Accumulation, Metabolism, and Depuration of Organotin Compounds in the Marine Mussels *Mytilus graynus* and *Mytilus edulis* under Natural Conditions

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The accumulation, transformation, and depuration of tri-*n*-butyltin (TBT) were studied over periods of ∼60−70 days using marine mussels, *Mytilus graynus* and *Mytilus edulis*, under natural conditions. *M. graynus* collected at a lightly polluted site were transplanted to a highly polluted site and *M. edulis* collected at a highly polluted site were transplanted to a lightly polluted site. TBT taken up in *M. graynus* showed a bioconcentration factor of 10 500 in the accumulation phase. Di-*n*-butyl-(3-oxobutyl)tin, which is a main metabolite of TBT in *M. edulis*, showed a longer half-life (8.13 days) than that of the parent compound (4.82 days) in the depuration phase. On the other hand, another metabolite, di-*n*-butyl(3-hydroxybutyl)tin, showed a shorter half-life (3.98 days) than that of the parent compound. The different half-lives among TBT and its metabolites are responsible for the different metabolic patterns in blue mussels at each sampling time.

Keywords: *Organotin; TBTO; TBT; accumulation; depuration; elimination; metabolism; blue mussels; Mytilus graynus; Mytilus edulis; half-life; bioconcentration factor; BCF*

INTRODUCTION

Organotin compounds have been used as antifouling paint biocides on ships and boats since the early 1970s. Because of the widespread use of tri-*n*-butyltin compounds (TBTs) and triphenyltin compounds (TPTs), these highly toxic organotin compounds have been frequently found in enclosed areas with heavy boat traffic (Maguire et al., 1986; Alzieu et al., 1989, 1990; Fent and Hunn, 1991; Tolosa et al., 1992) and also at elevated levels in commercially available foods (Takeuchi et al., 1987; Sasaki et al., 1988a,b; Mizuishi et al., 1989; Ishizaka et al., 1989a; Suzuki et al., 1992). Since TBTs and TPTs may have adverse effects on many aquatic organisms, not only on target organisms but also on nontarget organisms, at nanograms per liter levels (Laughlin and French, 1980; Waldock and Thain, 1983; Cleary and Stebbing, 1987; Bushong et al., 1988), they have been considered to be a group of the most toxic compounds to aquatic organisms ever introduced anthropogenically to aquatic systems. Due to the above reason, the use, manufacture, and import of organotin compounds have been prohibited or restricted in many countries.

In Japan the Fishery Union of Japan placed a voluntary ban on the use of bis(tri-*n*-butyltin) oxide (TBTO) for culturing nets in December 1987, as severe contamination of fishery products had been observed. Complete government control of TBTO (categorized as a class I chemical: prohibition on the sale, manufacture, and import of the chemicals without the permission of the Japanese government) was made possible by the passing of the Chemical Substances Control law in January 1990. This law in effect prohibited the use of TBTO for any purpose. However, 13 other tri-*n*-butyltin salts (e.g., chloride and fluoride) and 7 triphenyltin compounds (categorized as class II chemicals) were not prohibited, although their production and uses were restricted; that is, notification concerning the production and import of these chemicals must be given to the government, technical guidelines must be adhered to, and so on (September 1990). In response to this law, the government, in 1990, issued notices prohibiting the use of organotin compounds on culturing nets and on boats and ships, which need to be repainted in a dock within a year, and on those working on domestic lines. Under these guidelines the shipbuilding and painting industries have voluntarily regulated the production and use of organotin-containing antifouling paints. As a result, organotin-containing antifouling paints have been replaced by tin-free antifouling paints, although active ingredients have not been publicly stated. A decreasing trend in seawater concentration of organotin compounds in recent years, with the exception of international ports, has been confirmed by surveys over 10 years examining locations throughout Japan (Environment Agency, 1985−1995).

In previous papers, the authors reported that TBTs are degraded into hydroxylated and oxygenated metabolites at an alkyl moiety of TBTs and/or di-*n*-butyltin compounds in fish and shellfish under natural conditions (Ishizaka et al., 1989a; Suzuki et al., 1992) and in

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Table 1. Organotin Compounds and Their Abbreviations

compd	abbrev
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
diphenyltin dichloride	DPTC
di-*n*-butyl(4-hydroxybutyl)tin chloride	T4OH
di-*n*-butyl(3-carboxypropyl)tin chloride	TCOOH
triphenyltin chloride	TPTC
tri-*n*-propylethyltin	Pr_3SnEt

a given fish species under laboratory conditions (Yamamoto et al., 1997). Shiraishi et al. (1992) also reported the presence of a high amount of oxygenated products in blue mussels.

Mussels have heretofore been extensively studied as sentinel organisms for the assessment of organotin contamination (Farrington et al., 1983; Laughlin et al., 1986; Laughlin and French, 1988; Wade et al., 1988; Short and Sharp, 1989; Uhler et al., 1989). The objective of this study is to investigate the accumulation and depuration behaviors of TBTs in mussels under natural conditions and to provide information with reference to the suitability of marine mussels as biomonitor organisms. First, marine mussels (*Mytilus graynus*) were collected in a lightly contaminated area in northern Japan and transplanted to a highly contaminated area in central Japan to monitor the tissue concentration of TBTs and their metabolites over time under three environmental conditions. Second, naturally contaminated blue mussels (*Mytilus edulis*) were collected at a highly polluted site near Tokyo and transplanted to a lightly contaminated area in northern Japan, and depuration studies of TBTs and their metabolites were performed over a period of ∼70 days.

MATERIALS AND METHODS

The chemical names and their abbreviations used throughout are shown in Table 1. For the sake of brevity, each of the organotin species is referred to in the paper as if it existed only in chloride form, but this is not meant to imply the exact identities of these species in mussels.

All organotin values in mussels are reported as the chlorides on a wet basis (parts per billion) (water content: *M. graynus*, 77.3 ± 2.8%, $n = 10$; *M. edulis*, 76.8 ± 3.1%, $n = 10$) and those in seawater are reported as chlorides (nanograms per liter).

Sample Collection. Mussels (*M. graynus*) for accumulation experiments were collected on August 8, 1992, from wharves at a site (site G) 300 m off the coast of Oshoro Bay in Otaru City, located in northern Japan. Oshoro Bay has very little boat and ship traffic, and the contamination level of TBTC in the bay is thought to be extremely low. The mussels collected were sent by a chill-car at 4 °C on the sampling date to the National Research Institute of Fisheries Sciences in Kanagawa Prefecture near Tokyo, ∼1000 km from the collection site and near the site for accumulation experiments. Upon receipt in the laboratory in the evening, 2 days later, the mussels were dipped overnight into carbon-processed flowing seawater with continuous aeration, and accumulation studies were started the following day.

Mussels (*M. edulis*) for depuration studies were collected from wharves at site D in Moroiso Bay (Figure 1) on August 21, 1993. Moroiso Bay is adjacent to Aburatsubo Bay, which was used for the uptake experiments described above, and ∼120 recreational boats are moored throughout the year in Moroiso Bay, illustrated as an oblique-lined area. Mussels (*M. edulis*) collected from another two sites were used for a comparison of metabolic maps. One of these sites (site E, Figure 1) is in a narrow sea area between the Miura Peninsula and Jogashima Island (collected on August 2, 1993) with heavy boat and ship traffic, and the other (site F, Figure 1) is in Manadzuru Bay, Shizuoka Prefecture (collected on August 17, 1993). Manadzuru Bay, which is used as a fishing port, is next to a marina and shielded by a seawall from high waves and is located only 44 km away from Aburatsubo Bay, with Sagami Gulf lying between them (Figure 1).

Seawater Samples. Seawater samples were collected in glass bottles (2 L) at a depth of 1 m under the sea surface usually at 2−3 weeks intervals at sites A (for the accumulation experiment) and G (for the depuration experiment). The mean seawater temperatures were as follows: site A, 28.0 °C (August), 22.5 °C (September), and 22.0 °C (October); site G (the first, second, and last 10 days of the month), 19.5, 21.0, and 21.0 °C (August), 20.0, 20.2, and 18.7 °C (September), and 16.7, 15.3, and 14.0 °C (October). Hydrochloric acid (10 mL) was added to seawater samples as a preservative, and the samples were kept at 4 °C in a refrigerator and analyzed within 3 days of sampling.

Accumulation and Depuration Experiments. A marina in Aburatsubo Bay, Yokosuka City, Kanagawa Prefecture, which had been suspected as being a potential source of TBTC, was selected as the site for the accumulation experiment. This bay is surrounded by hills and is sheltered from the open sea. It is used as a refuge for fishing boats in typhoon seasons, and ∼100 recreational boats are moored throughout the year in the area shown by oblique lines (Figure 1). Three cages (40 × 15 × 15 cm, made of stainless steel) containing 30 mussels each (major axis = 78−90 mm, minor axis = 38−50 mm) were suspended at a depth of 1 m under the surface of the seawater with the aid of ropes at sites A (floating pier), B (float), and C (floating raft). Thus, the mussels always remained in water and never surfaced even at low tide. These conditions are clearly different from those in nature. Such conditions were established only from a technical point of view, and no data are available on the effects of such conditions on the physiological state of mussels, although the same methods have been used in culturing oysters, which exhibit a lifestyle similar to that of mussels. Therefore, no particular attention to possible effects of these conditions was given. The accumulation experiment was started on August 11, 1992, and the final sampling was completed 56 days later, on October 7, 1992. On days 0, 7, 16, 22, 30, and 56, ∼15 mussels were collected from each site to make three composite samples for butyltin analysis.

For the depuration experiment, blue mussels collected at site D in Moroiso Bay next to Aburatsubo Bay were transferred following the same method as that used for the accumulation experiments to a natural site (site G), 300 m off the coast of Oshoro bay in Otaru City, Hokkaido, located in northern Japan. Site G is close to the collection site of mussels for the accumulation experiment. The mussels were placed in round cages (i.d. 60 cm, depth 30 cm) that were separated into four compartments, and the cages were suspended on a float at a depth of 1 m with the aid of a rope. The depuration experiment was started on August 23, 1993, the samples were collected at 7−10 day intervals (∼15 mussels for each sampling over 10 times), and the final sampling was completed on October 30, 1993, after a duration of 68 days.

Gas Chromatography/Helium Atmospheric Pressure Microwave-Induced Plasma/Atomic Emission Detection System (GC/MIP/AED). The conditions for GC were established essentially as described by Yamamoto et al. (1997); namely, 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

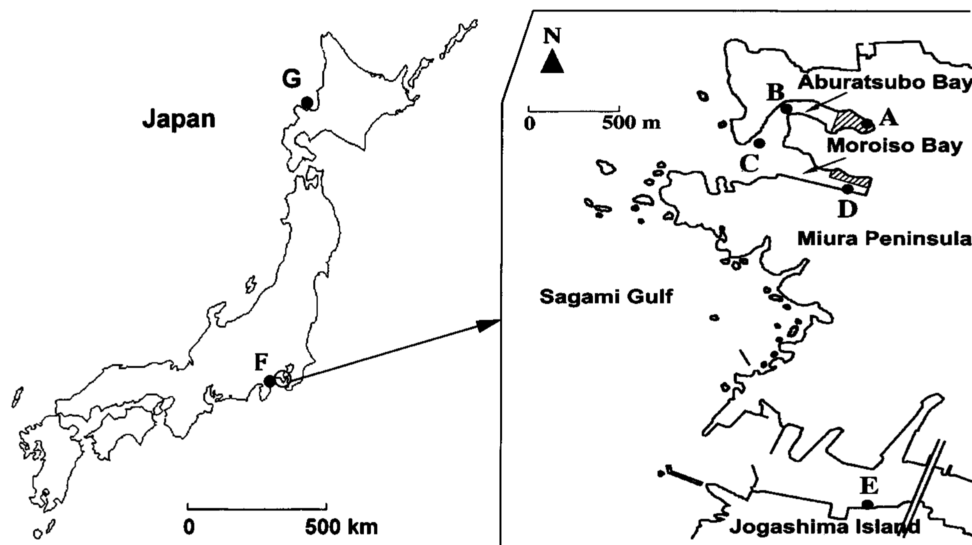

Figure 1. Sites used for accumulation and depuration experiments and for samplings.

Table 2. Optimal GC and AED Temperature Parameters

	column			
	DB-5	DB-225	DB-1701	HP-1
GC Parameters				
injection port temp (°C)	250	220	250	250
head pressure (kPa)	173	173	173	142
solvent vent off time (min)	4.0	3.6	4.0	3.0
oven program				
initial temp (°C)/ hold time (min)	35/2	35/2	35/2	35/2
ramp rate 1 (°C/min)	30	30	30	30
middle temp (°C)/ hold time (min)	200/0		200/0	200/0
ramp rate 2 (°C/min)	15		15	15
final temp (°C)/ hold time (min)	280/4	220/15	280/4	280/6
GC/AED Interface and AED Temperature Parameters				
transfer line (°C)	280	220	280	280
cavity temp (°C)	280	220	280	280

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)]. The operating conditions are shown in Table 2. The conditions for AED, except for temperature and solvent vent off time, were established essentially as described by Łobiński et al. (1992): 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.

Authentic Standards. All organotin standards were purchased or prepared according to the literature (Fish et al., 1976; Ishizaka et al., 1989b; Suzuki et al., 1992; Suzuki et al., 1996). They are listed in Table 1.

Preparation of Matrix Solution. To equalize, as far as possible, the matrix effects of sample solutions with those of standard solutions in GC/MIP/AED measurement, a matrix solution was added to both sample solutions and standard solutions, respectively. The preparation was described elsewhere (Yamamoto et al., 1997).

Extraction of Organotins from Mussels. The extraction method is essentially that of Suzuki et al. (1992). Briefly, after addition of a 0.9% NaCl solution (10 mL to 5 g of mussels) to samples, the mixture was homogenized with a Biotron (Biotrona 6403, Küssnacht, Switzerland). Hydrochloric acid (HCl) was added to the homogenate, and the mixture was shaken vigorously. After extraction with Et_2O, followed by centrifugation, the supernatant extract was evaporated under reduced pressure, and the residue was partitioned between *n*-hexane and MeCN. The *n*-hexane layer was concentrated nearly to dryness under reduced pressure, and the residue was dissolved in a small volume of Et_2O and then applied to a column prepared from a slurry of Florisil (Floridin Co., Hancock, WV) and Et_2O. The column was washed with Et_2O (40 mL) and then eluted with acetic acid (AcOH)/Et_2O (1:99 v/v; 40 mL), which was evaporated under reduced pressure. This residue was combined with the MeCN extract described above, the mixture concentrated nearly to dryness under reduced pressure, and the final volume of solvent removed under an N_2 atmosphere. The residue was transferred to a chromatographic column containing 5 g of HCl-treated silica gel (Wakogel C-100) (Hattori et al., 1984), prepared with *n*-hexane, with the aid of a small amount of *n*-hexane/EtOAc mixture (2:1 v/v). 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 taken up in Et_2O (20 mL) and subjected to methylation with methylmagnesium bromide (4 mL) [MeMgBr, Tokyo Kasei Kogyo Co., Ltd. (Tokyo), ~3 M in Et_2O] [*caution*: MeMgBr/Et_2O and ethylmagnesium bromide (EtMgBr)/Et_2O are corrosive and a flammable combination and react violently with water]. The reaction mixture was extracted with *n*-hexane, concentrated to exactly 1 mL, and kept at −20 °C after addition of Na_2SO_3 until analysis (Sasaki et al., 1988b).

Standard solutions for calibration, with the exception of tri-*n*-propylethyltin (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. The solvent was then distilled off under reduced pressure below 35 °C nearly to dryness; the residue was transferred to a 50-mL centrifuge tube with the aid of Et_2O and made up to ~20 mL with Et_2O. After methylation, the extract was concentrated to exactly 10 mL, and this solution was used as a stock solution. An internal standard, Pr_3SnEt (20 μg/mL as Pr_3SnCl), was prepared by using 200 μL of Pr_3SnCl (1000 μg/mL) and 2 mL of ethylmagnesium bromide (Tokyo Kasei Kogyo, ~3 M in Et_2O) in place of MeMgBr. To each 400, 200, 100, and 50 μL of the stock solution was added an internal standard (Pr_3SnEt, 0.5 μg/mL, 200 μL), the matrix solution (200 μL), and *n*-hexane to make the volume 2 mL, and these solutions were used as mixed working standards.

Standard and sample solutions thus prepared were stored at −20 °C.

To an aliquot of the sample solution in a small test tube was added Pr_3SnEt (0.5 μg/mL, 50 μL), matrix solution (50 μL), and *n*-hexane exactly to make up the volume to 500 μL, and then the mixtures were sent to autosampler vials of 100-μL capacity for GC/MIP/AED analysis. The organotin concentrations reported here are usually mean values from triplicate analyses and corrected for reagent blank.

Recoveries (percent, mean ± SD, n = 3) of organotin compounds spiked to mussels at a level of 0.1 μg/g of sample were 60.2 ± 3.5 (MBTC), 91.6 ± 7.5 (DBTC), 113 ± 9.1 (D3OH), 94.6 ± 3.8 (D3CO), 92.3 ± 9.0 (TBTC), 82.9 ± 9.4 (D4OH), 109 ± 8.6 (DCOOH), 97.6 ± 9.7 (T3OH), 83.1 ± 4.1 (T3CO), 89.9 ± 3.8 (T4OH), 106 ± 12.3 (TCOOH), 63.8 ± 4.5 (DPTC), and 76.2 ± 8.7 (TPTC), respectively.

Detection limits in the mussels were 0.6 ng/g for MBTC (DB-5), 0.2 ng/g for DBTC (DB-5), 0.6 ng/g for D3OH (DB-5), 0.6 ng/g for D3CO (DB-5), 0.3 ng/g for TBTC (DB-5), 0.5 ng/g for D4OH (DB-1701), 1.4 ng/g for DCOOH (DB-5), 0.7 ng/g for T3OH (DB-5), 0.5 ng/g for T3CO (DB-5), 0.5 ng/g for T4OH (DB-5), 1.5 ng/g for TCOOH (HP-1), 1.0 ng/g for DPTC (HP-1), and 0.8 ng/g for TPTC (DB-5).

Extraction of Organotins from Seawater. Extraction of organotin compounds was essentially performed by following the method proposed by Suzuki et al. (1996). To 800 mL of seawater sample in a 1-L separatory funnel, briefly, 200 mL of a citric acid/ phosphate buffer at pH 5.0 was added. If necessary, 1 N sodium hydroxide was added to adjust the pH 5.0, which was ultimately monitored by putting a drop of the sample solution on an indicator paper. Next, NaDDTC 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 to a Teflon-capped 50-mL centrifuge tube with the aid of Et_2O (20 mL), and then methylated with methylmagnesium bromide (2 mL). After addition of 10 mL of water (added drop by drop until violent bubbling ceased), tri-*n*-propylethyltin (Pr_3SnEt, 0.5 μg/mL, 50 μL), anhydrous sodium sulfite (~100 mg), saturated NH_4Cl (5 mL), and *n*-hexane (10 mL) were added, and then the mixture was shaken. After centrifugation, the organic layer was collected and the extraction procedure with *n*-hexane (20 mL) repeated again. The combined extracts were dried over Na_2SO_4 and then concentrated to 0.5 mL. All analyses were done in duplicate, and the data are reported as the mean of duplicate trials using two to four columns suitable for the analysis. Recovery test and detection limit were reported elsewhere (Suzuki et al., 1996).

Kinetic Model. To clarify the accumulation and depuration behavior of TBTC and its metabolites, the data were mathematically analyzed by the kinetic models shown in Figure 2 and by the following equations:

$$dC_1/dt = k_{w1}C_w - (k_{12} + k_{13} + k_{14} + ... + k_{e1})C_1 \quad (1)$$

$$dC_2/dt = k_{12}C_1 - (k_{25} + k_{26} + k_{27} + ... + k_{e2})C_2 \quad (2)$$

$$dC_9/dt = ...(...)C_9$$

In these eqs C_1 = TBTC, C_2 = T3CO, C_3 = T3OH, C_4 = T4OH, C_5 = D3CO, C_6 = DBTC, C_7 = D3OH, C_8 = D4OH, and C_9 = MBTC concentrations (ppb) in mussels at day t after the start of experiment, respectively; C_w = TBTC concentrations (ng/L) in seawater during the experiments (a constant); k_{w1} = accumulation constant of TBTC from seawater into mussels (mL g^{-1} day^{-1}); k_{12} = degradation constant (degrd const) of TBTC to T3CO in mussels (day^{-1}); k_{13} = degrd const of TBTC to T3OH in mussels (day^{-1}); k_{14} = degrd const of TBTC to T4OH in mussels (day^{-1}); k_{16} = degrd const of TBTC to DBTC in mussels (day^{-1}); k_{25} = degrd const of T3CO to D3CO in mussels (day^{-1}); k_{26} = degrd const of T3CO to DBTC in mussels (day^{-1}); k_{27} = degrd const of T3CO to D3OH in mussels (day^{-1});

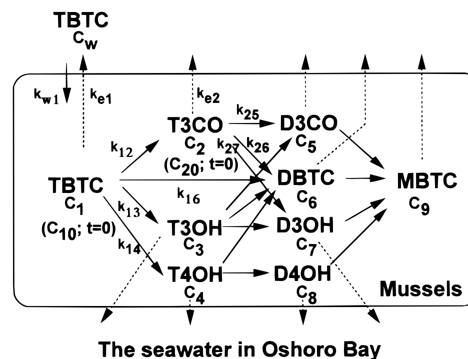

Figure 2. Models for the accumulation, metabolism, and depuration behavior of TBTC in mussels.

k_{e1} = elimination constant (elim const) of TBTC in mussels into seawater (day^{-1}); and k_{e2} = elim const of T3CO in mussels into seawater (day^{-1}).

When $k_{w1}C_w = k_0$ and $k_{12} + k_{13} + k_{14} + ... + k_{e1} = K_1$ (K_1 represents the sum of degradation constants of TBTC to metabolites in mussel tissue and an elimination constant of TBTC from mussel tissue to seawater.), eq 1 is reduced to

$$dC_1/dt = k_0 - K_1C_1 \quad (3)$$

(1) Accumulation Phase. Integration of eq 3 with boundary conditions of $C_1 = 0$ at $t = 0$ and C_w as a constant gives the following expression for tissue concentration in mussels as a function of time:

$$C_1 = (k_0/K_1)(1 - e^{-K_1t}) \quad (4)$$

At $t = \infty$, the steady-state expression is reduced to

$$C_1 = k_0/K_1 = k_{w1}C_w/K_1 = C_{ss}\ (t = \infty) \quad (5)$$

where C_{ss} is the TBTC concentration that has reached a state of equilibrium in mussel tissue. Accordingly, the steady-state tissue burden is the ratio of the accumulation constant multiplied by water concentration to a sum of degradation constants by metabolism and an elimination constant, and the steady-state bioconcentration factor (BCF_{ss}) is equal to k_{w1}/K_1. Values for K_1 and k_{w1} were calculated by a nonlinear least-squares regression analysis using a computer, and the value of C_w was obtained by actual surveillance. Biological half-life (half-life, $t_{1/2}$) was calculated by using the equation $t_{1/2}$ = ln $2/K_1$.

(2) Depuration Phase. Integration of eq 3 yields an expression for tissue concentrations as a function of time for elimination of TBTC from mussels

$$C_1 = A\,e^{-K_1t} + B \quad (6)$$

where $A = C_{10} - k_0/K_1$ [C_{10} = TBTC concentration (ppb) in mussels at t = 0], and $B = k_0/K_1$. The values of C_{10} and C_w were obtained by actual surveillance, and the values of K_1 and k_0 were determined by a nonlinear least-squares regression analysis. BCF_{ss} was determined by the equation $BCF_{ss} = B/C_w$. Half-life was calculated by using the equation $t_{1/2}$ = ln $2/K_1$.

For T3CO, the following relationship was obtained from eq 2:

$$dC_2/dt = k_{12}C_1 - K_2C_2 = k_{12}(A\,e^{-K_1t} + B) - K_2C_2 = K_3\,e^{-K_1t} + D - K_2C_2 \quad (7)$$

where $K_2 = k_{25} + k_{26} + ... + k_{e2}$, $K_3 = k_{12}A$, and $D = k_{12}B$.

Integration of eq 7 yields an expression for tissue concentrations as a function of time for elimination of T3CO from mussels:

$$C_2 = K_3/(K_2 - K_1)\ e^{-K_1 t} + (C_{20} - K_2/(K_2 - K_1) - D/K_2)\ e^{-K_2 t} + D/K_2 \quad (8)$$

where the values of C_{20} [T3CO concentration (ppb) in mussels at $t = 0$] were obtained by actual surveillance, and the values of K_2, K_3, and D were determined by a nonlinear least-squares regression analysis. Half-life was calculated by using the equation $t_{1/2} = \ln 2/K_2$.

TPTC, T3OH, and DPTC were analyzed in a similar manner as TBTC or T3CO, respectively.

RESULTS AND DISCUSSION

Identification of Accumulated Organotin Compounds in Mussels. Fish et al. (1976) showed that a TBT compound is metabolized in an in vitro system to hydroxylated and oxygenated products at a butyl moiety by P-450-dependent microsomal enzyme systems of rats. The authors also reported that TBTC and its related compounds absorbed into living organisms are subject to fairly rapid transformation in rats (Matsuda et al., 1993), fish (Ishizaka et al., 1989a; Suzuki et al., 1992; Yamamoto et al., 1997), or microorganisms in seawater (Suzuki et al., 1996). It is thought that a similar degradation pathway may also be present in mussels. The proposed metabolic patterns were simply summarized in Figure 2 by analogy with rats or fish, for which there is more information. Briefly, TBTC can be primarily transformed to di-*n*-butyl(1-hydroxybutyl)tin dichloride or di-*n*-butyl(2-hydroxybutyl)tin dichloride by 1- or 2-hydroxylation. However, these hydroxylated compounds at the 1-or 2-position of a butyl moiety are too unstable to be isolated and degraded to DBTC immediately after formation or during extraction (Fish et al., 1976). On the other hand, the products formed via oxidation at the 3- or 4-position of TBTC (T3OH, T4OH, and T3CO) are stable and can be isolated. Thus produced T3OH, T3CO, and T4OH can afford D3OH, D3CO, or D4OH by further 1- or 2-hydroxylation at an intact butyl moiety on them, respectively. T3OH, T4OH, and T3CO can also give DBTC by 1- or 2-hydroxylation at the oxygenated butyl moieties on them, followed by immediate elimination of 1- or 2-hydroxylated butyl moieties. It is expected that D3OH, D3CO, and D4OH can be subject to further 1- or 2-hydroxylation at the oxygenated butyl moiety to form MBTC and/or other unidentified polar products. Kinetic models shown in Figure 2 were designed depending on these stepwise debutylation pathways.

Confirmation and identification of organotin compounds in mussels were principally performed according to retention times (RTs) on a GC/MIP/AED system using three capillary columns (DB-5, HP-1, and DB-225) and an additional column (DB-1701) when necessary. TBTC and D4OH overlapped each other on HP-1, but DPTC and TCOOH were separated from each other. On the other hand, TBTC and D4OH were separated on DB-5, but DPTC and TCOOH were not. DB-225 was superior to DB-5 and HP-1 in separation and could analyze every organotin species on a single column, but it is unstable above 220 °C; that is, use at a low temperature caused inferior sensitivities and longer analytical times. A chromatographic peak was not accepted as the real peak unless it coincided on at least two columns. Confirmation of organotin compounds was further supplemented by the presence of four emission lines (300.914, 303.419, 317.505, and 326.234 nm) and their relative intensities as described in previous papers (Suzuki et al., 1994, 1996; Yamamoto et al., 1997).

A whole gas chromatogram on DB-225 from mussels after 56 days of culture at site A after being transferred from Oshoro bay to Aburatsubo Bay is shown in Figure 3 (site A), and enlargements of the gas chromatogram are shown in Figures 4 (4−7.5 min) and 5 (7.5−10 min). Each peak was monitored for the presence of tin emission lines and their relative intensities, and the peaks on the gas chromatogram were numbered in the order of short to long; those identified to be tin compounds (total of 14 peaks) are shaded.

Peaks 1, 4, 5, 13, and 14 (peaks 13 and 14 are not shown in Figure 4 or 5) were easily assigned to mono-*n*-butyltin trichloride (MBTC), di-*n*-butyltin dichloride (DBTC), tributyltin chloride (TBTC), diphenyltin chloride (DPTC), and triphenyltin chloride (TPTC), respectively, on the basis of the RTs on DB-225, and these assignments were also supported by the use of two other columns, DB-5 and HP-1. Two other small peaks (2 and 3) were observed in front of DBTC, but their concentrations were consistently too low to allow any comment on their origin. Peaks 6 and 7, which show the presence of emission lines characteristic of tin, were identified as *n*-butyl(3-oxobutyl)tin chloride (D3CO) and *n*-butyl(3-hydroxybutyl)tin chloride (D3OH). Peak 9 was identified as *n*-butyl(4-hydroxybutyl)tin chloride (D4OH) depending on the correspondence of this peak to the authentic standard on DB-225, DB-5, and DB-1701, although it could not be characterized on HP-1 since it overlapped with TBTC. The RT of the relatively strong peak 8 did not agree with those of any standard, and therefore further efforts to elucidate the structure were not made. Peaks 10 and 11 were identified as di-*n*-butyl(3-oxobutyl)tin chloride (T3CO) and di-*n*-butyl(3-hydroxybutyl)tin chloride (T3OH), respectively. The small peak 12, which appeared at 9.55 min in RT on DB-225, was identified as di-*n*-butyl(4-hydroxybutyl)tin chloride (T4OH) by further coincidence on DB-5, while the use of HP-1 or DB-1701 was less effective as a working column due to the presence of interfering peaks.

Time Course of Accumulation in Mussels. The concentration of TBTC and its degradation products DBTC and MBTC in seawater tended to be high in summer (August) and then to gradually decrease (Figure 6). This may be because the area surrounding the pier (site A) includes a marina and its boating activity is highest from July to August. These seasonal trends are consistent with the results of our earlier work in the same area (Suzuki et al., 1996). The relationships between TBTC levels in seawater and sources of pollution were also supported by the fact that TBTC levels in seawater are generally higher around site A than around the other sites. Site A is in close proximity to the marina. The next closest is the intermediate float (site B) and then the raft (site C), which is located farthest from the suspected source of organotin compounds and open to Sagami Bay with little or no restricted water circulation. These results indicate that the concentrations of TBTC decreased from the inner to the outer part of the marina coincide with the results of a previous study (Zuolian and Jensen, 1989) and that the marina constitutes a source of organotin contamination.

Accumulation of TBTC in mussels (*M. graynus*) at site A showed a peak around August 28 and then decreased

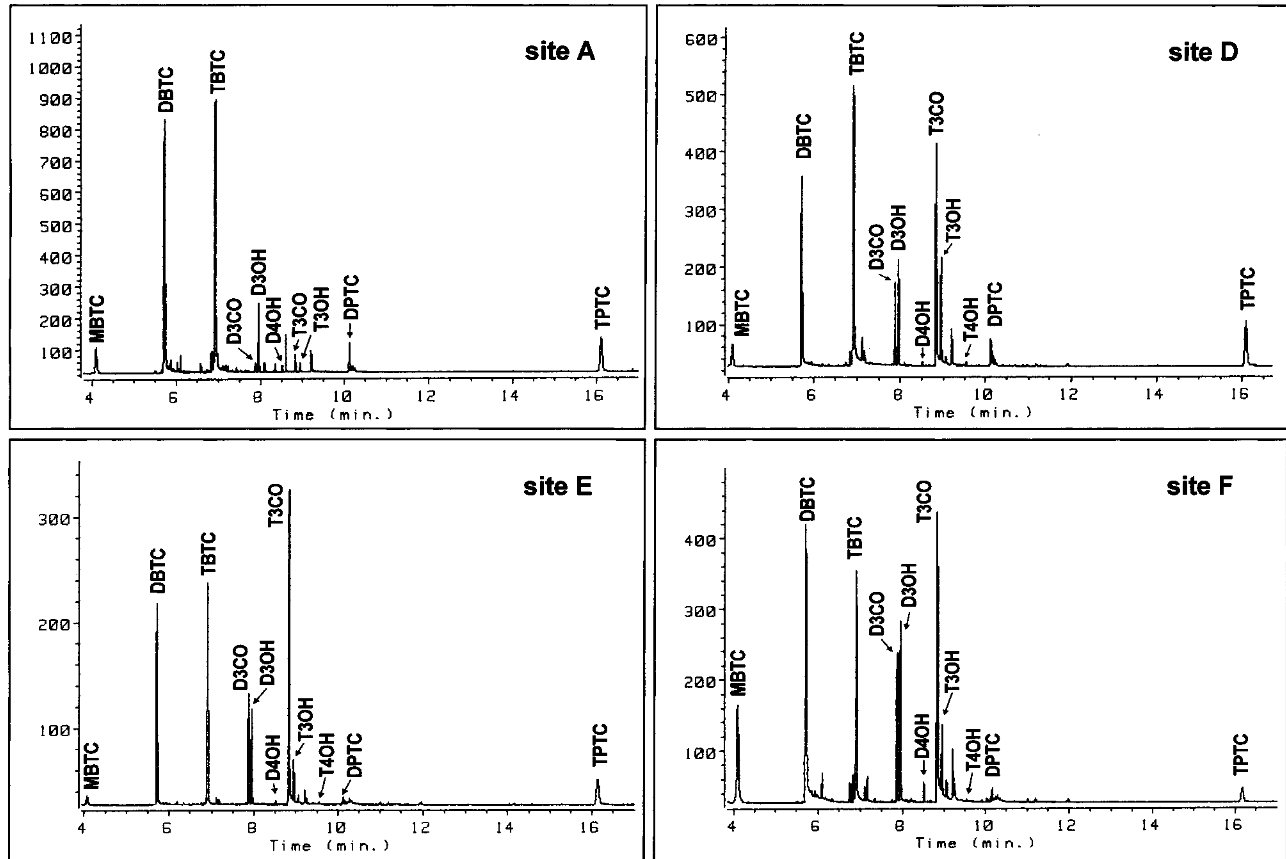

Figure 3. GC/MIP/AED chromatograms obtained from the mussel extracts at four sites on DB-225. Mussels *M. graynus* were collected at a noncontaminated area, site G (Oshoro Bay), transferred to a contaminated area, site A (Aburatsubo Bay), and reared for 56 days (site A). Mussels *M. edulis* were collected at site D in Moroiso Bay on August 21, 1993 (site D), at site E between the Miura Peninsula and Jogashima Island on August 2, 1993 (site E), and at site F in Manadzuru Bay on August 17, 1993 (site F).

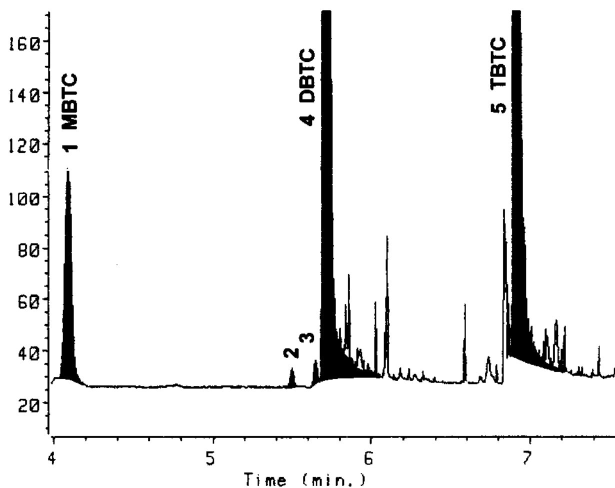

Figure 4. Enlargement of the GC/MIP/AED chromatogram (4–7.5 min) for site A in Figure 3.

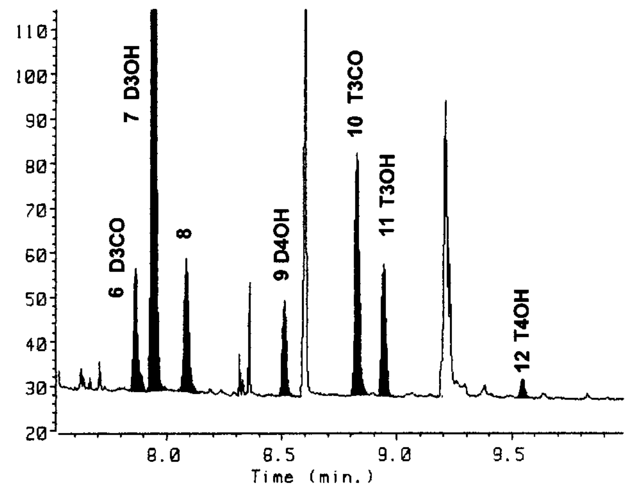

Figure 5. Enlargement of the GC/MIP/AED chromatogram (7.5–10 min) for site A in Figure 3.

slightly but again increased on October 7 (Figure 7) to a maximum concentration of ~2800 ppb. The same trends were also observed at sites B and C, and accumulated TBTC levels were in the order of sites A > B > C for the duration of the experiment. This result may more exactly reflect the exposure levels of mussels to TBTC than by water measurement as exemplified by a mussel watch program (Farrington et al., 1983). The TBTC level in seawater at site B was similar to that at site A on August 17 but thereafter approached the low levels at site C. In accordance with this trend, the levels of TBTC and its metabolites in mussels at site B increased from August 11 to 17 but thereafter approached the levels at site C more than those at site A. Most of the TBTC and TPTC homologues in mussels at site A increased linearly with time over the course of the first four sampling dates, when TBTC levels in seawater remained fairly stable. Furthermore, organotin metabolites such as MBTC, DBTC, D3OH and T3CO, which accumulated at relatively high levels in mussels, showed a relatively linear increase throughout the observation period at every site regardless of the

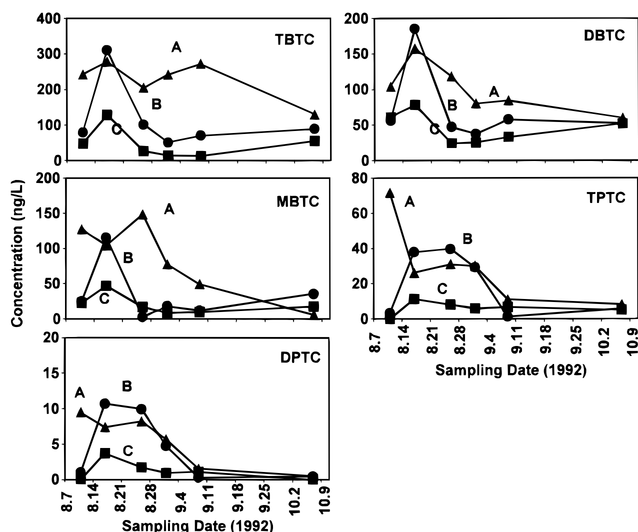

Figure 6. Seasonal variations in organotin compounds in the seawater at the three sites (A–C) in Aburatsubo Bay, Yokosuka City, Kanagawa Prefecture.

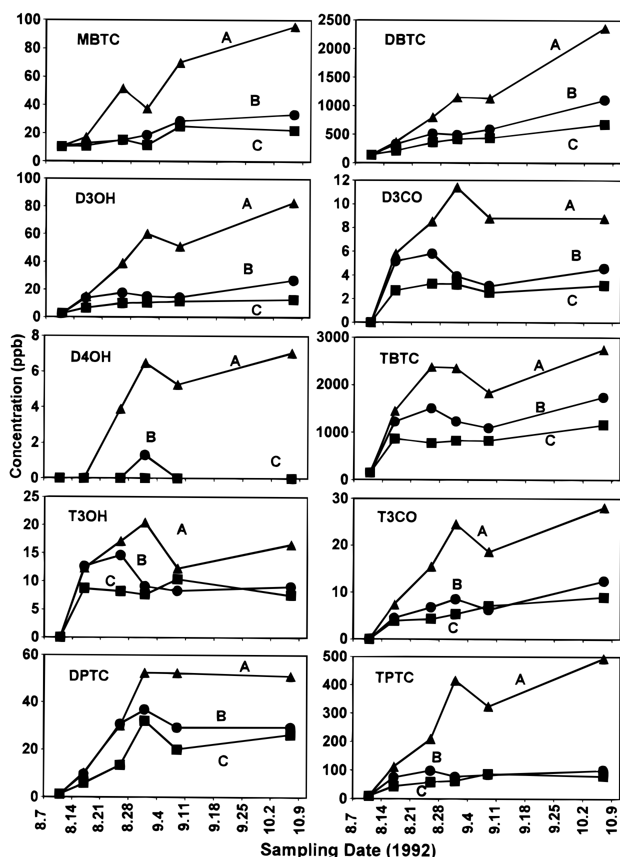

Figure 7. Time course of accumulation and metabolism of TBTC in mussels *M. graynus* at a contaminated area, site A (Aburatsubo Bay), after being transferred from a noncontaminated area, site G (Oshoro Bay, Otaru City).

nonlinear increase in TBTC. This phenomenon suggests that the TBTC level in mussels approached an approximate steady-state value and that the level reflected an integration of water column TBTC levels. It was thought that, as soon as mussels absorbed TBTC, they continuously transformed absorbed TBTC to these organotin metabolites but some of the metabolites in mussels still did not approach steady states. On the basis of seawater concentrations at relatively constant

Table 3. Kinetic Coefficients for Accumulation and Depuration Phases

	organotin compd				
coefficient	TBTC	T3CO	T3OH	TPTC	DPTC
Accumulation from Seawater (*M. graynus*)					
K_1 (day^{-1})	0.148	–	–	0.0107	–
k_{w1} (mL g^{-1} day^{-1})	1,560	–	–	–	–
C_w (ng/L)	242 ± 30	–	–	–	–
$t_{1/2}$ (days)	4.68	–	–	65	–
BCF_{ss} (mL g^{-1})	10500	–	–	43000	–
r^2	0.9913	–	–	0.9235	–
Depuration from Blue Mussels (*M. edulis*)					
K_1 (day^{-1})	0.1437	–	–	0.072	–
k_{w1} (mL g^{-1} day^{-1})	1,490	–	–	2,670	–
C_w (ng/L)	5.4 ± 3.0	–	–	0.8 ± 0.7	–
K_2 (day^{-1})	–	0.0852	0.1741	–	0.1126
$t_{1/2}$ (days)	4.82	8.13	3.98	9.63	6.15
BCF_{ss} (mL g^{-1})	10400	–	–	36000	
r^2	0.9744	0.9883	0.9642	0.9907	0.9373

[a] –, not determined.

levels from August 11 to September 1 (242 ± 30 ng/L, mean ± SD, $n = 4$) (Table 3), kinetic coefficients for the uptake for *M. graynus* were calculated by a nonlinear least-squares regression analysis in the uptake phase ($r^2 = 0.9913$). The half-life (4.68 days) and BCF_{ss} (10 500) in the uptake phase of TBTC in *M. graynus* were obtained.

TPTC and DPTC in the seawater showed relatively high levels on August 11 at site A but low levels at the other two sites (Figure 6), suggesting that pollution of seawater by TPTC occurred only recently, probably on the sampling day and near the sampling site. Thereafter, the TPTC levels at site A decreased and only very low levels were found in September and October, but the levels at the other two sites increased from mid-August to early September. These phenomena may reflect the relative geometrical relationships in environmental pollution among the three sites. The tissue burden of TPTC and DPTC in mussels increased at every site until September 1 and then remained at relatively constant levels (Figure 7).

Depuration of Organotin Compounds from Mussels. Figure 8 shows the exponential decline over time in concentrations of TBTC and its metabolites or TPTC and its metabolite DPTC from mussels that were collected at site D and transplanted to site G (Oshoro Bay). The order of tissue burden of the butyltin species on day 0 in the blue mussels *M. edulis* was TBTC > T3CO > DBTC > D3OH > D3CO > T3OH > MBTC. TBTC and D3OH levels in mussels decreased more rapidly than the other organotin species. The TBTC levels were higher at the start of the depuration experiment, comparable to after 3 days, and then less than those of T3CO. A similar relationship was also observed between D3OH and D3CO. Furthermore, the level of TBTC was lower than that of DBTC after 24 days, and the final order of tissue burden of the butyltin species, which remained in a steady-state equilibrium on about day 48 or later after the start of depuration experiment, was T3CO > DBTC > TBTC > D3CO > D3OH > MBTC > T3OH.

Kinetic coefficients of TBTC in the depuration phase were calculated by a nonlinear least-squares regression analysis in the depuration phase ($r^2 = 0.9744$). On the basis of these kinetic coefficients and the seawater concentrations (C_w) from August 19 to October 30 (5.4 ± 3.0 ng/L, mean ± SD, $n = 10$) (Table 3), half-life (4.82

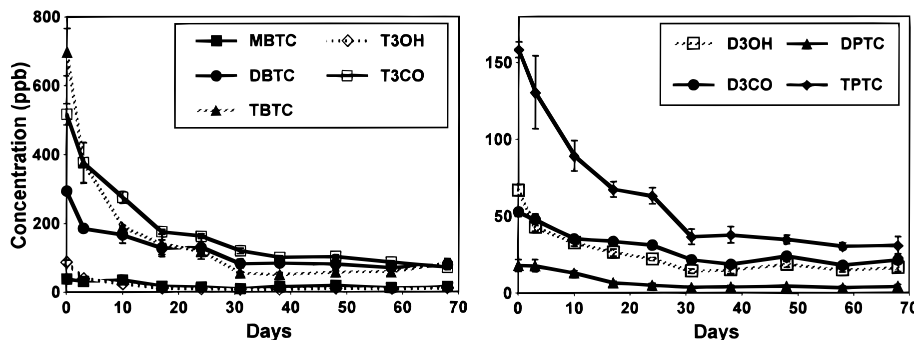

Figure 8. Depuration of organotin compounds from mussels *M. edulis* transferred from a heavily polluted site in central Japan, site D (Moroiso Bay), to a lightly polluted site in northern Japan, site G (Oshoro Bay, Otaru City).

days) and BCF_{ss} (10 400) were obtained. These values compare favorably with the half-life (4.68 days) and BCF (10 500) obtained from the accumulation phase of *M. graynus* despite species difference. However, the half-lives of TBTC in *M. edulis* and *M. graynus* were significantly shorter than those reported previously: 14 days under laboratory conditions (Laughlin et al., 1986) and 40 days under natural conditions (Zuolian and Jensen, 1989). The reported BCFs were 5 000 for TBTO (Laughlin et al., 1986) and 5 000−60 000 for TBTO at concentrations of 0.05−0.45 μg/L seawater (Zuolian and Jensen, 1989). Zuolian and Jensen showed that the value of BCF for TBTO in blue mussels is a function of the seawater concentration of TBTO; that is, BCF increases exponentially with decreases in the seawater concentration. When the concentration of TBTC in water (242 ± 30 ng/L) used in the present accumulation experiment was extrapolated to the exponential expression proposed by Zuolian and Jensen, a BCF value of ~15 000 was obtained for *M. graynus*. This value is in general agreement with the values of 10 400 and 10 500 that were obtained in the present accumulation and depuration phase experiments, respectively. However, there were consistent differences in the half-lives among our data and those by Laughlin et al. or those by Zuolian and Jensen. Although there is no substantial proof, this is probably due to the difference in temperature in the accumulation and depuration phases. There are still relatively few studies that contribute to a picture of the relationships between drug absorption by marine organisms and seawater temperature. Vieth et al. (1979) studied the effect of temperature on BCF for polychlorinated biphenyls (PCBs) in minnows, and they reported that significant differences in BCF are apparent between 5 and 25 °C but that there are not significant differences between 15 and 25 °C in the BCF values for PCBs in minnows and greensunfish. Yamada et al. (1996) also reported, from the results of an experiment using three kinds of fish, that BCFs for α-hexachlorocyclohexane were not affected by water temperatures of 20 and 25 °C, because a rise in the temperature caused a rise in the accumulation constant and also a rise in the depuration constant. Accordingly, the ratios of accumulation constant to depuration constant, i.e., BCFs, were not changed. On the other hand, the increases in depuration constants naturally resulted in the decreases in half-lives. The same is probably true for mussels in the present study. In addition, a higher temperature may entail a higher rate of metabolism for aquatic organisms if they are capable of metabolizing a given chemical and the temperature is within a suitable range for their lives. Consequently, it is thought that an increase in the seawater temperature will lead to a higher depuration rate in blue mussels, which are cold-blooded animals. In the experiment performed by Zuolian and Jensen (1989), there was no description of seawater temperature, but the accumulation experiment was started in late October in northern European cold weather. This might cause a low rate of metabolism in mussels due to the reason described above. As it is expected that mussels in the experiment by Zuolian and Jensen were raised at the same temperature as in the field experiment after they were collected and taken back to the laboratory, it might cause a delayed depuration, i.e., a long half-life such as 40 days. On the other hand, Laughlin et al. (1986) raised mussels at 18 ± 2 °C in a laboratory experiment and then in cages in San Francisco Bay for 6 months (it is not clear when the experiment was started and how high the temperature in San Francisco Bay was). In our present depuration study, the mean seawater temperatures at site G were 21 °C at the beginning of the experiment (August 21−31, 1993) and 14 °C at the end of the experiment (October 21−31, 1993). Therefore, it appears that differences in these conditions are reflected in the differences in half-lives, e.g., 40 days (Zuolian and Jensen), 14 days (Laughlin), and 4.82 days in the present study.

T3CO, a main metabolite, showed a longer half-life of 8.13 days, while T3OH, a minor metabolite, exhibited a shorter half-life of 3.98 days than that of the parent compound TBTC. Considering the exponentially decreasing curves in Figure 8, there seem to exist several rules: (1) Among the peak height ratios of T3CO/T3OH, D3CO/D3OH, TBTC/DBTC, and T3CO/TBTC, that of T3CO/T3OH appears ideally suited for estimation of input time because of the different half-lives of the two metabolites—a maximum half-life of T3CO and a minimum half-life of T3OH among the TBTC metabolites. Accordingly, the peak height ratio of T3CO/T3OH would significantly increase with time due to the differences in half-life in the depuration phase. (2) T3CO/TBTC ratios are at first <1 but later become >1 because TBTC decreases more rapidly than T3CO. (3) D3CO/D3OH ratios are at first <1 but become >1 due to a rapid decrease of D3OH. Considering that D3CO and D3OH are daughter molecules of T3CO and T3OH, respectively, the amounts of D3CO or D3OH should depend on the amounts of T3CO or T3OH, depuration constants (K_2) of T3CO or T3OH, and depuration constants of D3CO or D3OH, respectively. By assuming that depuration constants for D3CO and D3OH are provisionally equal, an increased ratio of D3CO/D3OH should be observed with time as shown in Figure 8. (4) T3CO/

DBTC ratios were about 5:3 at the beginning of the experiment but later approached 1 or became <1 as shown in Figure 8. However, DBTC is located at the central position of biological degradation of TBTC as shown in Figure 2. When the TBTC level is high, therefore, the DBTC level is strongly dependent on the TBTC level, but it later becomes dependent on the T3CO level, which remains at a higher level. Accordingly, it is expected that the formation of and decrease in DBTC are more complicated compared with those of TBTC, T3CO, and T3OH.

These different degradation rates among metabolites may produce different metabolic patterns depending on the sampling date, help to assess approximate TBTC input time, and estimate the degree of contamination. For example, the GC/MIP/AED gas chromatograms of extracts from *M. edulis* sampled at Jogashima Island (site E), Manadzuru Bay (site F), and Moroiso Bay (site D) and that of *M. graynus* (site A), which was collected at Oshoro Bay (site G), transferred to site A in Aburatsubo Bay and then cultured for 56 days, are shown and compared in Figure 3. *M. edulis* collected at site E showed relatively low levels of TBTs compared with the other sites. The mussels collected at site F showed relatively low levels of TBTC regardless of the high level of T3CO. In samples collected at site D, the TBTC peak was highest, followed by T3CO. As shown in Figure 3, the peak height ratios of T3CO to T3OH increased in the order of sites D, F, and E. For simplicity, assuming that only one input of TBTC occurred in the surrounding seawater environment, Figure 3 indicates that contamination by TBTC at site D occurred only recently and that those at sites E and F were relatively old. This was also supported by larger DBTC/TBTC or T3CO/TBTC peak height ratios at sites E and F than those at site D. Next, a comparison of mussels from sites E and F showed that the T3CO/T3OH peak height ratio at site E was larger than that at site F, suggesting that contamination at site E is older than that at site F. This was also supported by the smaller T3CO/D3OH and T3CO/D3CO peak height ratios at site F than at site E, although the T3CO/DBTC peak height ratio showed a value less close to 1 at site E than at site F. The higher ratio of T3CO/TBTC at site E than at site F also supports this order. Furthermore, the peak height level of D3CO exceeded that of D3OH only at site E among sites D−F. This suggests that the sample from site E is the oldest of the three, but the difference seems too small to state with confidence. In conclusion, it is thought that pollution at site E proceeded that at site D by 6−8 days and that the pollution time at site F is between those at site D and E. In addition to this, the higher levels of T3CO and DBTC in samples at site F than at site D indicates that contamination by TBTC at site F in the past was higher than that at site D despite the fact that the TBTC level at site D on the sampling date was higher than that at site F. The TBTC concentrations at sites A, D, E, and F in Figure 3 accounted for only 61.5, 39.5, 17.2, and 13.2% of total organotin compounds on the sampling date in each site, respectively. These observations also suggest that the TBTC concentration in mussels is not correlated with those of metabolites.

Although, as described above, there were only small differences in the composition of organotin compounds among the samples collected at sites D−F, much larger differences were observed between these three samples and those from Oshoro Bay, which were transferred and cultured for 56 days at site A (Figure 3, site A). In general, higher peak height ratios of T3CO, T3OH, D3CO, and D3OH to DBTC were observed in *M. edulis* in contrast to *M. graynus* as shown in Figure 3. For these phenomena, usually two reasons can be mentioned: (1) a difference in the metabolic activity between *M. edulis* and *M. graynus,* i.e., species difference; and (2) adaptation or induction of P-450-dependent drug metabolizing enzyme system in the blue mussels by contact with contaminants. Becker van Slooten and Tarradellas (1994) reported that mussels cultured in cages for accumulation contained more than twice the level of TBTC of mussels living naturally in the same marina due to reason 2. In the present study, however, the transferred and cultured mussels of *M. graynus* showed half-lives similar to those of *M. edulis* and almost equal K_1 values. Therefore, a large difference in the potential of metabolic activities does not seem to exist between the two species. Consequently, it is reasonable to assume that the differences in metabolic pattern are due to species difference in marine mussels, i.e., metabolic difference in the positions of hydroxylation of the butyl moiety on TBTC. Unfortunately, the mussels used for the accumulation experiment were not the same as those used for the depuration experiment, because the authors could not get *M. edulis* at site G, which is a relatively uncontaminated area and under well established experimental conditions. More experiments will be needed for further detailed discussion.

The TPTC levels in mussels were in the order site D > site E > site F, and this order was different from that of TBTC, which is in the order site D > site F > site E. The half-life and BCF of TPTC calculated for the depuration phase in blue mussels of *M. edulis* were 9.63 days and 36 000, respectively, and these values were not consistent with the values calculated for the accumulation phase of *M. graynus*, especially in half-life (Table 1). The longer half-life of TPTC compared with TBTC indicates that TPTC is more resistant to biological degradation by enzyme systems in mussels than is TBTC, which has three alkyl groups highly susceptible to biological oxidation. On the other hand, DPTC, a degradation product of TPTC, showed a shorter half-life (6.15 days) in the depuration phase.

In conclusion, it has been thought that bivalves have low or undetectable enzyme activity that metabolizes xenobiotics, and this idea has led to the use of bivalves as a sentinel organism for pollutants (Farrington et al., 1983; Laughlin et al., 1986). On the contrary to this assumption, TBTC was rapidly metabolized after being accumulated. Di-*n*-butyl(3-oxobutyl)tin chloride, which is a main metabolite of TBTC, showed a longer half-life than that of the parent compound. The different half-lives among TBTC and its metabolites are responsible for different metabolic patterns in blue mussels at each sampling time, and the high body burdens of these metabolites in mussels, which have been outside the scope of investigation, will be able to explain the differences between the results of total and organic tin analyses.

ABBREVIATIONS USED

TBTs, tributyltin compounds; BCF_{ss}, steady-state bioconcentration factor; TPTs, triphenyltin compounds; TBTO, bis(tri-*n*-butyltin) oxide; GC/MIP/AED, gas chromatography/helium atmospheric pressure microwave-

induced plasma/atomic emission detection system; AED, atomic emission detector; Et_2O, diethyl ether; MeCN, acetonitrile; MeMgBr, methylmagnesium bromide; EtMgBr, ethylmagnesium bromide; PCBs, polychlorinated biphenyls; $t_{1/2}$, biological half-life (half-life); other abbreviations are given in Table 1.

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