ELSEVIER

Environmental Pollution 120 (2002) 245–253

ENVIRONMENTAL POLLUTION

www.elsevier.com/locate/envpol

# Evaluation of mitogen-induced responses in marine mammal and human lymphocytes by in-vitro exposure of butyltins and non-*ortho* coplanar PCBs

H. Nakata[a,*], A. Sakakibara[b], M. Kanoh[c], S. Kudo[d], H. Watanabe[e], N. Nagai[f], N. Miyazaki[g], Y. Asano[c], S. Tanabe[b]

[a]*Graduate School of Science and Technology, Kumamoto University, Kurokami 2-39-1, Kumamoto, 860-8555 Japan*
[b]*Center for Marine Environmental Studies, Ehime University, Tarumi 3-5-7, Matsuyama, 790-8566 Japan*
[c]*Department of Microbiology and Immunology, Ehime University School of Medicine, Shigenobu-cho, Onsen-gun, Ehime, 791-0295 Japan*
[d]*Marine World Umino-Nakamichi, Saitozaki 18-28, Higashi-ku, Fukuoka, 811-0321 Japan*
[e]*Awashima Marine Park Co., Ltd., Shigedera 186, Uchiura, Numazu, Shizuoka, 410-0221 Japan*
[f]*Japan NUS Co., Ltd., Loop X Bldg., 8F, Kaigan 3-9-15, Minato-ku, Tokyo, 108-0022 Japan*
[g]*Otsuchi Marine Research Center, Ocean Research Institute, University of Tokyo, 028-1102 Japan*

Received 13 August 2001; accepted 25 January 2002

**"Capsule":** *Butyltins may affect the immune response in marine mammals.*

**Abstract**

The effects of exposure to butyltin compounds (BTs: tributyltin; TBT, dibutyltin; DBT and monobutyltin; MBT) and non-*ortho* coplanar PCBs (IUPAC 77, 126 and 169) on marine mammals and human lymphocyte were evaluated. Peripheral blood mononuclear cells (PBMCs) isolated from Dall's porpoises (*Phocoenoides dalli*), bottlenose dolphins (*Tursiops truncatus*), a California sealion (*Zalophus californianus*), a larga seal (*Phoca largha*) and humans (*Homo sapiens*) were exposed at varying concentrations of BTs and coplanar PCBs. Concanavalin A (Con A)-stimulated mitogenesis found significantly suppressed ($P<0.01$) when the cells were exposed at 300 nM (89 ng/ml) of TBT and 330 nM of DBT (77 ng/ml), while MBT showed little cytotoxicity at treatment levels of up to 3600 nM (620 ng/ml). BTs concentrations in the liver of Dall's porpoises from Japanese coastal waters ranged between 81–450 ng/g for TBT and 200–1100 ng/g (wet wt.) for DBTs, which is greater than the cytotoxic levels registered in this study. In contrast, non-*ortho* coplanar PCBs did not suppress cell proliferation at concentrations of up to 30 nM (10 ng/ml). The residue levels of coplanar PCBs in the blubber of Dall's porpoises were 0.12–1.3 ng/g, which were one order of lower than those levels that do cell proliferation. When cells were exposed to a mixture of TBT/DBTand coplanar PCBs, the proliferation was significantly reduced to 33 nM DBT plus 34 nM CB-77 and 33 nM DBT plus 28 nM CB-169 mixtures, respectively. The investigations relating the contaminant-induced immunosuppression in marine mammals have been focused on persistent organochlorines such as PCBs, pesticides and dioxin compounds. However, this study suggested the possibility of BTs could also pose a serious threat to the immune functions in free-ranging marine mammals and humans. 

*Keywords:* Immunosuppression; Peripheral blood mononuclear cells (PBMCs); Mitogen-induced response; Marine mammal; Butyltins; Non-*ortho* coplanar PCBs

## 1. Introduction

During the last two decades, approximately seven serious mass mortalities of marine mammals have been reported (Simmonds, 1992). Nearly, 20,000 harbour seals and several hundred grey seals were found dead in the North Sea in 1988 (Dietz et al., 1989). Similarly, mass mortalities of 2500 bottlenose dolphins and 8000 Baikal seals were found in the US east coast waters (Kuehl et al., 1991) and in the Lake Baikal (Grachev et al., 1989), respectively. When we consider such mortalities, three common features were observed to all these incidents. First, the animals were severely infected

* Corresponding author. Tel./fax: +81-96-342-3380.
*E-mail address:* nakata@aster.sci.kumamoto-u.ac.jp (H. Nakata).

0269-7491/02/$ - see front matter 
PII: S0269-7491(02)00155-0

by virus. Secondly, most of them occurred near industrial regions or semi-closed ecosystems. Thirdly, elevated concentrations of persistent toxic contaminants were found in the organs of the affected animals.

Immunotoxicological studies have been conducted with marine mammals such as harbour seals under semi-field conditions. Immune parameters such as T cell mitogen response, NK cell activity and delayed-type hypersensitivity have been investigated, and found that the contaminant levels in free-ranging harbour seals inhabiting polluted areas may be high enough to cause immunosuppression (Swart et al., 1994, Ross et al., 1995). Similarly, Shaw (1998) documented the impairment of immune functions in Pacific harbour seal pups by the accumulation of non- and mono-*ortho* coplanar PCBs and *p,p′*-DDE. In in-vitro assays, exposure to a mixture of PCB congeners (CB-138, CB-153 and CB-180: 5 ppm each) significantly reduced splenocyte proliferation in beluga whales, which were in the range of those found in the tissues of wild marine mammals (De Guise et al., 1998). Considering all these observations, it is likely that the chemical pollution cannot be ruled out as a cause of debilitating diseases and mass mortality that has occurred in the past.

In the experimental studies, butyltins (BTs) showed strong immunotoxicity, such as thymus atrophy, reduction in spleen weight and cytotoxicity to bone marrow and red blood cells at relatively low exposure concentrations (Boyer, 1989). Seinen and Penninks (1979) reported that $^{3}$H-thymidine incorporation into DNA in the rat thymus cells decreased at a concentration of 20 ng/ml DBT chloride, and it was completely inhibited at the concentration of 100 ng/ml DBT-Cl in the culture medium. Because BTs have been detected at parts per million levels in coastal cetaceans (Kannan et al., 1997; Tanabe et al., 1998), it is assumed that immunosuppression might have occurred in marine mammals. Based on these backgrounds, we examined the effects of the in-vitro exposure to BTs and coplanar PCB congeners on peripheral blood mononuclear cells (PBMCs) of marine mammals in this study. The human lymphocytes were also applied to this experiment to compare the results of immunological assay between marine and terrestrial mammals.

## 2. Experimental

### 2.1. Sample collection

Thirteen Dall's porpoises were collected from Off Sanriku, the northeast coast of Japan, during January and February 1998 (Fig. 1). Details of the samples used in this study were shown in Fig. 1 and Table 1. The liver and blubber samples were packed in polyethylene bags and stored at −20 °C until chemical analysis. The blood samples from three Dall's porpoises were collected in heparinized plastic tubes within 10 min following death of the animal, and kept under the ice (less than 4 °C) prior to the lymphocyte stimulation assay. The fresh blood samples were also collected from two bottlenose dolphins, one California sealion and one

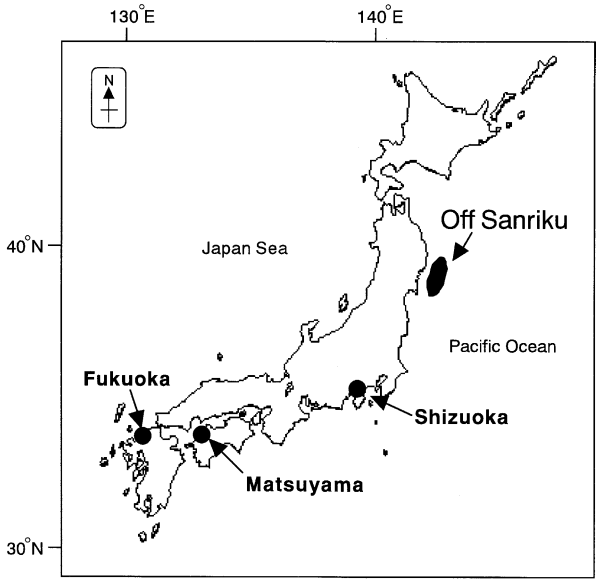


Fig. 1. Sampling sites of blood and tissue samples of marine mammals and humans.

Table 1
Details of samples analysed in this study

| Species | Date of collection (1998) | Location | *n* | Sex[a] | Tissues analysed[b] |
|---|---|---|---|---|---|
| Dall's porpoise (*Phocoenoides dalli*)[c] | January–February | Off Sanriku | 13 | M: 10, F:3 | Liver and blubber |
| Dall's porpoise (*Phocoenoides dalli*)[c] | January–February | Off Sanriku | 3 | M: 3 | Blood[d] |
| Bottlenose dolphin (*Tursiops truncates*) | July–August | Aquarium (Fukuoka) | 2 | M: 2 | Blood[d] |
| California sealion (*Zalophus californianus*) | July | Aquarium (Shizuoka) | 1 | M: 1 | Blood[d] |
| Larga seal (*Phoca largha*) | July | Aquarium (Shizuoka) | 1 | M: 1 | Blood[d] |
| Human (*Homo sapiens*) | April | Matsuyama | 2 | M: 2 | Blood[d] |

[a] M (male); F (female).
[b] Liver and blubber were analysed for butyltins and PCBs, respectively.
[c] The blood samples of three Dall's porpoises were analysed for lymphocyte proliferation assay.
[d] Fresh samples (see Section 2).

larga seal housed in aquariums (Awashima Marine Park, Shizuoka and Marine World Umino-Nakamichi, Fukuoka). These samples were taken by venipuncture of the extradural veins and approximately 20–40 ml of whole bloods were collected from each animal. Additionally, two human blood samples (30 ml) were collected from volunteers with consent at School of Medicine, Ehime University.

### 2.2. Cell culture

Lymphocyte stimulation assays were carried out within 35 h of blood collection because mitogen-induced proliferation responses significantly decrease after that period (data not shown). The PBMCs were isolated by density gradient centrifusion on Ficoll-Histopaque-1077 (Sigma, USA) for 30 min at 400 ***g*** under 4 °C. The PBMC layer was washed three times and resuspended in complete RPMI containing 10% heat-inactivated FCS (fetal calf serum), 100 unit/ml penicillin and 100 μg/ml of streptomycin, 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate and 50 μM 2-mercaptaethanol. Con-A (Wako Pure Chemical, Japan) was used to stimulate the PBMCs. The PBMCs adjusted to $1\times10^5$ cells/well, and Con-A (5 μg/ml) and varying concentrations of test chemicals were cultured in 96-well round-bottom culture plate at 37 °C in a humidified atmosphere of 5% $CO_2$. TBT–Cl, DBT–Cl, MBT–Cl and non-*ortho* coplanar PCBs, CB-77, 126, 169 were mixed into the culture medium at four to five concentrations, ranging from 0.30 to 3600 nM (0.062–890 ng/ml) of $BT^+$ and 2.8 pM–34 nM (0.001–10 ng/ml) of the PCB congeners, respectively. All compounds were dissolved in DMSO, and the final DMSO concentrations were prepared to be less than 0.5% in the medium. The PBMCs were incubated for 3 days for the human, California sealion and larga seal cells, and 4 days for the cetacean cells. $^3$H-thymidine (0.5 μCi/well) was added at the last 24 h of the cultivation. The cells were then filtered using a cell harvester, and the associated radioactivity was measured by liquid scintillation counter. A medium concentration containing 0.5% DMSO alone was used as control. The raw data were expressed as disintegration per minute (DPM), triplicates were averaged, and the results were presented as the relative percentage to the control.

### 2.3. Chemical analysis

PCBs and butyltin compounds (TBT, DBT and MBT) were analysed in the blubber and liver tissues of Dall's porpoises. PCB congeners including non-*ortho* coplanar homologues (IUPAC 77, 126 and 169) were determined by the method of Wakimoto et al. (1971) and Tanabe et al. (1987) with some modifications. Briefly, 3–5 g blubber was homogenized with anhydrous $Na_2SO_4$ and Soxhlet-extracted using diethyl ether:hexane (3:1) for 7 h. The fat content was determined after K-D (Kuderna-Danish) concentration of the extracts. An aliquot of the Soxhlet extract was refluxed in 1 N KOH-ethanol, followed by re-extraction into hexane, and purification by silica gel column chromatography. The eluates were concentrated and an aliquot was used for the determination of the PCB congeners except for the non-*ortho* coplanar PCBs. The remaining hexane extract was passed through a glass column packed with 125 mg activated carbon for separation of non-*ortho* coplanar PCBs (IUPAC 77, 126 and 169) from the other congeners.

Determination of total PCBs and non-*ortho* coplanar congeners were performed by a gas chromatograph with a mass selective detector (GC–MSD: Hewlett Packard 5890 Series II-5972 Series). A fused silica capillary column (30 m×0.25 mm i.d.) coated with DB-1 (100% dimethyl polysiloxane, J&W Scientific, Folsom, CA) at 0.25 μm film thickness was used for the quantification. The PCB homologues were identified by selective ion monitoring (SIM) at $m/z$ values of 254 and 256, 290 and 292, 324 and 326, 358 and 360, 392 and 394, 428 and 430 for the tri-, tetra-, penta-, hexa-, hepta- and octa-chlorobiphenyls, respectively. An equivalent mixture of Kanechlor 300, 400, 500 and 600 was used as the standard. PCB congeners were quantified from individual peak areas in the samples with the corresponding peak areas in the standard. Recoveries of PCBs and non-*ortho* coplanar congeners were examined by spiking 3.0 μg of total PCBs, 90 ng for CB-77, 91 ng for CB-126 and 30 ng for CB-169 into corn oil, and the percentages were 91, 113, 114 and 88%, respectively. The detection limits for the total PCBs and coplanar congeners were 0.1 and 0.03 ng/g, wet weight, respectively.

Butyltins were analysed by the method of Iwata et al. (1995) with some modifications. The liver tissues (1–2 g) were homogenized with 1 N HCl and 0.1% tropolone/acetone, and centrifuged at 3000 rpm for 15 min. The supernatant was transferred to 0.1% tropolone/benzene, and the moisture was removed by anhydrous $Na_2SO_4$. The BTs were propylated by adding propylmagnesium bromide as a Grignard reagent, and the excess of this reagent was decomposed by adding 1 N $H_2SO_4$. The extracts containing the BTs were passed through a Florisil packed wet column for cleanup. The BTs were measured using a gas chromatograph with a flame photometric detector (GC–FPD: Hewlett Packard Series II) and a fused silica capillary column (DB-1, described earlier). Monobutyltin trichloride, dibutyltin dichloride, tributyltin chloride of known amounts (0.16 μg each) were spiked into the liver of an Antarctic minke whale, containing trace levels of BTs for use as the external standard. Hexyltributyltin was added as the internal standard. A procedural blank was analysed with every set of six samples to check for interfering

compounds and to correct the sample values, if necessary. The average recovery rates of the BTs were 91±14, 121±14 and 115±5.2% for MBT, DBT and TBT, respectively. All the BTs concentrations in this study were presented as the corresponding ion, and they were not corrected for the recovery of the internal standard. The detection limits of MBT, DBT and TBT were 15, 4.0 and 2.0 ng/g wet weight, respectively.

### 2.4. Statistical analysis

For the proliferation assay, triplicates were averaged, and the mean and standard deviation were determined. A one-way analysis of variance (ANOVA) with Dunnett's test was used to compare the different experimental groups to the control, using $P<0.01$ (#) for statistical significance.

## 3. Results and discussion

### 3.1. Cytotoxic levels of lymphocytes

An evaluation of mitogen-induced proliferation has been conducted in marine mammals and human lymphocytes using Con-A, PHA (Phytohaemagglutinin-M), PWM (Pokeweed mitogen) and LPS (Lipopolysaccharide from *Salmonella typhimurium*). Among them, Con-A, PHA and PWM were relatively active in the lymphocytes proliferation of these animals. Particularly, Con-A proved to be a most powerful mitogen (data not shown).

The proliferation activity of three Dall's porpoises PBMCs in the presence of butyltin compounds and non-*ortho* coplanar PCBs were shown in Table 2. A significant decrease in the percentage of lymphocyte proliferation was found at concentrations of 300 nM for TBT (89 ng/ml) and 330 nM for DBT(77 ng/ml). The exposure to MBT resulted to a less significant decline at higher concentration levels (3600 nM; 620 ng/ml MBT), implying that MBT showed to less toxic than DBT and TBT. Similar observations were also reported in the values of $LD_{50}$ among the BTs (TBT–Cl, 122–349 mg/kg, DBT–Cl, 112–219 mg/kg, MBT–Cl, 2200–2300 mg/kg; Snoeij et al., 1987). On the other hand, the suppression of lymphocyte proliferation did not observe with the exposures of non-*ortho* coplanar PCBs (2.8 pM–3.4 nM; 0.001–1 ng/ml) in Dall's porpoises (Table 2). The proliferation activity of bottlenose dolphins when cultured with PCBs ($n=1$) and BTs ($n=2$) are presented in Fig. 2. All of the PCB congeners were less effective at concentrations of 2.8 pM–34 nM (0.001–10 ng/ml) in the culture medium. In contrast >300 nM of TBT and DBT prevented the proliferation completely, which is in accord with the results of Dall's porpoises. Besides, the effects on proliferation by the BTs and coplanar PCBs were also examined in a larga seal, a California sealion and humans. The results also showed significant suppressions in the cell proliferation when exposed to TBT and DBT concentrations at >300 nM, while exposure to MBT and coplanar PCBs has less effect (Figs. 3 and 4).

In the experimental studies, following the in-vitro BT treatment to immune cells, $^3$H-thymidine incorporation into the DNA of rat thymus cell has decreased at a concentration of 20 ng/ml of DBT–Cl, and was completely inhibited at a concentration level of 100 ng/ml (Seinen and Penninks, 1979). Whalen et al. (1999) suggested that TBT inhibited the tumour-killing capacity of NK cells at 200 nM. TBT concentration of 100 nM or greater in rat thymocytes increased the population of annexin V-positive live cells, suggesting the possibility of TBT-induced apotosis or/and direct affects to cell membranes (Nakata et al., 1999). These inhibition levels were more or less similar to those levels obtained in this study. Although it is difficult to identify the cytotoxic

Table 2
PBMCs proliferation of Dall's porpoises cultured with Con A and different concentrations of butyltin chlorides and non-*ortho* conlanar PCBs[a]

| Sample No. | Control[b] | Tested conc. (nM) | 3.0–3.6 | 30–36 | 300–360 | 3000–3600 | Tested conc. (pM) | 2.8–3.4 | 28–34 | 280–340 | 2800–3400 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| DP-9 | 30,786 | TBT | 33,285 | 38,520 | 1270[c] | 1983[c] | CB-77 | 29,939 | 26,533 | 33,823 | 32,689 |
| | | DBT | 28,860 | 27,398 | 1310[c] | 1478[c] | CB-126 | 30,163 | 24,660 | 33,041 | 27,583 |
| | | MBT | 27,697 | 34,059 | 34,712 | 20,832 | CB-169 | 29,416 | 27,761 | 32,586 | 31,755 |
| DP-10 | 28,543 | TBT | 28,992 | 22,798 | 1911[c] | 1688[c] | CB-77 | 27,973 | 25,146 | 23,168 | 22,097 |
| | | DBT | 27,857 | 22,870 | 1419[c] | 1155[c] | CB-126 | 27,825 | 21,349 | 22,294 | 23,793 |
| | | MBT | 26,573 | 28,379 | 24,686 | 14,924 | CB-169 | 28,306 | 27,356 | 26,572 | 23,093 |
| DP-11 | 37,015 | TBT | 36,599 | 40,201 | 2000[c] | 2089[c] | CB-77 | 33,146 | 30,756 | 35,217 | 36,187 |
| | | DBT | 38,222 | 34,436 | 3537[c] | 1921[c] | CB-126 | 34,999 | 33,159 | 35,857 | 34,598 |
| | | MBT | 38,072 | 35,358 | 34,082 | 29,129 | CB-169 | 37,089 | 39,204 | 31,752 | 39,278 |

PBMC, peripheral blood mononuclear cell; TBT, tributyltin; DBT, dibutyltin; MBT, monobutylin.
[a] Results are expressed in disintegrations per minute (dpm).
[b] 0.5% DMSO.
[c] $P<0.01$.

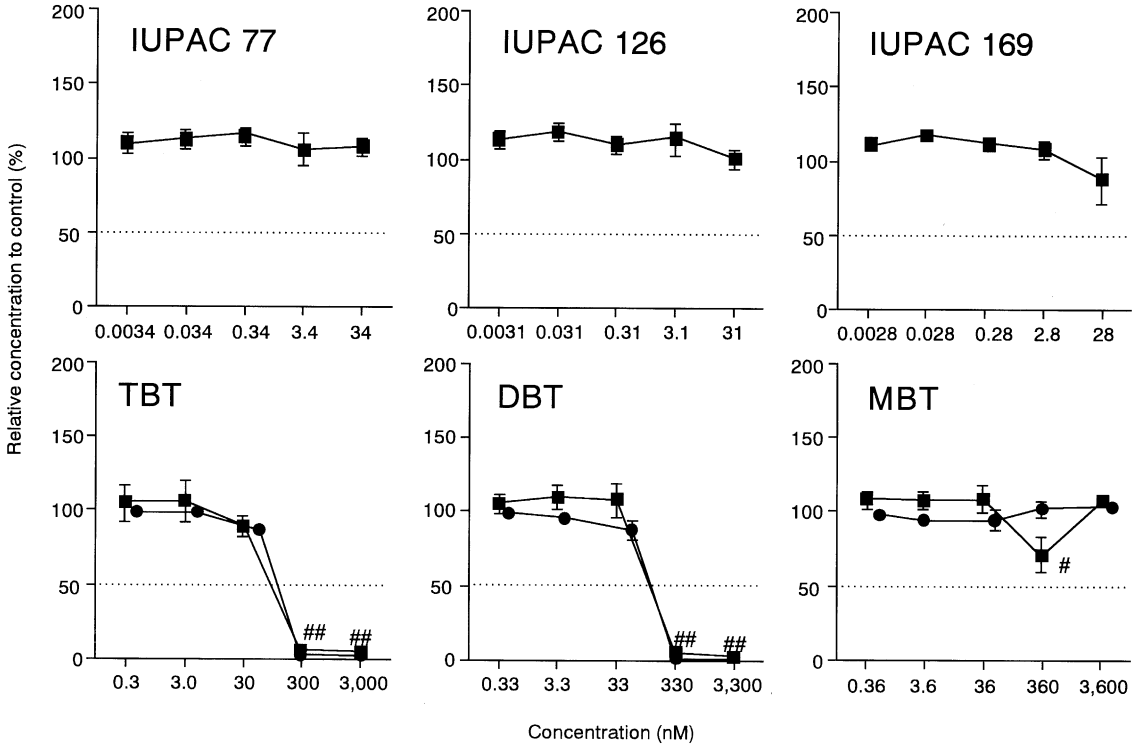


Fig. 2. Proliferation response of peripheral blood mononuclear cells (PBMCs) in two bottlenose dolphins following the treatment with non-*ortho* coplanar PCB congeners and butyltins. #: $P<0.01$.

mechanisms of PBMCs based on the present study, the effective concentrations of BTs to immune cells are in the range of several tens ppb of TBT and DBT, and its levels were less variable among mammalian species.

In-vitro studies of the exposure to PCBs congeners (IUPAC 138, 153, 180, 169) to beluga whale splenocytes showed reduced proliferation at concentrations of 20 and 25 ppm CB-138 (De Guise et al., 1998). Surprisingly, PCB 169 did not show any response to the exposed levels of 5–25 ppm, which are 500–2500 times greater than those tested levels. The lack of immunotoxicity of coplanar PCB congeners might be due to the possible low expression of the Ah receptor of beluga whale (Hahn et al., 1994). Few in-vivo studies suggested that the administration of 10 mg/kg CB-77 for 2 days prior and 2 days after immunization with SRBC (sheep erythrocytes) significantly decreased the percentage of thymus weight to body weight in C57BL/6 mice (Silkworth and Grabstein, 1982). Dose dependent inhibition of the splenic PFC (plaque-forming cell) response were observed when coplanar congeners such as CB-77, CB-126 and CB-169 were individually injected into B6C3F1 mice at concentrations of 120, 1.2 and 2.4 ng/g, respectively (Harper et al., 1995).

To assess any effects of combined chemicals, lymphocytes of bottlenose dolphins were exposed to a mixture of BTs and PCB coplanar congeners, and compared with the unexposed control (Fig. 5). When cells were exposed to mixtures of 30 nM TBT and 33 nM DBT and 2.8 pM–34 nM PCBs, proliferation was significantly reduced in mixtures of 33 nM DBT, and 34 nM CB-77 and 28 nM CB-169. In contrast, the mixture of 33 nM DBT and 31 nM CB-126 increased the proliferation. A mixture consisting of 5 ppm each of CB-138, 153 and 180 significantly reduced the splenocytes proliferation when compared with that of the control (De Guise et al., 1998). On the other hand, in-vitro exposure of rat leukocytes to low levels of methylmercury, PCDDs/DFs and PCBs mixtures had no suppressive effects on the immune functions analysed (Omara et al., 1998). While investigation should be conducted to examine any possible synergistic and/or antagonistic effects by mixture of these contaminants, this study might suggest the possibility of synergistic effects by the butyltins and coplanar PCBs in marine mammals.

### *3.2. Residue levels of PCBs and BTs in Dall's porpoises and their implications for immunotoxicological potentials*

PCBs and BTs were detected in the blubber and liver samples from all Dall's porpoises, respectively (Table 3). PCBs concentrations in Dall's porpoises were 6700± 1500 ng/g wet weight, and found that IUPAC-153 was the most prevalent congeners, followed by CB-138 and 180. CB-118 and 105 were predominant among mono-*ortho* PCB congeners, which were almost similar to those of other marine mammals (Minh et al., 2000). Non-*ortho* coplanar PCBs were detected with the mean

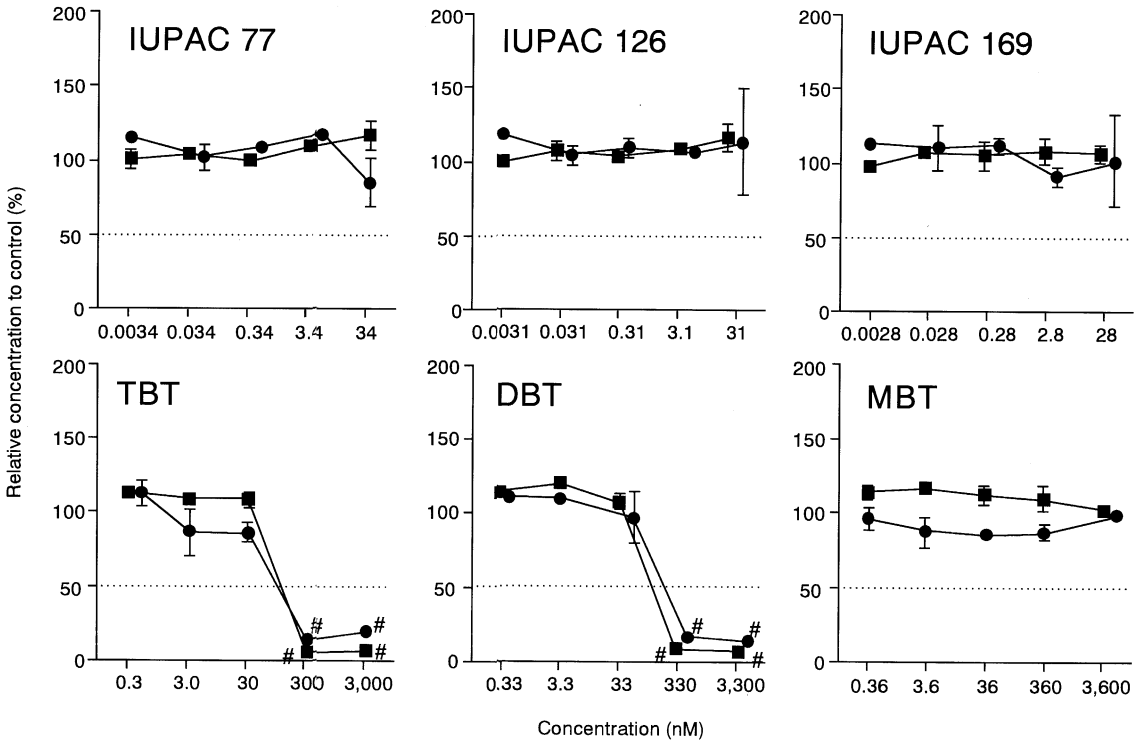


Fig. 3. Proliferation response of peripheral blood mononuclear cells (PBMCs) in California sealion (circle) and larga seal (square) following the treatment with non-*ortho* coplanar PCB congeners and butyltins. #: $P<0.01$.

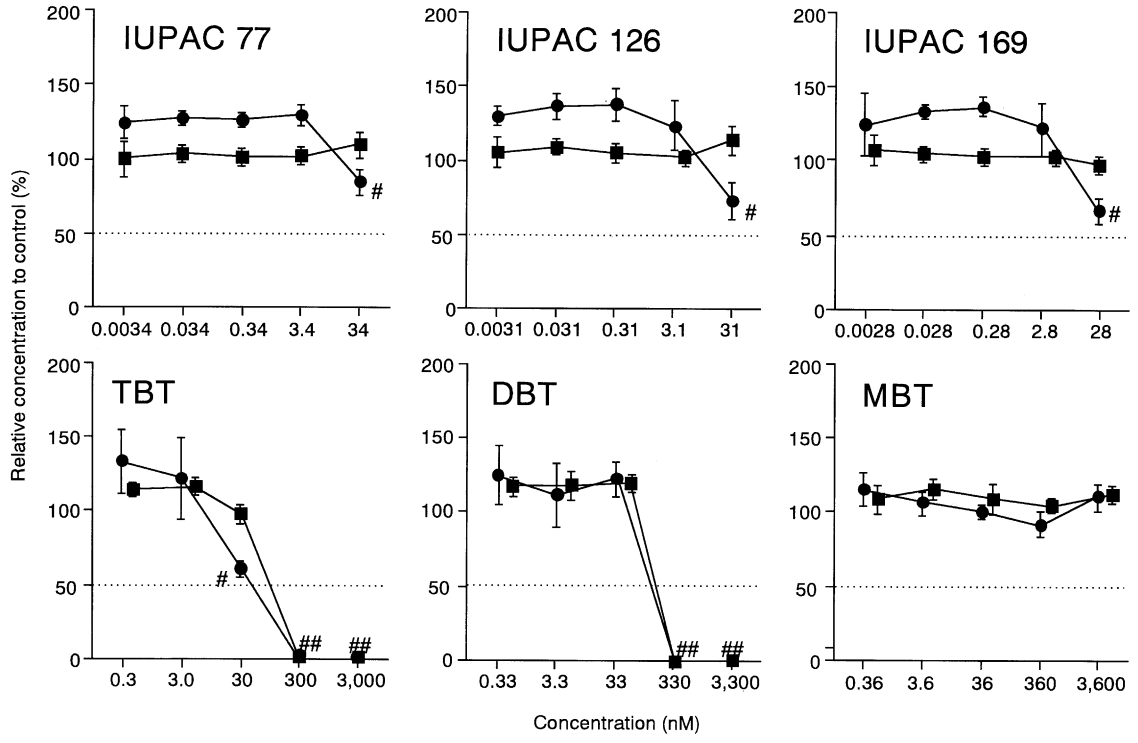


Fig. 4. Proliferation response of peripheral blood mononuclear cells (PBMCs) in humans following the treatment with non-*ortho* coplanar PCB congeners and butyltins. #: $P<0.01$.

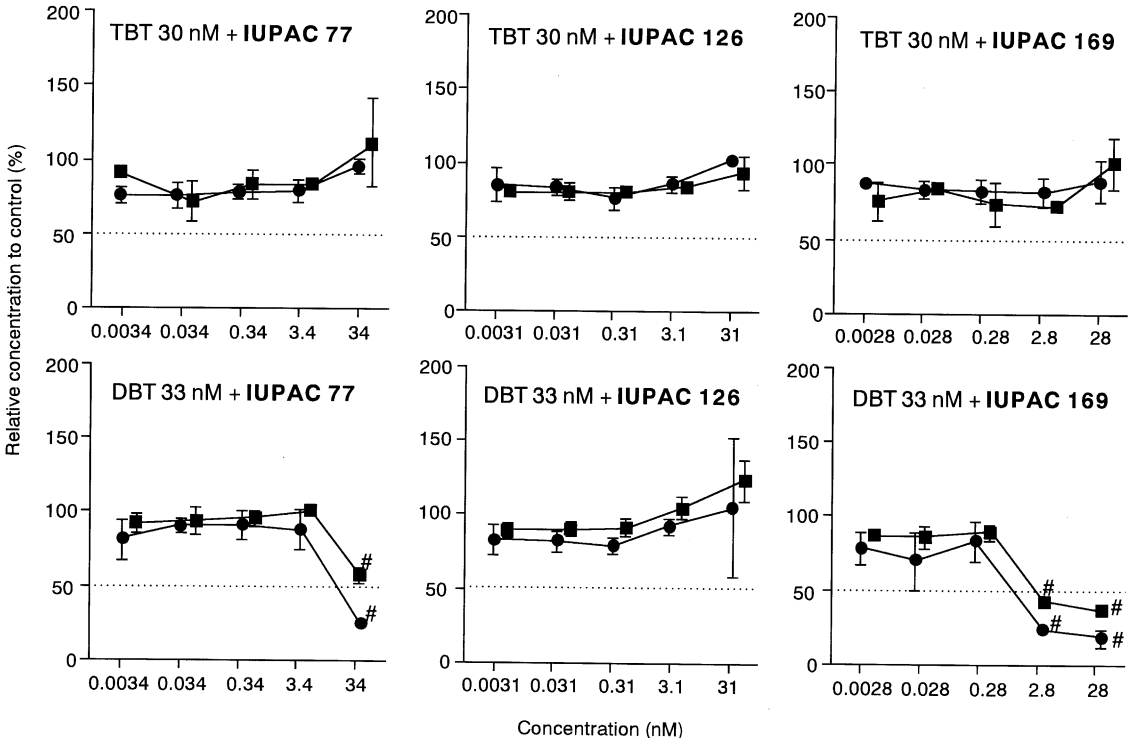


Fig. 5. Proliferation response of peripheral blood mononuclear cells (PBMCs) in bottlenose dolphins following the treatment with the mixture of butyltins and non-*ortho* coplanar PCB congeners and butyltins. #: $P<0.01$.

Table 3
Concentrations (ng/g wet wt.) of PCBs including non-*ortho* coplanar congeners (in blubber) and BTs (in liver) in Dall's porpoises collected from Japanese coastal waters

| Sample No. | Sex | Body length | Lipid % | ΣPCBs | CB.77 | CB-126 | CB-169 | ΣCOP | MBT | DBT | TBT | BTs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DP-1 | M | 152 | 89 | 5500 | 0.48 | 0.18 | 0.13 | 0.79 | 82 | 280 | 110 | 470 |
| DP-2 | M | 169 | 87 | 6800 | 0.36 | 0.12 | 0.15 | 0.63 | 210 | 700 | 410 | 1300 |
| DP-3 | M | 163 | 85 | 7500 | 0.36 | 0.11 | 0.12 | 0.59 | 210 | 730 | 370 | 1300 |
| DP-4 | M | 167 | 88 | 6300 | 0.65 | 0.18 | 0.15 | 0.98 | 200 | 1100 | 450 | 1800 |
| DP-5 | M | 176 | 88 | 6300 | 1.3 | 0.42 | 0.25 | 1.9 | 94 | 320 | 210 | 630 |
| DP-6 | F | 164 | 84 | 9300 | 0.41 | 0.13 | 0.15 | 0.70 | 220 | 780 | 220 | 1200 |
| DP-7 | M | 177 | 86 | 9800 | 0.44 | 0.18 | 0.18 | 0.79 | 59 | 430 | 160 | 650 |
| DP-8 | F | 154 | 80 | 5500 | 0.30 | 0.13 | 0.14 | 0.56 | 90 | 400 | 210 | 700 |
| DP-9 | M | 156 | 84 | 8000 | 0.43 | 0.14 | 0.16 | 0.72 | 50 | 270 | 100 | 420 |
| DP-10 | M | 156 | 84 | 6400 | 0.71 | 0.23 | 0.20 | 1.2 | 50 | 200 | 81 | 330 |
| DP-11 | M | 167 | NA | 4400 | 0.35 | 0.11 | 0.10 | 0.55 | 120 | 330 | 200 | 650 |
| DP-12 | F | 147 | NA | 5400 | 0.30 | 0.12 | 0.14 | 0.55 | 47 | 130 | 80 | 260 |
| DP-13 | M | 169 | NA | 6100 | 0.40 | 0.13 | 0.16 | 0.70 | 110 | 300 | 140 | 550 |
| Average | – | 163 | 86[a] | 6700 | 0.50 | 0.17 | 0.16 | 0.82 | 120 | 460 | 210 | 790 |
| S.D. | – | 9.2 | 2.7[a] | 1500 | 0.26 | 0.08 | 0.04 | 0.38 | 67 | 280 | 130 | 460 |

NA, not analysed; MBT, monobutyltin; DBT, dibutyltin; TBT, tributyltin; BT, butyltin.
[a] Values were calculated from the lipid % of DP-1 to 10.

levels of 0.50, 0.17 and 0.16 ng/g wet weight., for CB-77, 126 and 169, respectively (Table 3). Except for two individual porpoises, the animals showed less than 1 ng/g of Σnon-*ortho* PCBs congener concentrations.

Butyltin compounds were detected with a range of 260–1800 ng/g wet weight in the liver of Dall's porpoises. Among the BTs, DBT was predominant (mean: 460 ng/g), followed by TBT (mean: 210 ng/g) and MBT (mean: 120 ng/g). The compositions of BTs were 15% for MBT, 58% for DBT and 27% for TBT in this study. The residue levels and compositions of BTs in Dall's porpoises were almost similar to those reported for the same species collected from Japanese coastal waters in 1995 (Tanabe et al., 1998).

BTs residue levels in Dall's porpoises were greater than the cytotoxic levels (approx. 100 ng/g of DBT and

TBT) to Dall's porpoise lymphocytes. On the other hand, non-*ortho* coplanar PCB concentrations in the blubber of Dall's porpoises showed more than 10 times lower than those levels that suppress the proliferations (10 ng/g). The elevated concentrations of TBT and DBT (several μg/g) have been reported in marine mammals inhabiting Japanese coastal (Tanabe et al., 1998) and US (Kannan et al., 1997) waters. These findings may suggest that butyltins are more immunotoxic rather than PCBs in free-ranging marine mammals. On the other hand, PCB congeners, CB-138, 153 and 180 showed a significant reduction of splenocyte proliferations at concentrations of 5 ppm (De Guise et al., 1998), which are close to those measured in Japanese coastal cetaceans (Kim et al., 1996, Minh et al., 2000). The investigations of synergistic and/or antagonistic effects by individulals and mixture of these contaminants should be conducted to understand the realistic immunotoxicity by BTs and PCBs.

Butyltin compounds are environmental toxic contaminants with the potential for persistence in aquatic organisms. In particular, detrimental effects of DBT were greater than that of TBT through the phagocytic activity of hemocytes in marine bivalves (Bouchard et al., 1999) and of carboxylesterases inhibition in fish (Al-Ghais et al., 2000). These results may suggest that DBT is one of the important immunotoxic compounds in marine biota combined with TBT. The immunotoxic effects of environmental contaminants have been speculated to be persistent organochlorines, such as PCBs, pesticides and dioxins in marine mammals so far. In addition, this study suggested that BTs such as TBT and DBT could also pose a serious threat to the immune systems of free-ranging marine mammals. An epidemiological investigation is warranted in order to elucidate the potential effects of BTs as well as persistent organochlorines.

## Acknowledgements

The authors would like to thank Dr. K. Kannan (Michigan State University) and K. Senthilkumar (Yokohama National University) for critical reading of this manuscript. We also thank Mr. Ogasawara and the crew of the sampling vessel for collecting Dall's porpoises, and the staffs of the aquariums at Marine World Uminno-Nakamichi and Awashima Marine Park for providing blood samples from the marine mammals. The present study was supported by the Japan Society for the Promotion of Science (Project No. 13780435), a Grant-in-Aid for Scientific Research Program on Priority Area (A) (Contamination and Risk Assessment of Endocrine Disrupters in Wildlife) from Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant No. 13027101) and the Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of the Japanese Government, and "Assessment and Control of Risks to Progeny from Exposure to Complex Chemicals in the Environment" supported by "Environmental Technology Development Fund" from the Ministry of Environment.

## References

Al-Ghais, S.M., Ahmad, S., Abi, B., 2000. Differential inhibition of xenobiotic-metabolizing carboxylesterases by organotins in marine fish. Ecotoxicol. Environ. Safe. 46, 258–264.

Bouchard, N., Pelletier, E., Fournier, M., 1999. Effects of butyltin compounds on phagocytic activity of hemocytes from three marine bivalves. Environ. Toxicol. Chem. 18, 519–522.

Boyer, I.J., 1989. Toxicity of dibutyltin, tributyltin and other organotin compounds to humans and to experimental animals. Toxicology 55, 253–298.

De Guise, S., Martineau, D., Beland, P., Fournier, M., 1998. Effects of *in vitro* exposure of beluga whale leukocytes to selected organochlorines. J. Toxicol. Environ. Health 55, 479–493.

Dietz, R., Heide-Jorgensen, M.-P., Harkonen, T., 1989. Mass deaths of Harbor Seals (*Phoca vitulina*) in Europe. Ambio 18, 258–264.

Grachev, M.A., Kumarev, V.P., Mamaev, L.V., Zorin, V.L., Baranova, L.V., Denikina, N.N., Belikov, S.I., Petrov, E.A., Kolesnik, V.S., Kolesnik, R.S., Dorofeev, V.M., Beim, A.M., Kudelin, V.N., Nagieva, F.G., Sidorov, V.N., 1989. Distemper virus in Baikal seals. Nature 38, 209.

Hahn, M.E., Poland, A., Glover, E., Stegeman, J.J., 1994. Photoaffinity labeling of *Ah* receptor: phylogenetic survey of diverse vertebrate and invertebrate species. Arch. Biochem. Biophys. 310, 218–228.

Harper, N., Connor, K., Steinberg, M., Safe, S., 1995. Immunosuppressive activity of polychlorinated biphenyl mixtures and congeners: nonadditive (Antagonistic) interactions. Fundam. Appl. Toxicol. 21, 412–419.

Iwata, H., Tanabe, S., Mizuno, T., Tatsukawa, R., 1995. High accumulation of toxic butyltins in marine mammals from Japanese coastal waters. Environ. Sci. Technol. 29, 2959–2962.

Kannan, K., Senthilkumar, K., Loganathan, B.G., Takahashi, S., Odell, D.K., Tanabe, S., 1997. Elevated accumulation of tributyltin and its breakdown product in bottlenose dolphin (*Tursiops truncatus*) found stranded along the US Atlantic and Gulf coasts, USA. Environ. Sci. Technol. 31, 296–301.

Kim, G.B., Tanabe, S., Iwakiri, R., Tatsukawa, R., Amano, M., Miyazaki, N., Tanaka, H., 1996. Accumulation of butyltin compounds in Risso's dolphin (*Grampus griseus*) from the Pacific coast of Japan: Comparison with organochlorine residue pattern. Environ. Sci. Technol. 30, 2620–2625.

Kuehl, D.W., Haebler, R., Potter, C., 1991. Chemical residues in dolphins from the US Atlantic coast including Atlantic bottlenose obtained during the 1987/88 mass mortality. Chemosphere 22, 1071–1084.

Minh, T.B., Nakata, H., Watanabe, M., Tanabe, S., Miyazaki, N., Jefferson, T.A., Prudente, M., Subramanian, A., 2000. Isomer-specific accumulation and toxic assessment of polychlorinated biphenyls including coplanar congeners in cetaceans from the North Pacific and Asian coastal waters. Arch. Environ. Contam. Toxicol. 39, 398–410.

Nakata, M., Oyama, Y., Okada, Y., Yamazaki, Y., Chikahisa, L., Satoh, M., 1999. Flow cytometric analysis on tri-*n*-butyltin-induced increase in annexin V binding to membranes of rat thymocytes. Environ. Toxicol. Pharmacol. 7, 267–273.

Omara, F.O., Flipo, D., Brochu, C., Denizeau, F., Brousseau, P., Potworowski, E.F., Fournier, M., 1998. Lack of suppressive effects of mixtures containing low levels of methylmercury (MeHg), polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and Aroclor biphenyls (PCBs) on mixed lymphocyte reaction, phagocytic, and natural killer cell activities of rat leukocytes in vitro. J. Toxicol. Environ. Health—Part A 54, 561–577.

Ross, P.S., Swart, R.L.D., Reijnders, P.J.H., Loveren, H.V., Vos, J.G., Osterhaus, A.D.M.E., 1995. Contaminant-related suppression of delayed-type hypersensitivity and antibody responses in harbour seals fed herring from the Baltic Sea. Environ. Health Perspect. 10, 162–167.

Seinen, W., Penninks, A., 1979. Immune suppression as a consequence of a selective cytotoxic activity of certain organometallic compounds on thymus and thymus-dependent lymphocyte. Ann. N.Y. Acad. Sci. 320, 499–517.

Shaw, S.D., 1998. Organochlorines and Biomarkers of Immune and Endocrine Effects in Pacific Harbour Seal and Northern Elephant Seal Pups. PhD thesis of Columbia University.

Silkworth, J.B., Grabstein, E.M., 1982. Polychlorinated biphenyl immunotoxicity: Dependence on isomer planary and the Ah gene complex. Toxicol. Appl. Pharmocol. 65, 109–115.

Simmonds, M., 1992. Marine mammal epizootics worldwide. In: Pastor, X., Simmonds, M. (Eds.), Proceedings of the Mediterranean striped dolphin mortality, International Workshop, GREENPEACE, Palma de Mallorca, Spain, November. pp. 9–19.

Snoeij, N.J., Penninks, A.H., Seinen, W., 1987. Biological activity of organotin compounds—an overview. Environmental Research 44, 335–353.

Swart, R.L., Ross, P.S., Vesser, L.J., Timmerman, H.H., Heisterkamp, S., Loveren, H.V., Vos, J.G., Reijnders, P.J.H., Osterhaus, A.D.M.E., 1994. Impairment of immune function in harbour seals (*Phoca viturina*) feeding on fish from polluted waters. Ambio 23, 155–159.

Tanabe, S., Kannan, N., Wakimonto, T., Tatsukawa, R., 1987. Method for the determination of three toxic non-*ortho* chlorine substituted coplanar PCBs in environmental samples at part-per-tillion level. Intern. J. Environ. Anal. Chem. 29, 199–213.

Tanabe, S., Prudente, M., Mizuno, T., Hasegawa, J., Iwata, H., Miyazaki, N., 1998. Butyltin contamination in marine mammals from North Pacific and Asian coastal waters. Environ. Sci. Technol. 32, 193–198.

Wakimoto, T., Tatsukawa, R., Ogawa, T., 1971. Analytical method of PCBs. J. Environ. Pollut. Cont. 7, 517–522. (Japanese).

Whalen, M.M., Loganathan, B.G., Kannan, K., 1999. Immunotoxicity of environmentally relevant concentrations of butyltins on human natural killer cells *in vitro*. Environ. Reser. 81, 108–116.