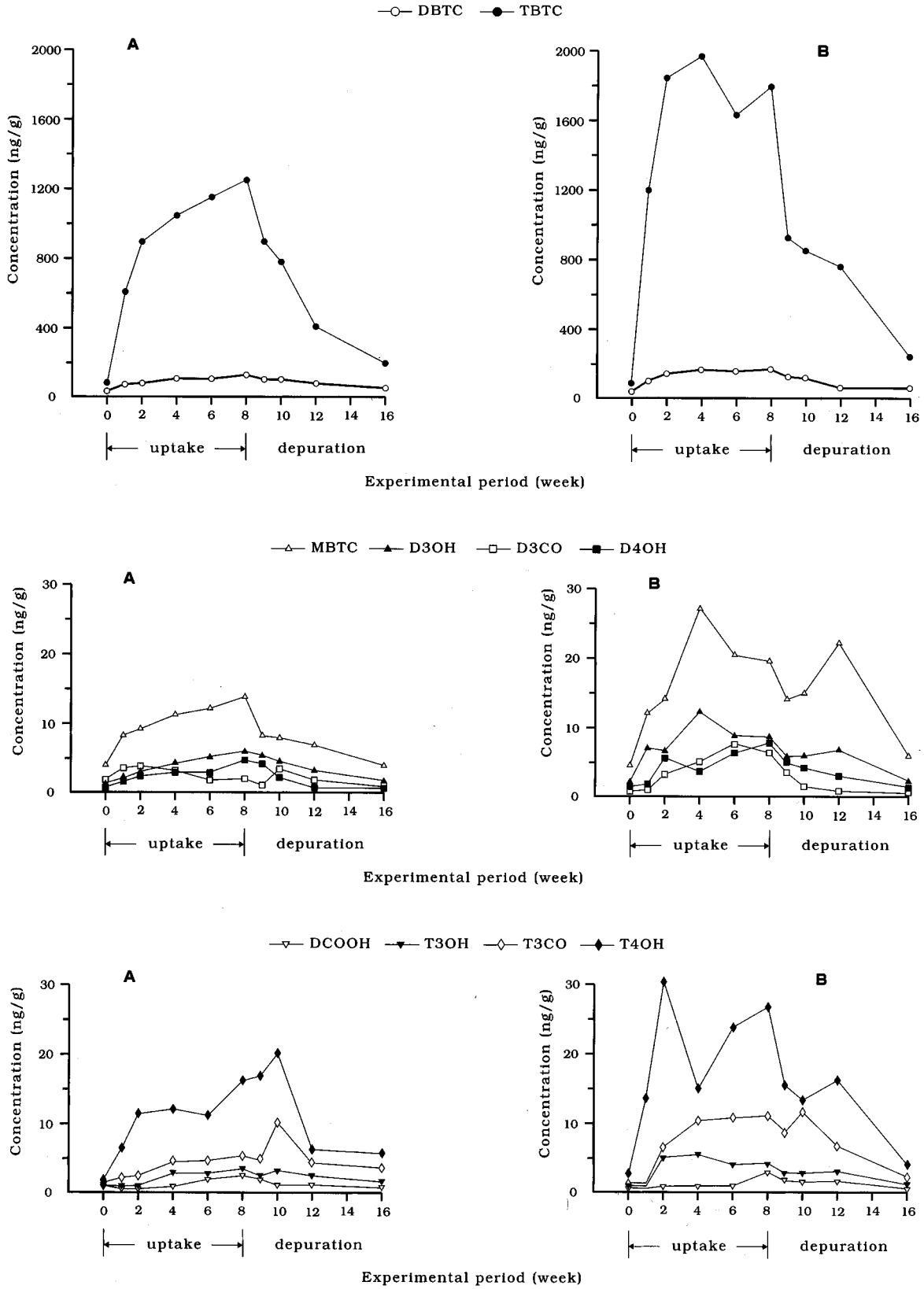

Figure 4. Accumulation and depuration of organotin compounds in the gills of red sea bream, *P. major*, exposed to TBTO-containing seawater: A, 163 ng/L as TBTC; B, 326 ng/L as TBTC.

TBTO groups: the TBTC levels plateaued at relatively early stages of uptakes compared with the other three tissues and there was not a great difference in the levels between two groups (top). On the other hand, the DBTC levels were similar in both the low- and high-TBTO groups to those of the gills and rose to a peak between 8th and 10th week in the low-TBTO group and between the 6th and 8th weeks in the high-TBTO group. The dibutyltin derivatives and MBTC showed almost the same trend in both the low- and high-TBTO groups, and the levels in the high-TBTO group were about twice those in the low-TBTO group (middle and bottom). Their levels decreased in the sequence MBTC, D3OH, D4OH, and D3CO in both groups, and this trend

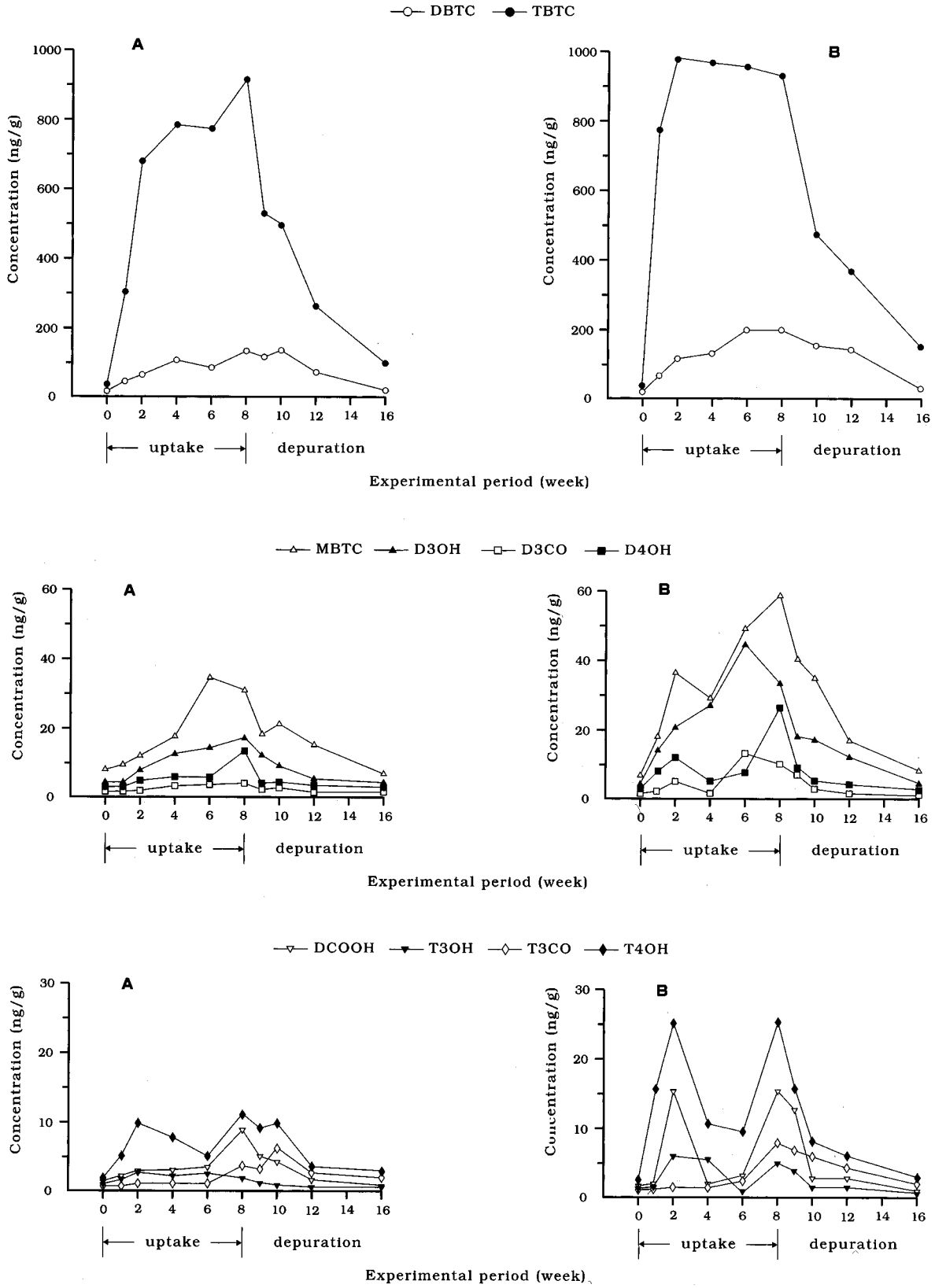

Figure 5. Accumulation and depuration of organotin compounds in the digestive tract of red sea bream, *P. major*, exposed to TBTO-containing seawater: A, 163 ng/L as TBTC; B, 326 ng/L as TBTC.

coincided with those in the liver and gills. On the other hand, DCOOH as well as tributyltin derivatives, especially T4OH, showed two peaks in high-TBTO group: one at the 2nd week and the other at the 8th week. Though the reason for the appearance of two peaks is not clear, it is speculated that the first peak is due to an increased tissue burden and this phenomenon was also observed for T4OH in the liver of the high-TBTO group (Figure 4, bottom). Taken together, the results suggest that the metabolic patterns in the gills and digestive tract are similar to that in the liver but that their activities are considerably weaker.

(5) BCF. Figure 6 shows the BCFs of TBTC in the muscle, liver, gills, and digestive tract in both the low- and high-TBTO groups up to the 8th week of uptake. Except for BCF in the muscle, there was almost no

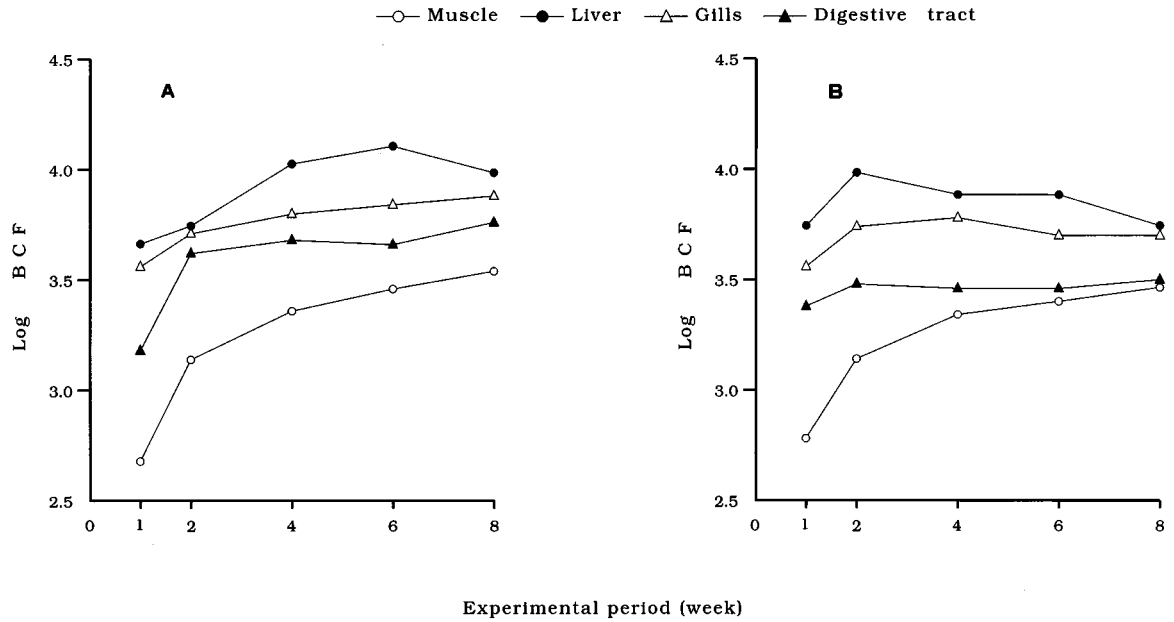

Figure 6. Changes in the bioconcentration factor of TBTO in tissues of red sea bream, *P. major*, exposed to TBTO-containing seawater: A, 163 ng/L as TBTC; B, 326 ng/L as TBTC.

Table 2. Biological Half-Life[a] of Tri-*n*-butyltin Chloride (TBTC) in Each Tissue under Low and High Exposure Conditions of Bis(tri-*n*-butyltin) Oxide (TBTO)

tissues	low-TBTO group (A)[b]	high-TBTO group (B)[b]
muscle	26.3	26.5
liver	20.7	23.7
gills	20.7	22.5
digestive tract	18.3	22.2

[a] Biological half-lives (day), $t_{1/2}$, were calculated by using the equation $t_{1/2} = \ln 2/k_d$, where k_d is a rate constant for depuration (d^{-1}). The value of k_d was determined by a least-squares linear regression of ln C vs t in the equation $C = C_0e^{-kdt}$, where C is tissue concentration on day t and C_0 is tissue concentration on day 0 (Becker and Tarradellas, 1994). [b] See legend to Figure 2.

change in BCF after the 4th week. BCFs were in the order of liver > gills > digestive tract > muscle in both the low- and high-TBTO groups, and the metabolic activities were in the order of liver > digestive tract > gills > muscle. Therefore, the differences in practical BCFs among the tissues would increase if metabolites were taken into account in the calculation of BCFs. This figure also clearly indicates that BCFs in the low-TBTO group were generally higher than those in the high-TBTO group in all tissues except in the first and 2nd week, where equilibrium distributions could not be fully attained. This observation suggests that BCF is inversely proportional to exposed concentration levels as pointed out by Laughlin and French (1988) and Yamada and Takayanagi (1992). It is noteworthy that BCFs in the liver did not converge even after 8 weeks of accumulation but rather rose to a peak, and the peak time varied depending on ambient TBTC concentrations. It is thought that these phenomena are linked to metabolic activation of the liver by TBTC and that BCF based on whole body, in the strict sense of the word, represents an averaged value of the BCF of each tissue.

(6) Half-Life. In the depuration experiment, the biological half-lives in muscles were calculated to be 26.3 and 26.5 days for TBTC in the low- and high-TBTO groups, respectively, depending on regression analyses (Table 2). These half-lives in the muscle were in general agreement with the half-life (28.8 days) in the whole body previously reported by Yamada and Takayanagi (1992), and they were longer than those of other fish species, *Mugil cephalus* (13.4 days) and *Rudarius ercodes* (7.4−8.9 days) in the whole body (Yamada and Takayanagi, 1992). On the other hand, *P. major* has a reported high BCF value of 8000−10000 (Yamada and Takayanagi, 1992). If the significance of the contribution of the TBTC level in the muscle to that in whole body is taken into account, this high BCF value and long half-life for TBTC indicates a low rate of decomposition of TBTC in *P. major*, and the high TBTC/DBTC ratios in the muscle are also thought to be symptomatic of low degradability of TBTC. This long half-life of TBTC in the muscle also suggests that the muscle provides a refuge for accumulated TBTC. Other tissues also showed a similar trend; the half-lives of the liver, gills, and digestive tract in the high-TBTO group showed similar values, and these values were longer than those in the low-TBTO group. The difference between the muscle and other tissues is attributed to differences in the function of tissues, i.e., the muscle serves as a store place for TBTC as described above and the other tissues degrade organs for xenobiotics. The fact that the high-TBTO group always showed a longer half-life than the low-TBTO group suggests that the xenobiotic metabolic capability per unit protein of tissue is limited.

CONCLUSIONS

Only two organotin compounds, TBTC and DBTC, were detected in the muscle, and 96% of these products were confirmed to be TBTC. In addition to the mother compound, TBTO (TBTC), 10 organotin compounds, including dealkylated, hydroxylated, and oxo compounds, were identified in the liver, gills, and digestive tract. The metabolic pattern in the red sea bream liver was significantly different from that in mammalian species' livers, where a carboxylic acid derivative of DBTC (DCOOH) is the main metabolite. DCOOH was found in only small amounts but hydroxy or oxo derivatives of TBTC, which were not found in rat liver, were clearly identified. This pattern is in general agreement with that found in the natural marine environment, which was surveyed in an earlier work (Suzuki et al., 1992). It was thought that the metabolic activity of fish liver was induced by raising fish in TBTO-containing

seawater, and this induction is strongly concentration-dependent.

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