Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission to assess the health risks to consumers associated with exposure to organotins in foodstuffs

(Question N° EFSA-Q-2003-110)

Adopted on 22 September 2004

SUMMARY

The Panel was asked to assess the possible risks to human health from the consumption of food contaminated with organotin compounds (OTC), based on intake estimates for Europe. The main source of OTC in food is likely to be tri-substituted compounds (e.g. tributyltin (TBT) and triphenyltin (TPT)), which have been used extensively as biocides in wood preservatives, in antifouling paints for boats and as pesticides. Mono- and di-substituted OTC (e.g. monomethyltin (MMT), dimethyltin (DMT), dibutyltin (DBT), mono-n-octyltin (MOT) and di-n-octyltin (DOT)) are generally used in mixtures in various amounts as polyvinyl chlorides (PVC) stabilizers, and dialkyltins have been approved as PVC stabilisers for food contact materials. OTC are lipophilic contaminants sparingly soluble in water and easily adsorbed to particulate matter in the aquatic environment. Hence, they accumulate in sediments where they are relatively persistent and can be taken up by benthic organisms such as clams. OTC tend to accumulate in fish and other aquatic organisms. There is indication that not only in laboratory animals but also in humans OTC are absorbed from the gastrointestinal tract and that triorganotins are bio-degraded to di- and monoorganotin compounds.

The Panel focused on the most toxic OTC: TBT, DBT and TPT, primarily found in fish and fishery products and for which the exposure databases were considered adequate. Furthermore, the Panel considered it appropriate to assess also the toxicity of DOT as they act by a similar mode of immunotoxic action, even though not found in fish and fishery products. In particular, TBT and TPT are highly toxic to aquatic organisms and show a complex toxicity profile in rodents. Furthermore, they tend to bioaccumulate through the food chain (in particular in fish and seafood). TBT and TPT cause masculinization in female snails ("imposex") and in fish at low concentrations (1 ng/L in water), suggesting that these compounds are endocrine disruptors. Reproductive and developmental toxicity in rodents at relatively low doses (around 1 mg/kg b.w./day) further supports this endocrine activity. The critical toxicological endpoint for risk assessment was considered to be immunotoxicity. Other endpoints of toxicological relevance considered in this opinion are reproductive and developmental toxicity, genotoxicity, carcinogenicity, and neurotoxicity.

A no observed adverse effect level (NOAEL) for immunotoxicity of 0.025 mg/kg b.w./day was identified for TBT oxide from chronic feeding studies. Because TBT, DBT, TPT and DOT exert their immunotoxic effects by similar mode of action and potency, the Panel considered it reasonable to establish a group tolerable daily intake (TDI) for these OTC. In the absence of specific studies on combined effects it seemed justified to consider the...
immunotoxic effects of these compounds as additive. By applying a safety factor of 100, a group TDI of 0.25 µg/kg b.w. for TBT, DBT, TPT and DOT compounds was established (based on TBT oxide molecular mass, this group TDI is 0.1 µg/kg b.w. when expressed as Sn content or 0.27 µg/kg b.w. when expressed as TBT chloride).

The statistical analysis of the SCOOP data (scientific co-operation on questions relating to food) collected by eight European member states has shown that OTC concentration distributions span over several orders of magnitude and are severely skewed. This is explained by the large variety of organisms taken into account, including farmed as well as wild fish, molluscs, crustaceous, cephalopodes, and echinoderms. Based on fully aggregated data for fish and fishery products, the estimated concentration medians of TBT, DBT, and TPT are 7.0, 2.5 and 4.0 µg/kg of fresh weight, respectively, and the corresponding mean values being about 4- to 7-fold higher. Concentrations of OTC in seafood other than fish are in general higher than in fish. The EU SCOOP report contains very few data on DOT, which were always below the limit of determination.

Intake calculations based on fish and seafood consumption in Norway, taken as paradigm of high consumption in Europe showed that the combined TBT, DBT and TPT intake estimated from median concentration was 0.018 µg/kg b.w./day (approximately 7 % of the proposed group TDI). The same intake calculated on mean basis was 0.083 µg/kg b.w./day (about 33 % of the proposed group TDI). The intakes for high consumers, calculated on median and mean concentrations were 0.037 and 0.17 µg/kg b.w./day, respectively, which represents approximately 15 % and 70 % of the group TDI. The Panel noted that the consumption of fish, mussels and other marine animals from highly contaminated area, such as the vicinity of harbors and heavily used shipping routes, may lead to OTC intake that exceed the group TDI.

**KEYWORDS**

Organotin compounds, fish, seafood, TBT, DBT, TPT, DOT, monitoring, occurrence, intakes, immunotoxicity, endocrine disruption, group TDI.
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LIST OF ABBREVIATIONS

Ac     Acetate
ADI    Acceptable daily intake
AFS-Convention International Convention on the control of harmful anti-fouling systems on ships
AR     Androgen receptor
Aq.    Aqueous
BHA    2-β-butyl-4-hydroxyanisol
BHT    3,5-di-β-butyl-4-hydroxytoluol
BGVV   Bundesamt für Gesundheitlichen Verbraucherschutz und Veterinärmedizin
B.w.   Body weight
CHO    Chinese hamster ovary
cPLA₂  Cytosolic phospholipase A₂
CSTEE  Committee on toxicity, ecotoxicity and the environment
DBT    Dibutyltin
DDT    Di-n-dodecyltin
DET    Diethyltin
DISC   Death-inducing signalling complex
DHT    Dihydrotestosterone
DMT    Dimethyltin
DOT    Di-n-octyltin
DOTC   Diocyltindichloride
DOTTG  Di-n-octyltin di-isoctylthioglycolate
ECCO   European Commission Co-ordination
EEG    Electroencephalogram
EPA    Environmental Protection Agency (US)
ERK    Eaq. racellular signal-regulated protein kinase
GFAP   Glial fibrillary acidic protein
IMO    International Maritime Organisation
i.p.   Intraperitoneal
I.R.I.S. Integrated Risk Information System
JNK    c-Jun NH 2 - terminal kinase
LD₅₀   Lethal Dose – 50 % death
LED    Lowest effective dose
LH     Luteinizing hormone
LOD    Limit of determination
LPS    Lipopolysaccharide
MBT    Monobutyltin
MDT  Mono-n-dodecyltin
MMT  Monomethyltin
MOT  Mono-n-octyltin
MOTTG Mono-n-octyltin di-isooctylthioglycolate
MPT  Monophenyltin
NCE  Normochromatic erythrocytes
NK   Natural killer
NOAEL No observed adverse effect level
OTC  Organotin compounds
OT-SAFE Research project on sources, consumer exposure and risk of organotin contamination in seafood
PCE  Polychromatic erythrocytes
PHA  Phytohemagglutinin
PND  Post-natal days
PPD  Pseudopregnant day
PWM  Pokeweed mitogen
QSAR Quantitative structure-activity relationship
ROS  Reactive oxygen species
SCE  Sister chromatid exchange
SCF  Scientific Committee of Food
SCOOP Scientific co-operation
SMA  Spontaneous motor activity
TBT  Tributyltin
TBBT Tributyltin oxide
TDI Tolerable daily intake
TET  Triethyltin
TeBT  Tetrabutyltin
TePT  Tetraphenyltin
THT  Trihexyltin
TOT  Triocyltin
TPT  Triphenyltin
UCL  Upper confidence limit
UDS  Unscheduled DNA synthesis
US EPA United States Environmental Protection Agency
WHO  World Health Organization
w.w.  Wet weight
BACKGROUND

The Commission is considering the possible need to establish maximum levels for organotin compounds in food at Community level, based on the legal framework of Council Regulation EEC 315/93 of 8 February 1993\(^1\). It therefore seeks the advice of the European Food Safety Authority (EFSA) on the risks to human health from exposure to these compounds.

The main source of organotins in food is likely to be trisubstituted compounds used in marine paints. The trisubstituted organotins are widely used as biocides, e.g. in wood preservatives and antifouling paints, and as pesticides. They are toxic to aquatic life and human exposure mainly arises from consumption of certain seafood. For tributyltin oxide (TBTO), a TDI of 0.25 µg/kg b.w. was established by WHO (1996). The database on the toxicity of the other tributyltin (TBT) compounds seems to be limited or rather old. For certain triphenyltin (TPT) compounds, JMPR (1992) established an ADI of 0.5 µg/kg b.w. In 1999 the SCF Working Group on Food Contact Materials evaluated octyltin compounds in food packaging materials and established group TDIs (EC, 1999).

In January 2001 the Commission proposed a scientific co-operation (SCOOP) task which should provide an assessment of the dietary exposure to organotin compounds, in particular trisubstituted compounds, e.g. TBTO, tributyltin chloride (TBTCI) and TPT compounds.

The Commission requested in 2002 that the Scientific Committee on Food (SCF) should give an opinion on the assessment of health risks to consumers associated with exposure to organotins in foodstuffs. In considering these issues the SCF was asked to take note of a proposed parallel assessment on organotins in consumer products by the Committee on Toxicity, Ecotoxicity and the Environment (CSTEE). It was indicated that a liaison between the two scientific committees might be appropriate, particularly where exposure to similar organotin compounds arises from foods as well as from other routes.

CSTEE has adopted during the 38\(^{th}\) plenary meeting of 12 June 2003 an “Opinion on the non-food aspects of assessment of the risks to health and the environment posed by the use of organostannic compounds (excluding use as a biocide in antifouling paints) and a description of the economic profile of the industry”. The task of the SCF concerning the organotin compounds in food has been transferred to the European Food Safety Authority, particularly to the Scientific Panel on Contaminants in the food chain (CONTAM).

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\(^1\) Council Regulation (EEC) No 315/93 of 8 February laying down Community procedures for contaminants in food. OJ No L37 13.2.1993, p.1

http://www.efsa.eu.int
TERMS OF REFERENCE

The European Food Safety Authority is requested to assess the health risks to consumers associated with exposure to organotins in foodstuffs.

The European Food Safety Authority is in particular invited to indicate, on the basis of current knowledge, which organotins are of most concern for public health and for which there may be an urgent need for measures to reduce their presence in foodstuffs.

In considering these issues the Scientific Panel is asked to take note of a proposed parallel assessment on organotins in consumer products by the Scientific Committee on Toxicity, Ecotoxicity and the Environment. Liaison between the two scientific committees might be appropriate, particularly where exposure to similar organotin compounds arises from foods as well as from other routes.

ASSESSMENT

1. Introduction

A list of organotin compounds (OTC) was identified by Scientific Committee on Food (SCF) for its toxicological assessments and survey of exposure. The list comprises the following OTC, detectable by analytical methods in fish and seafood:

- Tetrabutyltin (TeBT)
- Tributyltin (TBT)
- Dibutyltin (DBT)
- Monobutyltin (MBT)
- Triphenyltin (TPT)
- Diphenyltin (DPT)
- Monophenyltin (MPT)

A second group comprises the OTC which can migrate from food contact materials into foodstuffs:

- Monomethyltin/dimethyltin (MMT, DMT)
- Butylthiostannoic acid
- Dibutyltin (DBT)
Mono-n-octyltin/di-n-octyltin (MOT/DOT)
Mono-n-dodecyltin/di-n-dodecyltin (MDT/DDT)

In 1999 the SCF Working Group on Food Contact Materials evaluated octyltin compounds and established the following group TDIs: 0.6 µg/kg b.w. (as Sn) for DOTs and 20 µg/kg b.w. (as Sn) for MOTs.

The organotin compounds (OTC) are substances characterized by the presence of a carbon to tin bond and have the general formula: Rx Sn(L)(4-x) where R is an alkyl or aryl group (e.g.: methyl, butyl, phenyl, octyl) and L is an organic (or sometimes inorganic) ligand. Several types of OTC are manufactured and placed on the market. Commercially used OTC are characterized by the number of organic groups in the compound. Tetra-substituted OTC are used only as an intermediate in the synthesis of other chemicals. Tri-substituted OTC have been and are used for various purposes owing to their strong biocidal properties toward a range of aquatic organisms such as bacteria, fungi and algae as well as molluscs and crustaceans. Examples of their use are antifouling paints for boats and cooling towers, preservatives for wood, cotton, textiles, paper and stain for buildings, slimicides in industrial process, molluscicides to prevent schistosomiasis and, in the case of TPT, as a fungicide in agriculture. Mono- and di-substituted OTC are generally used in mixtures as PVC stabilisers, as catalysts and in glass coating. Several other consumer products such as clothes and shoes may also contain OTC.

Since 1960, the tri-substituted OTC (TBT and TPT) have been used extensively as biocides in antifouling paints for boats. This use has been restricted in many countries because of the recognised adverse effects of these compounds on the aquatic ecosystem. The European Union, Regulation 782/2003/EC (EC, 2003c) requires that the application and bearing on ships of organotin compounds which act as biocides in anti-fouling systems are prohibited as from 1 July 2003.

The uses of TBT and TPT, their persistence, their tendency to bioaccumulate through the food chain (in particular fish and seafood), their high toxicity to aquatic organisms and their complex toxicity profile in experimental animals cause concerns about risks to humans and nonhuman organisms. First concerns for these compounds arose in the 1970ies from observations of deformations and reproductive failure in oysters in France, with severe damage to their production. The discovery that TBT and TPT at very low concentrations (1 ng/L in water) causes “imposex” (masculinisation) in females of certain snails, suggested that these compounds are endocrine disruptors (WHO-IPCS, 1999a,b). More recently (Shimasaki et al., 2003), masculinization has also been observed in fish.

The SCOOP report on the assessment of the dietary exposure to OTC (EC, 2003b) is based on data provided by eight European Countries (Belgium, Denmark, Germany, France, Italy, Netherlands, Greek and Norway). Notwithstanding the limitations due to differences in the amount, detail and quality of the data submitted, this is the first European database with
information on concentrations of TBT, DBT, MBT, TPT, DPT and MPT in marine and freshwater organisms commonly consumed by humans. The SCOOP report includes data on mussels and other bivalve molluscs, fishes from both marine and freshwater, crustaceans, cephalopodes and others (fish oil, birds).

Concentrations of OTC are highly variable among different organisms, due to differences in exposure and metabolism. The highest observed levels are found in dolphins, which lack metabolism of OTC (Iwata et al., 1997; Lee, 1996). Levels are generally lower in invertebrates and even lower in fish.

In its assessments of OTC in food the Panel took note also of the evaluations made by SCF (EC, 1999), WHO-IPCS (1999a, 1999b, 2001), US EPA (1997, 1999), BGVV (2000), European Commission SCOOP Task (EC, 2003b), Risk and Policy Analysts Report for the European Commission (EC, 2002), and CSTEE (EC, 2003a, 2004a). The Committee primarily focused on those OTC for which both the toxicological and exposure databases were considered suitable.

2. Exposure assessment

2.1. Source of human exposure

Human exposure has four possible sources: i) food consumption; ii) ingestion of contaminated soil/sediments; iii) dermal absorption; and iv) inhalation. The most important source of exposure of the general population is from food (in particular fish and other seafood). Food contamination is primarily caused by the use of tri substituted OTC as biocides, components of antifoul ing paints and as agricultural pesticides. Water basins, especially those with a low water exchange, may accumulate OTC due to boating activities and agricultural runoff, with subsequent accumulation in the food chain.

2.2. Occurrence in food

Human exposure to TBT and TPT has been the subject of surveys conducted in Japan in the beginning of the 1990s. In 1991 and 1992, the average TBT intake was estimated at 4.7 ± 7.0 and 2.2 ± 2.2 µg/day/person, respectively. The market-basket method was used in Japan to estimate the national average daily intake of TBT from 1990 through 1997, with intake-levels between 1.5 and 9.9 µg/day/person (WHO-IPCS, 1999a). More recently, TBT intake was estimated in Asia, Australia, Europe, and the USA from the analysis of seafood species purchased from markets in eight cities (Keithly et al., 1999). Based on national diets and geometric means of seafood contamination, TBT doses were estimated to range from 0.18 (United Kingdom) to 2.6 (Korea) µg/day/person (Keithly et al., 1999). Similar results were given in another study on seafood collected in the USA (Cardwell et al., 1999). Between 1990
and 1997, TPT intake was estimated in Japan by the market-basket method in the range of 0.7 – 10.4 µg/day/person (WHO-IPCS, 1999b).

2.2.1. The database of the EU SCOOP Task 3.2.13 report

In October 2003, the final report of Scientific Co-operation (SCOOP) Task 3.2.13, entitled “Assessment of Dietary Exposure to Organotin Compounds of the Population of the EU Member States”, was released (EC, 2003b). This project was carried out at the request of the Commission of the European Communities and co-ordinated by Italy. The objective of this specific task was to provide the Commission with information on dietary exposure to OTC in European countries. Eight countries - Belgium, Denmark, France, Germany, Greece, Italy, Norway, and The Netherlands, further referred to as “participating countries” - delivered the available data on the occurrence of the aforesaid chemicals in food products, i.e. fish and seafood products. As can be deduced from the SCOOP report, country-specific samples appear to have been collected from various sites including heavily contaminated areas during the years 1993 – 2002. The analytical data selected for statistical assessment cover the period 1995 – 2002. Along with the data, relevant supporting information was collected on their quality together with an evaluation of whether they were representative of the country that had released them, i.e. suitable to estimate national dietary intakes.

The Panel noted that there were large differences in the amount of data submitted by the participating countries, and that results appeared to have been obtained with monitoring plans/activities not specifically designed for evaluating human intake. In particular, the data submitted concern the following OTC: TBT, DBT, MBT, TPT, DPT, and MPT. Data related to these compounds constitute the bulk of the SCOOP report, although those on other OTC are sometimes reported. The national dietary exposures to the six OTC selected were calculated by the participating countries, but different methods were used for these estimates. Two studies on intakes of specific population segments - children and high consumers living in coastal municipalities - were also reported.

2.2.2. Analysis of raw occurrence data of the EU SCOOP report

The SCOOP report is associated with a comprehensive database containing a large and rather unique set of OTC concentrations (raw data) in many fresh, semipreserved, and fully preserved fish and fishery products. The latter - hereafter also referred to as “seafood other than fish” — include molluscs, crustaceans, cephalopods, and echinoderms. The study of TBT, DBT, MBT, TPT, DPT, and MPT occurrence in fish and fishery products altogether, and in two coarse food aggregations thereof (“seafood other than fish” and “fish”), is described in detail in Annex 1. In the present opinion, only data officially identified as representative of food contamination have been used.

There are large differences in the amount, detail, and quality of the data from the participating countries. Germany provided by far the largest group of data (N > 5000 or approximately 86
% of the entire data set). Furthermore, analytical results appear to have been obtained largely without adequate harmonization of analytical procedures and/or intercalibrating processes of the different laboratories, hampering comparability of the data.

For the analysis of the raw data, the WHO (or “medium bound”), approach was used (GEMS/Food-EURO, 1995). Due to the characteristics of representative data set frequency distributions, these were subjected to non-parametric statistical analysis (Annex 1). In the SCOOP report database, many concentration values appear to have been released by the countries after averaging of analytical results, so that the number of data (or “true observations”, N) is actually less than the number of physical samples they represent (N_{WEIGHTED}). The N_{WEIGHTED} values were used to obtain the weighted estimates in Tables 3 – 5 of Annex 1. For each OTC, cut-off values (95th and 99th percentiles) for risk management may be derived from the same tables, where all relevant statistical descriptors are summarized. In addition to spreading over several orders of magnitude, OTC concentration distribution patterns are also severely skewed toward high values. This is readily explained by the large variety of organisms taken into account, including farmed as well as wild fish, fish from various regions of the world, fish belonging to different levels of the trophic web, molluscs, crustaceans, cephalopods, and echinoderms. In several cases, arithmetic means are substantially greater than the corresponding medians: these differences are largely associated with the aforesaid distribution high value tails.

In the SCOOP report, the ranking of food groups related to their contribution to total intake of OTC differs from country to country. These differences may result from diverse food consumption habits in the participating countries. On the other hand, other factors may be involved. These factors include the applied sampling strategy (e.g., differences in the coverage of products collected to represent a whole food group), and the large variations in concentrations of OTC in fish and fishery products. To facilitate the identification of cut-off values and possibly improve exposure estimates, the aforesaid two coarse food aggregations (“seafood other than fish” and “fish”) were considered: the OTC occurrence levels in seafood other than fish are in general higher than those detected in fish. For instance, calculated median and mean concentration values for TBT in seafood other than fish are 14 and 60 µg/kg fresh weight, respectively, whereas in fish the corresponding estimates are 5 and 17 µg/kg fresh weight. DBT and TPT concentrations are in general lower than those observed for TBT. Based on fully aggregated data for fish and fishery products, the estimated concentration medians for TBT, DBT, and TPT are 7.0, 2.5 and 4.0 µg/kg of fresh weight, respectively, and the corresponding mean values being about 28, 17, and 17 µg/kg fresh weight (i.e. 4- to 7-fold higher). The EU SCOOP report contains very few data on DOT, which were always below the limit of determination.

Preliminary investigations have shown that TBT is not significantly reduced in seafood by cooking (European Commission Research Project (QLK1-2001-01437) on sources, consumer exposure and risks of organotin contamination in seafood (OT-SAFE)).
2.3. Assessment of the European dietary exposures

2.3.1. Assessment of the average European dietary exposure

Indicatively, the average consumption of fish and fishery products in European countries ranges between 10 (The Netherlands) and 80 (Norway) g/day/person for adults. As shown in Annex 2 for both, average and high consumers, the Norwegian people represent a conservative paradigm of the exposed European population as they show a high consumption pattern. With reference to Norway, examples of mean consumption rates of seafood other than fish and fish are 10 and 70 g/day/person, respectively (Bergsten, 2004, personal communication). It may be noticed that the variability in the composition of food classes considered by the Member-countries participating in the EU SCOOP Task 3.2.13, as well as the species-specific consumption rates, limit the data available for a comparative estimation of country-specific intakes based on the average international occurrence data and consumption patterns.

Calculations based on the above Norwegian consumption values and the median international concentrations found in fish and fishery products (see Annex 1, Tables 3 – 5) provide average dietary exposures for three OTC, i.e. TBT, DBT, and TPT (Table 1, columns “median based“): their individual contributions are within a factor of 3 from each other (respectively, 0.0093, 0.0033, and 0.0053 µg/kg b.w./day,). The resulting TBT + DBT + TPT cumulative intake is 0.018 µg/kg b.w./day. The outcome of similar calculations carried out with the same Norwegian consumption patterns but with the mean international concentrations (see Annex 1, Table 3) provides rather different estimates of the average dietary exposures to TBT, DBT, and TPT (table 1, columns “mean based”) such as 0.038, 0.022, and 0.023 µg/kg b.w./day, respectively. With reference to the values reported, the resulting TBT + DBT + TPT cumulative intake is 0.083 µg/kg b.w./day.

2.3.2. Assessment of the high European dietary exposure

In line with the above evaluations, the exposures of high consumers have been assessed based on the pertinent 95th percentile (Q_{95}) figures of the Norwegian consumption distributions (Bergsten, 2004, personal communication). The estimation of high consumption for Q_{95} consumers (Annex 2) gives the following rates (food aggregation, g/person/day, g/kg b.w./day): seafood other than fish, 16, 0.27; fish, 149, 2.5; total fish and fishery products, 165, 2.8.

Combining these figures with the median international TBT, DBT, and TPT occurrence levels (see Annex 1, Table 3) yields the high (Q_{95}) consumer compound-specific total intakes reported again in Table 1 (columns “median based“). The resulting TBT + DBT + TPT cumulative intake is 0.037 µg/kg b.w./day. The outcome of similar calculations carried out with the same Q_{95} Norwegian consumption patterns but with the mean international concentrations (see Annex 1, Table 3) provides the Q_{95} dietary exposures to TBT, DBT, and TPT reported in the same table, columns “mean based”. With reference to the values shown,
the resulting TBT + DBT + TPT cumulative intake is 0.17 µg/kg b.w./day. In both cases - Q.95 exposures derived from median or mean occurrence data - the individual contributions to exposure of TBT, DBT, and TPT appear to be within a factor of 3 or less from each other (0.019, 0.0069 and 0.011, respectively or 0.078, 0.046 and 0.047, respectively µg/kg b.w./day).

The above calculations do not include the high consumers eating fish and fishery products with high OTC concentrations. For example, the combination of TBT Q.95 occurrence distribution figure (see Annex 1, Table 3) and the total consumption for high consumers (2.8 g/kg b.w./day) gives an exposure estimate of approximately 0.30 µg/kg b.w./day.

2.3.3. Fish and seafood other than fish contributions to dietary exposure

With reference to Norwegian dietary paradigm and the data in Tables 4 and 5 of Annex 1, the relative contributions of seafood other than fish to total exposure are estimated as 15 – 20 % and 27 - 34 % when occurrence medians and means are utilized respectively. It may be observed that the role of fish is in general predominant due to the large fraction of fish present in the Norwegian diet.

Table 1. Intakes (x 10⁻³ µg/kg b.w./day) of TBT, DBT and TPT by aggregated food groups (“fish and fishery products”), and median/mean concentrations (see Table 3 of Annex 1). Rounding off to an effective three-figure format.

<table>
<thead>
<tr>
<th>EXPOSURE LEVEL</th>
<th>MEDIAN BASED</th>
<th>MEAN BASED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TBT</td>
<td>DBT</td>
</tr>
<tr>
<td>Population (consumers)</td>
<td>9.3</td>
<td>3.3</td>
</tr>
<tr>
<td>Total</td>
<td>18.0</td>
<td></td>
</tr>
<tr>
<td>High (95th percentile) consumers</td>
<td>19.2</td>
<td>6.9</td>
</tr>
<tr>
<td>Total</td>
<td>37.1</td>
<td></td>
</tr>
</tbody>
</table>

3. Hazard identification and characterization

3.1. Absorption, distribution, metabolism and excretion of OTC

OTC can hydrolyse in aqueous media. For instance TPT compounds containing an anionic group such as chloride (TPTCl) or acetate (TPTAc) can hydrolyse at ambient temperatures in the pH range of 3 - 8 to its hydroxide (TPTOH). Accordingly, hydrolysis of OTC can occur in
food and in the organism. Dietary studies with e.g. TPTAc (or TPTCl) would therefore predict the oral toxicity of TPTOH (Buerkle, 1985). After oral uptake, the various OTC may be converted in part to their chlorides (OTCl) which are absorbed in the gastro-intestinal tract (DFG, 2001). For instance DOTbis(ethylmaleate) and DOTbis(2-ethylhexylthioglycolate) were almost completely hydrolyzed (~ 95 % and ~ 98 %, respectively) by human gastric juice \textit{in vitro} at 37 °C to the corresponding chlorides (Figge \textit{et al.}, 1983). OTC possess both lipophilic and ionic properties; while the former may favour their accumulation in lipids, the latter enables OTC to bind to proteins and glutathione.

**Tributyltin (TBT)**

\textbf{Absorption and Distribution}

In rats, tributyltinoxide (TBTO) was absorbed incompletely and slowly from the gastro-intestinal tract. The degree of absorption was 20 – 55 % depending on the vehicle. Maximum plasma levels were reached after about 1 day. Distribution in the organism was rapid. Following a single oral administration of $^{113}$Sn-labelled TBTO (25 mg/kg b.w., oily solution) high levels of radiolabel were found in the liver and kidney, 1 to 3 days after dosing. The liver contained more than 95 % of radiolabel as TBTO metabolites. Other organs and tissues (e.g. brain and fatty tissue) showed lower concentrations but the fraction of unchanged TBTO was higher. Whole body autoradiography did not demonstrate high levels of radiolabel in peripheral tissues as compared to excretory organs and contents of gastro-intestinal tract. TBTO may cross the placenta to some extent, as was shown by the presence of radiolabel in rat fetuses after a single oral dose of $^{113}$Sn-labelled TBTO to the mother (25 mg/kg b.w.). The concentration in fetal tissue was comparable to that of the mothers muscle tissue (equivalent to 0.38 µg $^{113}$Sn/g wet tissue) (Hümpel \textit{et al.}, 1986).

Daily intragastric administration of labeled TBTO (5 mg/kg b.w., aqueous suspension) for 14 days to rats resulted in steadily increase of radiolabel in all tissues. Steady state levels were not reached. After 14 treatments high levels were found in kidneys and liver corresponding to a 3.5 and 2.8 fold increase respectively in relation to a single dose, respectively. In other tissues like testicles at most a 7.4 fold increase was observed. It was estimated that steady state conditions would be reached in the rat after 3 to 4 weeks of daily treatment leading to higher accumulation factors of about 10 (Hümpel \textit{et al.}, 1986). In a similar experiment Evans \textit{et al.}, (1979) administered $^{14}$C-labelled TBTO to mice (COBS strain) in the drinking-water at low doses continuously for up to 30 days. Tissues that exhibited the highest accumulation of radioactivity were kidney, liver, spleen and fat. Levels were low in muscle, lung and brain. Blood levels were extremely low. For instance administration of 17.6 µg TBTO/ml drinking water leads to concentrations in the kidney equivalent to 2.1 µg TBTO/g wet tissue and in the brain to 0.36 µg TBTO/g wet tissue.

\textbf{Metabolism}

http://www.efsa.eu.int
TBT-compounds are substrates for mixed function oxidases. Casida et al., (1971) and Fish et al., (1975, 1976) using hepatic microsomes from various laboratory animals demonstrated hydroxylation in the alpha and beta position, followed by the formation of 1-butanol and butene. In vivo, the process of biotransformation particularly in the liver is characterized by cytochrome P-450 dependent hydroxylation and dealkylation leading to DBT, MBT and inorganic tin. Metabolites are detectable in liver within 3 hours of TBTAc administration (1.2 mg/kg b.w.) to Swiss Webster mice. Liberated carbon fragments were further oxidized. $^{14}$CO$_2$ appeared in the exhaled air in low amounts in addition to butene (Kimmel et al., 1977). In addition to the metabolites generated by mixed function oxidases, mercapturic acid derivatives were found in the urine of Wistar rats intraperitoneally injected with 4.5 mg/kg b.w. TBTCI (Suzuki et al., 1999). Despite extensive metabolism in the liver, a first pass effect does not seem to reduce the systemic availability to a great extent (Hümpel et al., 1986).

Excretion

On cessation of dosing with $^{14}$C-labelled TBTO for a period of 31 days, the examination of the mice (COBS strain) for further 15 days demonstrated loss of radiolabel retained in various tissues; TBTO-derived radiolabel had disappeared completely from the blood. The loss of radiolabel reached 97 % in liver and 73 % in kidney. The fat showed relatively high retention, with clearance of only 30 % and the lung tissue showed complete retention. The principal route of excretion was via the feces (Evans et al., 1979).

After injecting $^{113}$Sn-labelled TBTO to Swiss mice in high doses data from whole body radioactivity demonstrated a rapid decrease which became progressively slower with increasing time. Since the curve appeared to become asymptotic to that of inorganic tin, TBTO may be converted to inorganic tin in the body. The biological half-life in the mouse was estimated to be 29 days (Brown et al., 1977).

Dibutyltin (DBT)

On administration of $^{14}$C-labelled DBT(Ac)$_2$ to Swiss Webster mice (1.1 mg/kg b.w.) the feces contain a large amount of non metabolized compound (41 % of dose) and some MBT (3.5 % of dose). The formation of MBT from DBT may include both nonenzymatic dealkylation and cytochrome P-450 dependent hydroxylation reactions. The rate of debutylation is low. DBT, like TBT binds extensively to some tissue fractions (Kimmel et al., 1977).

Triphenyltin (TPT)

http://www.efsa.eu.int
Absorption and distribution

Following a single oral dose of $^{14}$C-TPTOH (10 mg/kg b.w.) to Wistar rats maximum blood levels occurred 2 - 8 hours after dosing. However enteral absorption was incomplete: comparison of the renal excretion following oral and intravenous administration of $^{14}$C-TPTOH (2 mg/kg b.w.) indicated that only 40 % of the orally administered dose was absorbed. Total tissue levels 7 days after single or repeated daily dosing were still 2 - 3 % of the administered dose. Maximum concentrations were detected in the liver (e.g. 0.42 µg TPTOH equivalents/g w.w. after 10 mg/kg b.w./day) and kidneys. Levels in other tissues were low (less than 0.1 µg equivalents/g w.w. or not detectable) (Kellner and Eckert, 1986).

When $^{113}$Sn radiolabelled TPTOH (2 mg/kg b.w.) was orally administered to Wistar rats for one or 7 consecutive days enteral absorption was calculated to be only 12 – 28 % within 30 hours (Kellner et al., 1989). One day after the administration of a single dose, maximum levels of radiolabel were observed in the liver, kidneys, gastro-intestinal tract wall, spleen, heart muscle and brain in descending order. Higher tissue levels were detected after repeated dosing. In this case highest radiolabels were found in kidney, followed by liver, brain and gastro-intestinal tract walls. One day after the last dose, the increase for kidney was 3.4 and for testis 4.3 fold compared to one day after a single dose. It was estimated that plateau levels would be reached after 3 - 4 weeks of dosing.

Similar results were obtained after a single s.c. administration of $^{113}$Sn-TPTAc (2 mg/kg b.w.) to guinea pigs. After 20 days, distribution of radioactivity was the highest in liver, kidney, brain and adrenal glands (Nagamatsu et al., 1978).

Transfer into milk was studied in 19 lactating Holstein cows, dosed orally with 1, 5 or 20 mg/kg b.w. $^{14}$C-labelled TPTOH for up to 60 days. Residue levels measured as total radioactivity in milk reached a plateau level around day 28 for all doses. With the lowest dose, total radioactivity in milk did not exceed 0.01 mg/kg TPTOH (Smith, 1981). In another study carried out in cattle, one animal was dosed once orally with 5.055 g $^{14}$C-labelled TPTOH. The majorities of the radioactivity was found in tissues, and were mainly associated with liver and kidney. Radioactivity was not analysed for metabolites or the parent compound. In milk radioactivity reached a maximum equivalent to 0.48 mg/kg TPTOH 2 - 3 days after dosing (Bakke et al., 1982).

Metabolism

The metabolism of TPT derivatives by the mixed function oxidase system seems to be not quite clear. Originally it was observed that they are not dearylated in vitro (Kimmel et al., 1977). In recent studies TPT was shown to be dearylated in microsomal systems in low amounts when dithiothreitol was added and an incubation period of at least 120 min was used (Ohhira et al., 2003a; 2004). In addition, several isoforms of cytochrome P-450 were assayed
to determine TPT metabolism. An increased dearylation of TPT was observed with CYP2C6 (Ohhira et al., 2004). In in vivo studies with rats TPT undergoes extensive metabolism to DPT and MPT by an unknown dearylation mechanism (Fish et al., 1976; Kimmel et al., 1977). Degradation of TPT derivatives starts in the gastro-intestinal tract by cleavage of successive phenyl groups before absorption takes place. By investigating radioactive $^{113}$Sn residues in the stomach and the intestinal wall, it was shown that a large portion of radioactivity was not extractable and therefore tissue-bound. In feces, unchanged TPT accounted for a large fraction of the radioactivity. DPT together with MPT were identified. After a single oral dose of tetrphenyltin (TePT) to male Wistar rats (55.4 mg/kg, oily solution), the highest concentrations of DPT were observed in both liver and kidney. DPT levels did not decrease within the studied time period of 4 days (Ohhira et al., 2003a). After oral administration of TPT to rats, benzene was detected as a volatile metabolite. Recovery studies suggest that up to 25 % of the dose was excreted in feces as benzene while in exhaled air less than 1 % of the administered $^{14}$C-TPTOH was attributable to benzene. TPT, DPT and MPT were also identified in bile, together with a complexed form of inorganic tin. Sulphate conjugates of hydroquinone, catechol, phenol and resorcinol, as well as the mercapturic acid conjugates of phenol, were found in urine. This pattern of urinary metabolites was consistent with the metabolism of benzene. In addition it would appear that some inorganic tin was also excreted in urine. Although it is known, that benzene is reported to be readily absorbed orally, a significant amount formed from TPTOH in the gastro-intestinal tract was excreted in the feces. Indeed the limited fraction of the dose exhaled as benzene (< 1 %) suggests that only a small amount of benzene was absorbed from the gastro-intestinal tract (Buerkle et al., 1986).

Excretion

After oral and i.v. administration of $^{14}$C-labelled TPTOH (2 mg/kg b.w.) to Wistar rats, fecal excretion was predominant (32 – 53 %). Renal excretion was about 12 % following a single oral dose of 2 mg/kg b.w. rising to 24 % following repeated administration of the same dose. About 30 % of the dose was excreted renally following a single i.v. administration of 2 mg/kg b.w.. Prolonged fecal excretion of radiolabel was suggestive of biliary elimination and enterohepatic recirculation. Biliary excretion however accounted for only 2.8 % of the dose for the $^{14}$C label at 7 days. Total recoveries varied considerably, ranging from 48 % - 84 %. After trapping benzene in the feces and radioactivity in the exhaled air total recovery was 99.9 %.

When $^{113}$Sn-labelled TPTOH (2 mg/kg b.w.) was given orally to Wistar rats for either one day or 7 consecutive days, 88 % was excreted fecally within 48 hours after both single and repeated doses and 96 - 100 % was excreted in the feces within 7 days. As less than 1 % of the radiolabel was excreted renally after a single dose and biliary excretion accounted for at most 6 % for the tin label at 30 hours, most of the radiolabel in feces had not been absorbed. Total recovery in excreta was 97 – 102 % (Eckert et al., 1989).
Similar results were obtained after oral administration of $^{113}$Sn-labelled TPTAc (1.6 mg/kg b.w.) to rats. Fecal excretion was the predominant route of elimination. Most of the radiotin appeared in the feces within 10 days. Although some of the administered TPT was excreted unchanged, a significant portion was excreted as DPT, MPT and other polar products. A large amount of "bound" radiotin was not recovered from particulate material by extensive extraction (Kimmel et al., 1977).

Di-n-octyltin (DOT)

Absorption and distribution

In orally treated rats (Wistar derived) only a small part of the dioctyltindichloride (DOTC) was absorbed as approximately 80 % of the $^{14}$C-labelled DOTC radioactivity was already excreted in the feces during the first day after administration. This is in accordance with the observation that after i.v. administration of 1.2 mg $^{14}$C-labelled DOTC/kg b.w./day the tissue radioactivity was about 3 - 4 times higher than after oral administration with 6.3 mg $^{14}$C-DOTC/kg b.w./day. Absorption was calculated to be approximately 20 % of the dose.

The highest amount of radioactivity was found in liver and kidney, and to a lesser degree in adrenal, pituitary and thyroid glands. The lowest activity was recovered from blood and brain. No selective accumulation was observed in the thymus, although thymus atrophy is the most sensitive parameter of DOT toxicity in rats. It was suggested that DOT is rapidly distributed and diffuses poorly into the brain (Penninks et al., 1987).

Metabolism

No data are available about the metabolism of DOT. It is however concluded that hydroxylation and dealkylation in vivo of octyltin compounds will be even lesser than of the butyltins because the extent of in vitro microsomal metabolism of various tri-n-alkyltins decreased with increasing chain length. Furthermore it was observed in mice that DBT(OAc)$_2$ was dealkylated much more slowly than TBTOAc and with TOT no other detectable products than trace amounts of DOT were observed in vitro (Kimmel et al., 1977). Together with the slow rate of DBT metabolism it is assumed that DOT is probably hardly metabolized. The results of radioactivity measurements after administration of DOTC will therefore largely reflect DOT concentrations and are not represented by the radioactivity of metabolites.

Excretion

After a single oral dose of $^{14}$C-labelled DOTC (6.3 mg/kg b.w./day) a time dependent decrease in radioactivity was found for all tissues investigated, except for the kidney. At day
25 after exposure, a total of about 10% of the administered radioactivity was excreted in the urine, while 89% of the dose was excreted in the feces. So at this time about 99.9% of the oral administered radioactivity was recovered from excreta. A half-life value of 8.9 days was estimated from the fecal excretion of radioactivity. The urinary excretion of radioactivity appeared to be independent of the body burden, since the daily urinary excretion of radioactivity was nearly constant during the 25 days experimental period after both oral and i.v. administration (Penninks et al., 1987).

Investigations in Humans

Toxicokinetics and internal dose

Human liver microsomes have been shown to be able to metabolize TBT in low amounts (Ohhira et al., 2003b). Uhl (1986) dissolved TBTO (9.88 or 5.54 mg) in a mixture of 3 ml cherry brandy and 7 ml ethanol and gave it orally to a volunteer. Only 5.1% to 5.4% of the dose was found in the urine, mainly as DBT. Metabolites in the urine decreased rapidly during the first days after administration.

Butyltins were detected in 9 liver samples from males and females in Poland (Kannan and Falandysz, 1997), in 4 male Japanese (Takahashi et al., 1999) and in the liver of 18 Danish males (Nielsen and Strand, 2002). Total butyltin burden ranged from 1.1 to 96 µg/kg wet weight (w.w.) (Table 2). DBT appears to be the main butyltin compound deposited in human liver. TBT and TPT levels were below the detection limits (0.3 ng/g w.w. and 3.0 ng/g w.w. respectively in the study of Nielsen and Strand, 2002). The hepatic concentrations of butyltin compounds in the Danish men were generally comparable to those reported from Poland, however some of the Danish men had considerably higher concentrations. Highest concentrations were reported from Japan. Total butyltin concentrations in human blood (central Michigan/USA) ranged from not detectable to 101 µg/L. MBT, DBT and TBT were detected in 53, 81 and 70% of the 32 blood samples examined (Kannan et al., 1999, Table 2). TPT was the major species found in blood of 8 healthy German adults (0.17 - 0.67 µg/L). Only minor concentrations of TBT could be found, whereas MBT, DBT, TeBT, MOT and DOT were not detectable (Lo et al., 2003).
Table 2. Internal doses of butyltins measured in human samples (µg/L for blood and µg/kg for liver).

<table>
<thead>
<tr>
<th>Country</th>
<th>Number of samples</th>
<th>Min</th>
<th>Mean</th>
<th>Max</th>
<th>Chemical</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denmark</td>
<td>18 livers</td>
<td>1.1</td>
<td>10.7</td>
<td>33.0</td>
<td>Total butyltins</td>
<td>Nielsen and Strand, 2002</td>
</tr>
<tr>
<td>Denmark</td>
<td>18 livers</td>
<td>0.3</td>
<td>1.6</td>
<td>14.7</td>
<td>MBT</td>
<td>Nielsen and Strand, 2002</td>
</tr>
<tr>
<td>Denmark</td>
<td>18 livers</td>
<td>0.8</td>
<td>9.0</td>
<td>28.3</td>
<td>DBT</td>
<td>Nielsen and Strand, 2002</td>
</tr>
<tr>
<td>Denmark</td>
<td>18 livers</td>
<td>n.d.</td>
<td>—</td>
<td>n.d</td>
<td>TBT</td>
<td>Nielsen and Strand, 2002</td>
</tr>
<tr>
<td>Germany</td>
<td>8 blood</td>
<td>0.17</td>
<td>—</td>
<td>0.67</td>
<td>TPT</td>
<td>Lo et al., 2003</td>
</tr>
<tr>
<td>Japan</td>
<td>4 liver</td>
<td>59</td>
<td>—</td>
<td>96</td>
<td>Total butyltins</td>
<td>Takahashi et al., 1999</td>
</tr>
<tr>
<td>Japan</td>
<td>4 liver</td>
<td>14</td>
<td>—</td>
<td>22</td>
<td>MBT</td>
<td>Takahashi et al., 1999</td>
</tr>
<tr>
<td>Japan</td>
<td>4 liver</td>
<td>45</td>
<td>—</td>
<td>76</td>
<td>DBT</td>
<td>Takahashi et al., 1999</td>
</tr>
<tr>
<td>Japan</td>
<td>4 liver</td>
<td>n.d.</td>
<td>—</td>
<td>n.d</td>
<td>TBT</td>
<td>Takahashi et al., 1999</td>
</tr>
<tr>
<td>Poland</td>
<td>9 liver</td>
<td>2.4</td>
<td>—</td>
<td>11.0</td>
<td>Total butyltins</td>
<td>Kannan and Falandysz, 1997</td>
</tr>
<tr>
<td>U.S.A</td>
<td>32 blood</td>
<td>n.d.</td>
<td>—</td>
<td>101</td>
<td>Total butyltins</td>
<td>Kannan et al., 1999</td>
</tr>
</tbody>
</table>
Aromatase (CYP 19) inhibition \textit{in vitro}

TBT was found to be a partial competitive inhibitor of human placental aromatase activity \textit{in vitro}. DBT was a less potent inhibitor of human aromatase activity and TeBT and MBT had no effect (Heidrich \textit{et al.}, 2001). Similarly, TBT inhibited aromatase activity competitively \textit{in vitro} at 12 \(\mu\)M and DBT at 74 \(\mu\)M while MBT and TOT, DOT and MOT were without effect (Cooke, 2002).

5 alpha-Reductase inhibition \textit{in vitro}

Androstenedione and testosterone are metabolized to androstanedione and dihydrotestosterone (DHT). These reactions are catalyzed by 5 alpha-reductase. Two human isoenzymes of 5 alpha-reductase have been identified. Human brain tissue was used for measurement of 5 alpha-reductase type 1 activity, whereas 5 alpha-reductase type 2 activity was determined in prostate tissue. 5 alpha-reductase 1 was inhibited by TBTCl (IC\textsubscript{50} = 19.9 \(\mu\)M) and DBTCl\textsubscript{2} (IC\textsubscript{50} = 32.9 \(\mu\)M), whereas 5 alpha-reductase type 2 was only inhibited by TBTCl (IC\textsubscript{50} = 10.8 \(\mu\)M). TBT inhibited 5 alpha-reductase type 1 competitively whereas an irreversible inhibition was evident for the type 2 enzyme. Both isoenzymes were not affected by TeBT or MBT (Doering \textit{et al.}, 2002).

3.2. Acute Toxicity

\textbf{Triphenyltin (TPT), diphenyltin (DPT) and monophenyltin (MPT)}

DPT and MPT are known metabolites of TPT. Only limited toxicity studies of DPT and MPT are available but as they occur normally during the metabolism in the animal, the toxicity studies with TPT implicitly would test for DPT and MPT. TPT hydroxide (TPTOH, Fentin hydroxide) has been reviewed for the European Commission in 1996 by the European Commission Co-ordination (EC, 1997). No relevant new information has been provided since.

The oral LD\textsubscript{50} in the rat was 140 - 298 mg/kg b.w. (fentin acetate and hydroxide) and in the mouse 81 - 93 mg/kg b.w.

\textbf{Tributyltin (TBT) and dibutyltin (DBT)}

Tributyltin is most often studied as its oxide (TBTO); other anionic moieties are mainly chloride, fluoride, oleate and benzoate.

The oral LD\textsubscript{50} in the rat of TBTO was between 94 and 234 mg/kg b.w.. With other cations, data were in the same order of magnitude. The LD\textsubscript{50} for the mouse was between 44 and 230 mg/kg b.w. (WHO-IPCS, 1990).
DBT is a metabolite of TBT and has a similar action profile and potency as TBT derivatives. Treatment with the mixed function oxidase inhibitor SKF 525 A (i.p. saline) before oral administration of 58.6 mg/kg b.w. TBTCI in corn oil (180 µmol/kg b.w.) to ddY mice resulted in an inhibition of hepatotoxicity by TBT up to 24 hours but did not affect hepatotoxicity induced by 54.7 mg/kg b.w. DBTCI₂ (180 µmol/kg b.w.). Therefore, DBT and not TBT may be the hepatotoxic agent (Ueno et al., 1997). The induction of hepatotoxicity by TBT and DBT in vivo was closely associated with the depression of mitochondrial respiration and DBT seems to have a higher affinity for hepatic mitochondria than TBT in mice (Ueno et al., 2003).

The oral LD₅₀ of DBT chloride (DBTCI) was 100 mg/kg b.w. in male rats and that of DBTO 520 mg/kg b.w.. In mice the oral LD₅₀ was 25 and 24 mg/kg b.w. for DBTCI and DBTO, respectively (WHO-IPCS, 1980).

**Di-n-octyltin (DOT)**

The octyltin stabilizers have been assessed by the EC Scientific Committee on Food in September 1999 and no additional data on this endpoint have supplied since. For a range of DOT compounds LD₅₀s have been summarised in WHO-IPCS (1980), being in general higher than 1000 mg/kg b.w.. For DOTC for instance an oral LD₅₀ of 5500/8500 mg/kg is given.

**3.3. Repeated Dose Studies**

**Triphenyltin (TPT)**

Subchronic feeding studies have been conducted in the rat, mouse and dog. In a 3 month study, NMRI mice (n = 10/group) were fed 0, 4, 20 and 100 mg TPTOH/kg diet (Suter and Horst, 1986a). At 100 mg/kg, haematological and biochemical parameters were affected, including a reduction in erythrocyte count and haemoglobin level, an increase in platelet count, and a decrease in IgG, IgA, and IgM (females only). Liver weight was increased in both sexes, and relative weights of ovaries, adrenals, kidneys, heart, and brain were decreased in females. A NOAEL was established at 20 mg/kg diet, equal to 3.44 - 4.12 mg/kg b.w., based on haematological and biochemical parameters.

The same authors also conducted a 13 week study in Wistar rats (15/sex/group) using the same concentration range, and with a recovery period of 4 weeks for 5 rats per group (Suter and Horst, 1986b). Serum enzymes were increased at 100 mg/kg feed (AST, ALT and AP). In females, white blood cells decreased in the mid- and high dose groups. The effects seen with 20 mg/kg in the diet were not considered of toxicological relevance by the ECCO group and a NOAEL based on haematological and biochemical parameters was established at 20 mg/kg diet, equivalent to 1.56 - 1.72 mg/kg b.w..
A one year study was conducted in Beagle dogs (n = 10) fed 0, 2, 6, and 18 mg TPTOH/kg diet. Results indicated some haematological and plasma protein changes but these were not considered of toxicological significance by the ECCO group. Based upon haematological and biochemical parameters the NOAEL was therefore established at 18 mg/kg diet, equivalent to 0.593 mg/kg b.w./day (Sachose et al., 1987).

**Tributyltin (TBT)**

In Wistar rats fed for 4 weeks TBTO at levels of 0, 5, 20, 80, or 320 mg/kg diet (equivalent to 0, 0.25, 1.0, 4.0 or 16.0 mg/kg b.w./day) reduction in body weight, lower feed consumption, weakness, emaciation, roughened fur, but no mortality were seen within 1 week in the group fed 320 mg/kg diet (Krajnc et al., 1984). Some of these signs were seen already at the lower doses after 4 weeks. In the 320, 80 and occasionally in 20 mg/kg diet group serum enzymes (ALAT and ASAT) and IgM were increased; a decrease was seen in white blood cell counts (mainly lymphocytes), serum glucose, liver glycogen, and haemoglobin / haematocrit. In the 320 mg/kg group the weights of brain, heart, liver, spleen and kidney were decreased. The thymus weight was already decreased at 20 mg/kg in males. Treatment-related histopathological changes were found in the liver, thymus, spleen, mesenteric lymph node and liver. In the liver, mainly in the 320 mg/kg group, hepatocellular atrophy was observed, and necrotic areas with bile duct hyperplasia and inflammation. The thymus changes included cortical atrophy with lymphocyte depletion, and similar depletion was found in T-dependent areas in spleen and mesenteric lymph nodes. In addition, splenic iron stores were depleted and in the mesenteric lymph nodes erythrocyte rosettes (sinus erythrocytosis) were seen at all doses.

A 6 week oral study in Wistar rats with 0, 20 and 80 mg TBTO/kg in the diet, showed decreased serum insulin, and decreased thyroid activity as assessed by TSH and thyroxin levels and corresponding histology of pituitary and thyroid at the highest dose, while an increase was found for LH producing pituitary cells (Krajnc et al., 1984).

Other toxicity studies in rats with TBTO reported similar effects at a similar dose range. Sinus erythrocytosis in the mesenteric lymph node was also the most sensitive endpoint in a short-term rat study with a LOAEL of 0.4 mg/kg b.w./day (Bressa et al., 1991).

Cynomolgus monkeys were given a daily (6days/week) oral dose of 0 (n = 3) and 0.160 (n = 4) mg TBTO/kg b.w. for 22 weeks. Total leukocyte count was lower during weeks 8 - 10 and 16 - 20. In the interim period, the leukocyte count had returned to control values. No changes in differential count, immunoglobulins and other parameters were observed (Karrer et al., 1992).

**DIBUTYL Tin (DBT)**
Subacute and subchronic studies with DBT revealed non-specific effects, e.g. reduced bodyweight gain or changes in various biochemical and haematological parameters, as well as specific effects in the liver and bile ducts (50, 1500, 2000 mg/kg in feed) and the immune system (50 mg/kg in feed). Liver and bile duct damage (but no immunologic findings) were also found in a 6-month feeding study with DBTCI at concentrations of 50 to 100 mg/kg, equivalent to 2.5 and 5 mg/kg b.w. (Barnes and Stones, 1958).

**Di-n-octyltin (DOT)**

The octyltin stabilizers have been assessed by SCF in September 1999 and no additional data on repeated dose studies have supplied since.

The evaluated performed 90-day dietary studies in rats showed dose-related increase in kidney weights accompanied by macroscopical and microscopical changes in two studies (NOAEL was 300 mg/kg diet or ~15 mg/kg b.w.), dose-related decrease in thymus weight in one study (decrease was significant at 3000 mg/kg diet or ~150 mg/kg b.w.) and an increase in adrenal/brain weight ratio significant at 100 and 500 mg/kg diet, returning to normal within a 30-day recovery period at the 500 mg/kg level.

**3.4. Carcinogenicity**

**Triphenyltin (TPT)**

Several carcinogenicity studies have been reported with TPT. A 2 year study was conducted in Wistar rats (25/sex/group) at dietary concentrations of 0, 0.5, 1, 2, 5, and 10 mg TPToH/kg diet corresponding to 0.025, 0.05, 0.1, 0.25 and 0.5 mg/kg b.w./day (Til *et al.*, 1970). There were slight changes in the immune system, such as spleen weight reduction, white blood cell counts, and reduction in thymus weight. The relative thyroid weight was slightly decreased at 10 mg/kg diet in the females only. No increased incidence of tumours up to the top dose of 0.5 mg/kg b.w./day were observed. A NOAEL was established at 2 mg/kg diet, equivalent to 0.1 mg/kg b.w., based on effects on the immune system.

A 2-year feeding study was conducted in KFM-Han Wistar rats, 70/sex/group, with concentrations of 0, 5, 20, and 80 mg TPToH/kg diet, equivalent to 0.4, 1.6, 3.2 mg kg b.w./day. (Tennekes *et al.*, 1989b). A dose related mortality was seen in females. Decrease in IgG concentrations was reported in all dosed groups, and IgM was increased in the two higher doses. An increase in the incidence of pituitary adenoma in females and an increase in Leydig cell tumours at higher doses were accompanied by non-neoplastic lesions in these organs. A NOAEL could not be established because of increased mortality in females and reduced serum immunoglobulin levels at the lowest dose tested. The tumour incidences were evaluated as being not significant (JMPR, 1992).
An 80 week study was conducted in KFM-Han NMRI mice, 50/group, with dietary concentrations of 0, 5, 20 and 80 mg TPTOH/kg. Body weight was decreased in females in the 20 and 80 mg/kg group and in males in the high dose group only. A decrease in immunoglobulin concentrations was seen in treated mice (IgM decrease even in the lowest concentration). The incidence of hepatocellular adenomas in both sexes and the incidence of hepatocellular carcinoma in females only were increased at the highest dose (80 mg/kg diet, equivalent to 21.76 mg/kg b.w./day) (Tennekes et al., 1989a). The NOAEL in this study was 5 mg/kg diet based on decreased body weight gain in females. Data on serum IgM was not used for derivation of NOAEL, as the method of analysis has not been evaluated in rodents (JMPR, 1992).

In the ECCO assessment, it was proposed that TPTOH be classified as “category 3 carcinogen” under Commission Directive 93/21/EEC (EC, 1997). This proposal was justified by the types of tumours observed in rats (pituitary and testicular tumours), which may have occurred by hormonal imbalance, and liver tumours in mice considered of doubtful relevance for humans, as well as by the lack of convincing evidence of in vivo genotoxicity. TPT was not assessed for carcinogenicity by IARC; US EPA has evaluated TPTOH as category B2 (“probable human carcinogen”).

**Tributyltin (TBT)**

In a 2-year chronic toxicity and carcinogenicity study, Wistar rats (60 animals per dose and sex, 10 of which were used for an interim kill at 12 months) were fed 0, 0.5, 5 and 50 mg TBTO/kg diet, equivalent to 0, 0.025, 0.25 and 2.5 mg/kg b.w./day (Wester et al., 1988; 1990). In the high dose group, mortality was increased near the end of the study and body weight was reduced. Increased incidence of benign pituitary tumours were observed in both sexes at low and high doses. Other endocrine-related tumours (pheochromocytomas in the adrenal medulla and parathyroid adenomas) were found at high dose only. There were variable and sometimes transient effects on various parameters (endocrine system, haematology, immunoglobulin levels) mainly in the top dose group. As there were also marginal effects in the 5 mg/kg group, the NOAEL was established at 0.5 mg/kg (equivalent to 0.025 mg/kg b.w.), based on changes in haematological and immunological parameters.

CD-1 mice (50 mice per group and sex) were fed TBTO at concentrations of 0, 5, 25 or 50 mg/kg diet, equivalent to 0, 0.75, 3.75 or 7.5 mg/kg b.w./day, during 18 months. Survival was reduced in treated animals, but tumour incidences were not increased (Daly, 1992).

TBTO was not evaluated for carcinogenicity by IARC and the US EPA assigned TBTO as “cannot be determined” (US EPA, 1997).

**Dibutyltin (DBT)**
A two-year carcinogenicity study has been carried in F344 rats and B6C3F1 mice fed diets containing 66.5 and 133 mg/kg dibutyltin diacetate for rats and 76 and 152 mg/kg dibutyltin diacetate for mice (NCI, 1979). Groups of 50 males and 50 females of each species were used. No statistically significant increases in tumour incidences were observed in dosed rats or mice compared to the controls. An accidental loss of tissues from high dose female rats precluded an evaluation of the carcinogenicity of dibutyltin diacetate in female rats.

**Di-n-octyltin (DOT)**

A 2-year carcinogenicity feeding study in rats performed by Ciba-Geigy Ltd. in 1982 was evaluated by SCF (EC, 1999). A 2:1 mixture (67.5, 32.5) of mono-n-octyltin trichloride (MOTS) and di-n-octyltin dichloride (DOTC) was administered to F3-hybrid of RII 1/Tif x RII 2/Tif rats (60 animals per sex). Dosage levels were: 0, 4.95, 14.5, 45.5 and 115.4 mg/kg feed, corresponding approximately to 0, 0.24, 0.69, 2.2 and 5.5 mg/kg b.w./day for males and to 0, 0.26, 0.74, 2.3 and 6.0 mg/kg b.w./day for females. The main adverse effects were in the immune system. A significant higher incidence of thymus lymphomas was observed in the female at the highest dose. A slight increase of generalized malignant lymphomas was observed in females at the highest dose and in males at the two highest doses. Doses of 14.5 mg/kg feed (equivalent to about 0.72 mg/kg b.w./day for the mixture or 0.23 mg of DOTC/kg b.w./day or 0.067 mg/Sn/kg b.w./day) and below did not produce higher tumour incidence.

**3.5. Genotoxicity**


**Tributyltin (TBT)**

The genotoxic potential, as reported by US EPA (1997) and WHO-IPCS (1999b) was evaluated in multiple *in vitro* and *in vivo* short-term tests by Davis *et al.*, (1987). The preponderance of the data shows that TBTO is not genotoxic in short-term tests using a wide variety of genetic endpoints. There were limited positive findings at cytotoxic concentrations (mutagenic in *S. typhimurium* strain TA100, elastogenic in cultured Chinese hamster ovary cells). Overall, the weight of evidence suggests that TBTO is devoid of significant genotoxic potential. More details on the findings are provided in Annex 3 of this document.

**Dibutyltin dichloride (DBT)**
Dibutyltin dichloride was positive in the Chinese hamster ovary cell (CHO)/HGPRT gene mutation assay (Li et al., 1982).

**Triphenyltin (TPT)**

TPTOH is not genotoxic in several studies *in vitro* for genetic end-points like gene mutations, gene conversion and UDS. It is a weak *in vitro* clastogenic agent in human lymphocytes. So far, its capacity to induce chromosome aberrations has not been clearly shown *in vivo*. Similarly, TPTAc is not genotoxic *in vitro* for genetic end-points like gene mutations in bacterial or mammalian cells and gene conversion in yeast. *In vitro* it induces micronuclei and sister chromatid exchanges (SCEs) in Chinese hamster ovary cells (CHO). Its capacity to induce *in vivo* micronuclei in bone marrow cells of mice is equivocal. However, it is able to induce micronuclei in peripheral erythrocytes in mice (Chao et al., 1999). More details on the studies are provided in Annex 3 of this document.

**Di-n-octyltin**

Octyltin stabilizers have been assessed by the EC Scientific Committee on Food (SCF) for their genotoxic potential (EC, 1999). Based on an overall evaluation of the results of a large battery of *in vitro* and *in vivo* assays it was concluded that di-n-octyltin dichloride does not possess genotoxic properties.

### 3.6. Immunotoxicity

**Triphenyltin (TPT)**

Immunotoxicity studies have been conducted with TPT in rats, mice and guinea pigs. Vos et al., (1983; 1984b) studied immune parameters in male Wistar rats fed 5, 25 or 100 mg/kg diet (equivalent to 0.25, 1.25 and 5 mg/kg b.w./day) TPTOH for 3 weeks. Even at the lowest concentration tested, blood lymphocytes and eosinophils were significantly decreased. At 25 mg/kg diet also the thymus weight was reduced, delayed type hypersensitivity was reduced, as was the mitogen response in spleen cells. Thus the LOAEL in this study was 5 mg/kg (0.25 mg/kg b.w./day).

Snoeij et al., (1985) studied male Wistar rats exposed to 0, 15, 50 and 150 mg TPTCl/kg diet (equivalent to 0, 0.75, 2.5 and 7.5 mg/kg b.w.) for two weeks. Thymus weight was reduced at all doses, and spleen weight was decreased in a dose dependent fashion, while signs of general toxicity were observed at 150 mg/kg diet.

In a study by McCormick and Thomas (1990), mice were given 0, 1, 5, 25, 50, or 125 mg TPTOH in the diet for 28 days, (equivalent to 0, 0.15, 0.75, 3.75, 7.5 or 18.75 mg/kg b.w./day) various signs of immunotoxicity (weight reduction of spleen and thymus, reduction
in white blood cell counts, B cells in spleen, T cells in thymus, immunoglobulins) were seen at the higher doses. These effects appeared to be reversible. However, the effects occurred at doses where also parameters for general toxicity (food consumption, liver weight) were affected. The NOAEL level in this study was 5 mg/kg diet, equivalent to 0.75 mg/kg b.w.

15 mg/kg TPTAc in the diet of guinea pigs (equivalent to 1.5 mg/kg b.w./day decreased thymus weight and the number of plasma cells of the spleen and lymph nodes in females examined on days 47 and 77. After 104 days the immunological reaction against tetanus toxoid was inhibited. The dosed group had a lower antibody count and fewer antitoxoid-producing cells in the popliteal gland than the controls when examined immunohistologically (Verschuuren et al., 1970).

Tributyltin (TBT)

The immunotoxicity of TBTO was apparent as thymus atrophy and related downstream lesions already in various toxicity studies described above (e.g. Kranjc et al., 1984). Wistar rats fed for up to 6 weeks with 0, 20 and 80 mg/kg TBTO/kg diet (equivalent to 0, 1 and 4 mg/kg b.w./day) showed suppression of the delayed type hypersensitivity responses, of antibody responses, of resistance to Trichinella spiralis infection, and in spleen cells, the viability and mitogenic response to PHA and ConA (but not to PWM and LPS) (Vos et al., 1984a, 1985). Various parameters were affected already at the lowest dose tested.

A similar set of parameters was assessed in male rats exposed to 0, 0.5, 5 or 50 mg/kg diet (equivalent to 0, 0.025, 0.25 and 2.5 mg/kg b.w./day) for 4 - 6 and 15 - 17 months (Vos et al., 1990). Some of the reported short-term immune effects were not observed in this longer-term study but the reduced thymus weight and mesenteric lymph node T-cells, as well as the reduced resistance to L. monocytogenes were seen at 50 mg/kg diet. Resistance to T. spiralis was apparent already at 5 mg/kg diet (based on IgE titers, larvae count and inflammatory reaction). There were no major differences in the effects between the animals tested at 4 - 6 or 15 - 17 months. From this study a NOAEL of 0.5 mg/kg diet, equivalent to 0.025 mg/kg b.w., was established. Based on this study, the US EPA has calculated a benchmark dose (10 % response, 95 % confidence limit) at 0.68 mg/kg diet, (0.03 mg/kg b.w./day; US EPA, 1997).

Two recent papers dealt with systemic toxicity (Cooke et al., 2004) and immunotoxicity (Tryphonas et al., 2004) of TBTCI in rats following in utero and postnatal exposure. In both studies, pregnant CD rats were gavaged daily with 0, 0.025, 0.25 or 2.5 mg TBTCI/kg body weight in olive oil (16 or 9 in the first and 10 dams per dose group in the second) 7 days/week from day 8 of gestation until weaning. Post weaning, randomly selected pups (n = 12 or 22 in the first and 10 per dose group in the second) were gavaged daily with the same dose of TBTCI administered to their mothers and sacrificed on post-natal days (PND) 30 (males and females), 60 (females) or 90 (males). Two litters from each dose group from in the first study were sacrificed at day 7 and the stomach contents removed and stored for later organotin content assessment.
Stomach contents of suckling pups contained undetectable levels of TBT and DBT was detectable only in the highest TBTCI dose group, indicating negligible lactational transfer to pups. TBTCI had no effects on dams' body weights, food consumption, litter size, sex ratio or survival of pups to weaning. However, all doses of TBTCI significantly affected parameters of the growth profile of the pups and the ratio of weekly food consumption to weekly body weight gain indicated enhanced food conversion to body mass in females but a decreased conversion in males. In male pups dosed at 2.5 mg/kg/day, reduced serum thyroxine levels were evident, indicating that the thyroid is a target for TBTCI toxicity. Significant decreases in liver weights in female pups exposed to 0.025 and 2.5 mg/kg/day TBTCI but not at 0.25 mg/kg/day were observed at PND 60 and in males at PND 90 at top dose. No histopathological lesions were seen in the liver but some markers were altered such as reduced serum triglycerides, creatinin or magnesium (females PND 60, highest dose-level) and reduced amylase (> 0.25 mg/kg, males PND 90). Decreases in spleen and thymus weights are present at 2.5 mg/kg (all age groups).

A detailed examination of immune function revealed increased mean percent and absolute natural killer (NK) cell numbers in male and female rats at 2.5 mg/kg and a non-linear dose-response increase in NK cell activity at all dose levels. Furthermore, increased mean serum IgM levels at the low and high TBTC dose and increased IgG levels at the middle and high doses were seen, as well as an increased mean percentage of CD4(+)8(+) (immature) T lymphocytes at the middle and high dose, increased mean numbers of L. monocytogenes colony-forming bacteria on day 2 post-infection at all dose levels (significant for trend: females, 60 days) and on day 3 only in the middle dose and the delayed-type hypersensitivity response to oxazolone was increased in the low and middle doses and decreased in the high dose males at 90 days.

All together, there was a clear NOAEL of 0.025 mg/kg b.w. for functional immune response (host defence reaction against Listeria). The Panel considered the minor effects observed at the lowest dose level on pup growth, liver enzymes and on some immune cells as biologically not relevant as most of these effects were small and not dose-related.

**Dibutyltin (DBT)**

As with TBT, immunotoxicity of DBT is the critical effect. In a comparative assessment, TBT was about 40 % less active than DBT in reducing relative thymus weight. Also the delay in the effects of TBT compared to DBT suggests that TBT-induced thymus atrophy may be induced by its metabolite DBT and with a lower activity of TBT itself. Dose levels calculated to cause 50 % reduction of relative thymus weight were 59 \(\mu\)mol (18 mg/kg b.w.) DBTCI\(_2\) and 89 \(\mu\)mol (29 mg/kg b.w.) TBTCI per kg b.w. (Snoeij et al., 1988). 50 and 150 mg/kg DBT in feed for up to 6 weeks reduced thymus weight and various parameters for humoral and cellular immune responses in Wistar rats (Seinen et al., 1977a). However, mice and guinea pigs were not responsive in this study. In pre-weaning exposure studies, gavage of 0, 1 and 3 mg/kg b.w. three times per week, affected immune responses at all doses. In a comparative study Snoeij et al., (1988) showed that the mode of action of TBT and DBT were
similar and proposed that the thymus atrophy from TBT was actually caused by its metabolite DBT. From all these studies, no NOAEL could be established, the LOAEL being 50 mg/kg feed, equivalent to 2.5 mg/kg b.w.

**Di-n-octyltin (DOT)**

Immunotoxicity of DOT has been studied by Seinen et al. (1976, 1977a,b. 1979), showing thymus atrophy in rats at the similar extent as DBT at 50 mg/kg (equivalent to 2.5 mg/kg b.w.), and thymus dependent immune suppression: decreased delayed type hypersensitivity response (50 and 150 mg/kg), retarded rejection of skin transplants (150 mg/kg), and decreased graft-versus-host response (50 and 150 mg/kg), while thymus independent humoral immune response and macrophage function were unaffected. Such effects were absent in mice and guinea pigs.

**In vitro studies**

The effects of in vitro exposure to a range of butyltin concentrations on human natural killer (NK) lymphocytes obtained from adult male and female donors were assessed. TBT inhibited the tumour-killing capacity of NK cells when the NK cells were pre-treated at 200 nM for 1 hour. Inhibition of NK-cell cytotoxic function ranged from 40 to greater than 90 %. Loss of NK cell cytotoxicity induced in the 24, 48 and 96 hours periods following a 1 hour exposure to 5 µM DBT is accompanied by a measurable loss of NK-cell tumour binding capacity. The toxic potency followed the order of TBT > DBT > MBT. Conjugation assays revealed that after a 24 hour exposure to TBT, there was about a 50 % decrease in NK cell binding to tumour cells, indicating alterations of the NK cell receptors for tumour cells. An altered pattern of cell surface marker proteins involved in NK cell interactions with possible target cells could be detected. NK cells show decreased expression of as many as five of the seven cell surface proteins tested following DBT exposure. There are very significant decreases in CD 16 and CD 56 expression that accompany decreases in tumour binding function of NK cells (Whalen et al., 1999; Whalen et al., 2002; Odman-Ghazi et al., 2003).

**3.7. Reproductive and developmental toxicity**

**Triphenyltin (TPT)**

No additional data are available to those evaluated by ECCO (EC, 1997) and WHO-IPCS (WHO-IPCS, 1999a). Triphenyltin appears to cause reproductive effects in rats and developmental toxicity in rat, rabbits and hamsters at low doses. In a two-generation study
reduced litter size, weight gain and survival as well as reduced spleen and thymus weight were observed in rat pups at exposure levels not inducing toxicity in adults (Young, 1986). The NOAEL was 0.4 mg/kg b.w.. The lowest NOAEL for maternal and prenatal toxicity were 0.1 and 0.3 mg/kg b.w., respectively, in rabbits. More details on studies are provided in Annex 4 to the document.

**Tributyltin (TBT)**

In the most recent two-generation study (Ogata et al., 2001; Omura et al., 2001) a NOAEL for long-term exposure could not be established because of the dose-related decrease of testis weight and increase of ano-genital distance observed in female pups at all dose levels in the most recent two-generation study. Therefore, the dietary concentration of 5 mg/kg diet, equivalent to 0.25 mg/kg b.w., has to be considered as a LOAEL. Details of study are described in Annex 4 to the document. Overall, the studies on TPT already evaluated by ECCO and WHO/IPCS indicate effects consistent with those induced by TBT, from both a qualitative and a quantitative point of view.

**Dibutyltin (DBT)**

DBT elicits teratogenicity and embryotoxicity in rats at dose levels inducing also maternal toxicity (Farr et al., 2001). The higher impact on older dams and dependency on chemical structure (DBTAc being more potent than DBTCl) need further clarification. A NOAEL for both maternal and developmental toxicity of DBTCl was established at 5 mg/kg b.w. For more details see Annex 4 to the document.

Supportive in vitro studies (see also Annex 4 to the document) indicate that TBT and, with lesser evidence, DBT and TPT may disrupt sex steroid metabolism by affecting both androgen activation and aromatase-mediated synthesis of estrogens (Heidrich et al., 2001; Doering et al. 2002; Yamabe et al., 2000). Moreover, TBT can activate AR-mediated transcription in mammalian cells without a direct interaction with the ligand-binding site of androgen receptor. No such evidence is yet supported for TeBT and MBT.

**Octyltins**

Octyltin compounds have been assessed by SCF in 1999 based on the following studies.

In a two-generation reproduction toxicity study, Sprague-Dawley rats were fed diets containing 0, 20, 60 or 200 mg/kg mixture of 80 % di-n-octyltin di-isoocysthioiglycolate (DOTTG) and 20 % mono-n-octyltin di-isoocysthioiglycolate (MOTTG), corresponding to about 0, 1.9, 5.4 or 17.1 mg/kg b.w./day (Mitterer, 1997). For the mixture DOTTG/MOTTG,
the dose level without adverse effects (NOAEL) was 20 mg/kg feed, equal to 1.9 mg/kg b.w./day (1.5 mg/kg b.w./day for DOTTG), based on thymus effects.

Two teratogenicity studies, one in rats and one in rabbits, were also evaluated by SCF (EC, 1999);

- A formulation of 80 % DOTTG and 20 % MOTTG was administered by gavage from day 6-15 of gestation, to 4 groups of Wistar rats, at dose levels of 0, 1, 5 or 25 mg/kg b.w./day (Pharma Research Report, 1993, unpublished). The percentage of dead foetuses at the high dose was increased. No irreversible structural changes were observed. The dose level without maternal and/or embryo-foetotoxicity was 5 mg/kg b.w./day (equivalent to 0.77 mg Sn/kg b.w./day), based on decreased body weight in dams and on pup mortality.

- The same formulation of 80 % DOTTG and 20 % MOTTG was administered by gavage from day 6 - 18 of gestation to four groups of NZW rabbits, at dose levels of 0, 1, 10 or 100 mg/kg b.w./day (Pharma Research Report, 1992 unpublished). The dose level without maternal toxicity was 100 mg/kg b.w./day (highest dose tested) and that without embryo-foetotoxicity was 1 mg/kg b.w./day (equivalent to 0.15 mg Sn/kg b.w./day), based on minor visceral and skeletal anomalies.

After the SCF evaluation a new developmental toxicity study in mice was made available on octyltin stabilizers (Faqi et al., 2001). A mixture of 80 % DOTTG and 20 % MOTTG was used to treat orally NMRI mice with doses of 20, 30, 45, 67, or 100 mg/kg b.w./day from gestation day 6 through 17. The study showed that the mixture DOTTG/MOTTG produces embryo-foetotoxicity and birth defects in mice, with a NOAEL of 45 mg/kg b.w./day/day for malformations. The maternal NOAEL was 30 mg/kg b.w./day, whereas no NOAEL was identified for skeletal anomalies (supernumerary lumbar ribs).

3.8. Neurotoxicity

Trimethyltin (TMT) and Triethyltin (TET) are well-known potent neurotoxic compounds and induce a consistent picture of damage in humans and animals alike. Both compounds have different targets. TET primarily targets myelin sheaths and causes interstitial oedema throughout the white matter of the central nervous system, particularly the brain; less marked damage occurs in the peripheral nervous system. Intoxications in humans and in animals are associated with seizures, visual disturbances and parapareses. TMT also causes severe damage to the central nervous system; however, the effect is neuronal necrosis, rather than oedema. It targets preferentially neurons, in particular of the limbic system. TMT is mainly associated with limbic seizures and memory deficits (Krinke, 2000a,b).

Recently also TPT has been shown to possess neurotoxic potential. In a few case reports intoxications with TPT were described to be associated for instance with a cerebellar syndrome, hearing impairment and loss of consciousness with paroxysmal activity in the EEG.
(Wu et al., 1990; Lin et al., 1998). However there is no evidence for human neurotoxicity of TBT. After acute exposures toxic effects unequivocally occur but the reported symptomatology was non-specific. Although some kinetic experiments demonstrate that the blood brain barrier is not preventing transfer of TBT/DBT into the central nervous system existing animal studies do not convincingly show that TBT is neurotoxic in non-lethal doses. There is at present no satisfactory mechanistic explanation for that. Obviously the neurotoxic potency of OTC differs substantially and for some OTC it is partially unexplored as systematic experimental studies in laboratory animals (dose-response) on their comparative in vivo neurotoxicity are not available.

Also on the mechanistic level, not much is known on the pathogenesis leading to cell damage. However convincing in vitro data show, that various OTC seem to share the mechanism of interruption of chemical energy supply; a supply critical for excitable tissue, in particular neurons (Snoeij et al., 1986a,b, 1987). In various studies different trialkyltins including TMT, TET and TPT were shown to decouple oxidative phosphorylation and inhibit mitochondrial ATPase (Stockdale et al., 1970; Aldridge, 1977b; Jakobs et al., 1983). Furthermore in liver mitochondria TMT, TET and TBT among other OTC caused an inhibition of ATP production (Aldridge, 1976). Also the dialkyltin compounds, such as dimethyltin (DMT), diethyltin (DET), dibutyltin (DBT) and di-n-octyltin (DOT) have similar biochemical effects in vitro (Snoeij et al., 1987; Boyer, 1989). Consistent with this view are the results of the studies on calcium load and apoptosis in rat neuronal cells. OTC elicited a sustained increase in intracellular Ca$^{2+}$ concentration and induced apoptotic cell death. But the results do not reflect the in vivo situation as TBT and TPT exhibited the most toxic potency in these in vitro cell systems (Viviani et al., 1995; Thompson et al., 1996).

While there is clear-cut evidence for neurotoxicity with regard to TMT, TET and recently also TPT other tri- and also dialkyltin are less or – possibly - not neurotoxic at all. Obviously a lack of systematic experimental data exists in the open literature comparing their in vivo neurotoxicity. However there are some indications for neurobehavioral effects. With regard to risk assessment NOAELs cannot be derived from the studies presently available. The lowest effect levels reported for rats were 1 mg TBTCI/kg b.w./day in a neurodevelopmental study and 0.36 mg TPTOAc/kg b.w. and day in the maze learning test.

Details of all studies are described in Annex 5 to the document.

3.9. Observations in humans

There are several case reports claiming various health effects following acute inhalation, dermal or oral exposure to TBT and TPT and case studies are described in more detail in Annex 6 to this document. However causality can not be established and none of these reports contain sufficient information to characterize a dose-response relationship for the reported
effects. No information was found regarding the toxicity of organotin compounds following long term exposure.

**Tributyltin (TBT)**

There are no reliable and consistent reports on TBT neurotoxicity in humans and toxic effects are unequivocally reported in patients (Shelton et al., 1992; Wax and Dockstader, 1995; Benya 1997). Human data summarized by Boyer (1989) suggest that TBTO is a potent dermal non-allergic irritant. There are several case reports claiming irritation of the respiratory tract following acute inhalation exposure of people to TBTO (Knobeloch et al., 1991; Wax and Dockstader, 1995; Shelton et al., 1992).

**Triphenyltin (TPT)**

Two cases of inhalation poisoning by triphenyltin acetate (TPTAc) were reported (Manzo et al., 1981). Abnormal neurological findings were not mentioned and symptoms (dizziness, nausea, photophobia, general malaise, weakness and dryness of the mouth) disappeared within days. However in other reports about severe intoxications with TPT various neurotoxic symptoms were described (Wu et al., 1990; Lin et al., 1998). Hypersensitivity reaction to a series of 36 TPT containing pesticide formulations was surveyed among 652 subjects in Italy (Lisi et al., 1987) and it was shown that TPT is a moderately strong irritant but not an allergen. Three male patients developed acute nephropathy following ingestion of TPTAc. The acute nephropathy appears to result mainly from proximal renal tubular damage with a benign and reversible course (Lin et al., 1993).

**3.10. Mechanistic data**

In cell culture systems OTC, especially DBT, TBT and TPT, exhibit strong effects on cell viability and cellular functions. Tri-organotin compounds seem to be somewhat more toxic than di-organotin compounds. Their cytotoxic potency is mainly determined by the length of the carbon side chains, with TBT being the most toxic compound. Administration *in vivo* points to additional characteristics that influence the toxic potency of OTC. Here the balance between their lipophilic properties and their affinity to dithiols or sulfhydryl groups may be important. Dialkylated OTC exhibit a stronger affinity to sulfhydryl groups than trialkylated OTC (Merkord et al., 2000; Penninks and Seinen, 1984) and, consequently, their distribution throughout the organism and availability for soft tissues is elevated and hence they are proved to be more toxic in several animal studies. Exemplary this has been shown for DBT that exhibits a stronger toxicity in comparison to TBT when administered per os (Ueno et al., 2003).
On the cellular level OTC interact directly with proteins in a variety of cell types from different origin and organisms and show effects on the calcium homeostasis. Fundamentally, cytosolic free calcium is highly regulated in all cellular systems. Upon stimulation many cells respond with an immediate increase of intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)), and this is obtained via two different pathways: firstly, various types of calcium channels in the plasma membrane can be opened leading to a dramatic influx of calcium from extracellular space, and secondly independent from extracellular calcium, calcium can be released from intracellular stores (Orrenius et al., 2003). Both pathways seem to play a role in the scenario after OTC-treatment of cells. Some experiments point to the opening of calcium channels within the plasma membrane (Ade et al., 1996; Kawanishi et al., 1999; Krug et al., 2003; Miura and Matsui 1991), but other studies with PC12 or CCRF-CEM human T-lymphoblastoid cells revealed the intracellular stores as the important calcium source after OTC-treatment (Reader et al., 1994; Viviani et al., 1995; Yu et al., 2000). With the exception of TMT that exerts cytotoxicity and calcium-influx only at millimolar concentrations, all other tested OTC induce enhancement of [Ca\(^{2+}\)]\(_i\) in the micromolar range with LC\(_{50}\) values between 2.5 \(\mu\)M and 160 \(\mu\)M for TBT and TET, respectively (Ade et al., 1996; Zaucke et al., 1998).

High concentrations of OTC induce necrosis via inhibition of important mitochondrial functions, e.g. ATP-synthesis and the subsequent collapse of ion gradients at the plasma membrane. The breakdown of ion gradients is responsible for the irreversible swelling of the cytoplasm and its organelles, a hallmark of necrosis. The cells then disintegrate and noxious cellular constituents were released. Consecutively, this leads to exudative inflammation in the surrounding tissue. Dependent on the cell type and the OTC, threshold values exist above that acute alterations such as expression of immediate early genes, the activation of kinase cascades via the inhibition of phosphatases, the induction of phospholipases and the disturbance of the cytoskeleton were induced resulting in necrotic cell death and inflammation.

The limit for acute toxic effects in vitro after treatment between 1 to 24 hours with the most toxic congeners TBT and TPT is around 3 \(\mu\)M, and the threshold for DBT is in the same range (Cima et al., 1998; De Santiago and Aguilar-Santelises 1999; Gennari et al., 2002a; Odman-Ghazi et al., 2003) or somewhat higher (Cima et al., 1996; De Vries et al., 1991; Whalen et al., 2002). Below these concentrations more specific mechanistic effects of OTC can be detected. Mostly nanomolar concentrations enhance stress kinase activity, decrease proliferation of cell populations and induce apoptotic cell death in a variety of different cell types.

Apoptosis as a genetically controlled form of cell death, important for embryonic development and for the maintenance of tissue homeostasis in the adult organism, can be triggered by extrinsic, receptor-mediated, or intrinsic, mitochondria-dependent, signalling pathways that induce death-associated proteolytic and nucleolytic activities. This well-organised sequence of events leads to the total breakdown of the cellular constituents without disruption of the plasma membrane. At last the cells break up into membrane-enclosed...
fragments, so-called apoptotic bodies, which are recognised and engulfed by neighbouring cells or macrophages. OTC induced alterations are the impairment of mitochondria and the influence on receptor molecules like death receptors, both important parameters for the onset of apoptosis. *In vitro* membrane blebbing, chromatin condensation and cell fragmentation could be directly observed, whereas *in vivo* the most frequent observed effect is the internalisation of apoptotic bodies by the surrounding cells.

These *in vitro* mechanisms could be demonstrated for most of the OTC, but not all investigations were carried out for all tin compounds in the same way. Especially for DOT, *in vitro* studies are rare and available studies concentrated on endpoints different from calcium homeostasis, apoptosis or mitochondrial alterations (Smith *et al.*, 1995; Tam and Hinsdill 1984; 1990; Webber *et al.*, 1985). *In vivo* investigations carried out with DOT however suggest similar or comparable effects to the other OTC congeners (Miller *et al.*, 1986b; Smialowicz, 2002).

With regard to the induction of thymus atrophy DBT, DOT, TBT and TPT are the most potent congeners. Dialkylated OTC with shorter side chains did not (DMT) or only slightly (DET) reduce thymus weights, and congeners with longer alkylchains (didodecyltin, dioctadecyltin) were ineffective in reducing thymus weights (Seinen *et al.*, 1977b; Snoeij *et al.*, 1987). The same is true for the trialkylated congeners. TBT and TPT have strong immunotoxic properties in the rat (Krajnc *et al.*, 1984; Snoeij *et al.*, 1985; Vos *et al.*, 1984b), but also in mice (Boyer 1989; Nishida *et al.*, 1990) and guinea pigs (Verschuuren *et al.*, 1966; 1970). Trialkyltin congeners with longer carbon side chains exhibit limited (triexylyltin) or no (TOT) immunotoxicity. Overall, good correlations between *in vitro* cytotoxicity data and *in vivo* toxicity of the OTC were found (Brüscheheimer *et al.*, 1995). The biochemical mechanisms, especially the induction of the programmed cell death in immunocompetent cells by OTC, accounts for their immunosuppressive effects. The opinion that apoptosis induced *in vitro* is not relevant for the *in vivo* observed thymus atrophy (Gennari *et al.*, 1997) could be disproved by *in vivo* cytopathology findings demonstrating treatment-related increases in phagocytes with engulfed apoptotic thymocytes (Raffray and Cohen 1993). Additionally, increased macrophage clearance activity in the thymus following OTC administration has been independently noted by several laboratories (De Waal *et al.*, 1993; Kempston *et al.*, 1993; Raffray and Cohen 1998). Moreover, histologic studies demonstrated that the decrease in lymphoid organ weights was associated with a depletion of lymphocytes in the thymus and thymus-dependent areas of spleen and lymphnodes (Penninks *et al.*, 1985; Seinen and Willems 1976).

Additional effects have been described that may enhance the immunosuppressive potency of OTC. Shortly listed are the inhibition of the cytotoxic function of human natural killer cells (Whalen *et al.*, 1999), the decrease of yeast phagocytosis at low doses of OTC in the cultivated clam *Tapes philippinarum* (Cima *et al.*, 1998) and the disturbance of the binding between thymocytes and thymic epithelial cells (Pieters *et al.*, 1995), a process normally initiating the maturation of T cells.
OTC are distributed all over the body with some preference to liver and kidney and no selective accumulation in the thymus (Iwata et al., 1997; Penninks et al., 1987). The apparent sensitivity of the thymus and other organs and cells of the immune system may be dependent on the occurrence of sensitive structures within these cells, e.g. the death receptors. Activation of the extrinsic but also of the intrinsic apoptotic machinery in immunocompetent cells makes these cells the primary target of OTC-activity resulting in severe immunosuppression.

4. Risk characterisation and derivation of a TDI

There are no epidemiological studies on chronic low level oral exposure to OTC available for human risk assessment. In contrast, a large number of experimental studies in vivo and in vitro demonstrate various effects of toxicological relevance after multiple exposures with dose levels as low as 0.25 mg/kg b.w./day. A summary table outlining the most relevant toxicological data for TBT, DBT TPT and DOT is provided in Annex 8.

TBT, TPT, and DOT compounds induce neoplastic lesions in various organs and tissues of experimental animals. Endocrine organs are the targets of TBT and TPT. With TPTOHT an increase in the incidence of pituitary adenomas in females and an increase in Leydig cell tumours in males at higher doses accompanied by non-neoplastic lesions in these organs were observed. Also with TBTO tumours of the anterior pituitary among others were found in both sexes, which also appeared in the lowest dose tested. However, the mechanism of tumour induction has not been established yet. OTC have been tested in various test systems for genotoxicity. As they did not exhibit any significant genotoxic potential in vivo, with the exception of weak effects at chromosome level exerted by TPT, likely due to the formation of benzene as a metabolite, it is concluded that tumour formation may be triggered by hormonal and/or immunologic actions.

In conclusion with regard to human risk assessment and in the absence of convincing evidence of genotoxicity the biological relevance of these tumour types is uncertain.

In a two-generation reproduction study in rats fed diets with a mixture of DOTTG (80 %) and MOTTG (20 %) involution of the thymus was the most sensitive toxic effect. The same mixture produced embryo-foetotoxicity and birth defects in mice, while did not cause irreversible structural changes in teratogenicity studies in rats and rabbits.

Results from studies on reproductive toxicity of OTC make reduced fecundity and developmental abnormalities another relevant endpoint. Increased implantation failure, decreased litter seize, pup weight and relative spleen/thymus weight in weanlings were observed. In two generation studies female offspring showed a delay in vaginal opening and impaired oestrus cyclicity in the higher dose. An increase in anogenital distance in pups was found in all TBT dose groups on PND 1. In male offspring the weights of testis and
epididymis were decreased in the lowest dose tested. Overall, the results indicate that TBT, TPT and to a lesser extent DBT may disrupt sex steroid metabolism. Serum estradiol concentrations were decreased in the F1 generation and in the F2 generation. *In vitro* studies reveal that both androgen activation and aromatase-mediated synthesis of estrogens were affected. Aromatase inhibition has not yet been confirmed *in vivo* and other mechanisms affecting sexual steroids metabolism could also be relevant e.g. an irreversible inhibition of 5-alpha reductase type2 by TBT as was demonstrated in human prostate tissue *in vitro*.

Reports on OTC neurotoxicity in humans exist for TMT and TET and more recently for TPT. There is no evidence for human neurotoxicity of TBT. Animal experiments seem to suggest that other OTC are less neurotoxic than TET and TMT. There is at present no satisfactory explanation for that since *in vitro* studies have shown that TMT, TET, TBT and TPT seem to share the common mechanism of interruption of chemical energy supply, which is critical for neurons and TBT/TPT were among the most toxic compounds in neuronal cell systems. Obviously a lack of systematic experimental data exists in the open literature comparing their *in vivo* neurotoxicity. However there are some indications for neurobehavioural effects. The lowest effect levels in rat studies were reported for TBT in a neurodevelopmental study and for TPT in a maze learning test. Conclusions cannot be drawn without further investigations.

Although the observations with regard to carcinogenicity, developmental and reproductive toxicity and neurotoxicity give some support for concern - NOAELs or LOAELs are often below 1 mg/kg b.w./day - the critical endpoint for risk safety assessment for TBT, DBT, TPT and DOT was considered to be their immunotoxic action. These compounds cause thymus atrophy with lymphocyte depletion in the thymus, spleen and peripheral lymphoid tissues, decreases in immunoglobulin concentrations, lymphopenia and decrease in white blood cells in rodents. As a result the thymus dependent immunity is depressed. These effects observed in rats have relevance for humans as *in vitro* studies with human thymocytes indicated that these cells are sensitive to OTC. Furthermore, it is reasonable to assume that similar downstream effects can be expected for all those OTC which typically affect the thymus since they all activate apoptotic mechanisms probably related to the occurrence of death receptors in the immune system. Based on similar mode of action the effects of OTC can be considered additive. Besides TBT and TPT this also applies for DBT and DOT compounds. Due to their similar potency based on the dose-response curves for DBT and DOT in short-term experiments, TBT, DBT, TPT and DOT compounds are of toxicological concern whereas according to the present state of knowledge MBT, DPT, MPT and MOT are less potent and are present in food in equal or lower levels and are therefore not further considered.

Table 3 outlines relevant immunotoxicological data for TBT, TPT, DBT and DOT indicating similar potencies. TBT is extensively investigated and NOAELs (0.025 mg/kg b.w./day) for immune parameters could be identified in two chronic studies for TBTO. For TPTOH a reduction of immunoglobulin and white blood cells was observed in a long term study with a LOAEL at the same order of magnitude as TBTO. Chronic experiments with DBT are not available. Short term experiments however demonstrate that TBT induced thymus atrophy and hepatotoxicity is preferentially generated by its metabolite DBT with a lower activity of
TBT itself. From this it is concluded that DBT is at least as potent as TBT. In addition in comparative toxicity studies investigating the structure activity relationship regarding thymus toxicity in the rat, DBT and DOT were the most potent dialkyltin compounds producing dose-related thymus atrophy in similar extents.

Table 3: Critical immunotoxicological data for derivation of the group-TDI

<table>
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<tr>
<th>OTC</th>
<th>Species/ exposure period</th>
<th>Effects</th>
<th>NOAEL/LOAEL (mg/kg b.w./day)</th>
<th>References</th>
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<td>TBTO</td>
<td>Wistar rats/2 years</td>
<td>Changes in haematological parameters and immunoglobulin levels</td>
<td>NOAEL 0.025</td>
<td>Wester et al., 1988; 1990</td>
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<tr>
<td>TBTO</td>
<td>Wistar rats/4-6 or 15-17 months</td>
<td>Reduced resistance to <em>T. spiralis</em> infection</td>
<td>NOAEL 0.025</td>
<td>Vos et al., 1990</td>
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<tr>
<td>TPTOH</td>
<td>Mice (strain unspecified), 28-day, diet</td>
<td>Reduced liver and thymus weight and decreased IgM</td>
<td>NOAEL 1.0</td>
<td>McCormick and Thomas, 1990</td>
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<td>TPTC</td>
<td>Wistar rats/2 weeks</td>
<td>Reduced thymus and spleen weight</td>
<td>LOAEL 0.75</td>
<td>Snoeij et al., 1985</td>
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<tr>
<td>TPTOH</td>
<td>Wistar rats/3 weeks</td>
<td>Decreased blood lymphocytes and eosinophils</td>
<td>LOAEL 0.25</td>
<td>Vos et al., 1983; 1984b</td>
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<tr>
<td>TPTOH</td>
<td>Wistar rats/2 years</td>
<td>Reduction in immunoglobulin and white blood cells</td>
<td>LOAEL 0.3 (males) 0.4 (females)</td>
<td>Tennekes et al., 1989b</td>
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<tr>
<td>DBTC</td>
<td>Wistar rats/2 weeks</td>
<td>Reduction in thymus weight and various parameters for humoral and cellular response</td>
<td>LOAEL 2.5</td>
<td>Seinen et al., 1977 a,b</td>
</tr>
<tr>
<td>DOTC</td>
<td>Wistar rats/2 weeks</td>
<td>Reduction in thymus weight and various parameters for humoral and cellular response</td>
<td>LOAEL 2.5</td>
<td>Seinen et al., 1977 a,b</td>
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</table>

As these compounds have a similar profile of action and potency in terms of immunotoxicity (lymphocyte depletion in the thymus and peripheral lymphoid tissues) a group TDI for TBT, TPT, DBT and DOT can be considered, which would also protect from the other toxic effects

Based on these chronic immunotoxicity studies in rats the Panel established a TDI of 0.25 µg/kg b.w. by applying an uncertainty factor of 100 to the NOAEL of 0.025 mg/kg b.w./day per day in that study to account for interspecies and interindividual variability (to put into the
The Panel also considered the 2003 EU SCOOP report on the assessment of the dietary exposure to OTC, which is based on data provided by eight European Countries. The statistical analysis of the data has shown that, based on full aggregation of fish and fishery products, the estimated concentration medians of TBT, DBT, and TPT distributions were 7, 2.5, and 4 µg/kg of fresh product, respectively, and the corresponding mean values being about 4- to 7-fold higher. The EU SCOOP report contains very few data on DOT, which were always below the limit of determination. For specific food aggregations, seafood other than fish is in general more contaminated than fish. Intake calculations based on the Norwegian fish and seafood other than fish consumption pattern, taken as paradigm of high consumption in Europe, showed that the combined TBT, DBT, and TPT intake of the general adult population is approximately 3-fold lower than the proposed group-TDI, when calculated from mean values. The corresponding intake for high consumers was approximately 70 % of the group-TDI. However, the Panel noted that the consumption of fish, mussels, and other marine animals from highly contaminated areas, such as the vicinity of harbours and heavily used shipping routes, may lead to intakes of OTC that exceed the proposed group TDI.

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2 Based on this same study the US EPA has calculated a benchmark dose (10 % benchmark response, with a 95 % confidence limit) at 0.68 mg/kg, i.e. 0.03 mg/kg b.w./day (USEPA Integrated Risk Information System).
CONCLUSIONS AND RECOMMENDATIONS

• The Panel established a group TDI of 0.25 \( \mu g/kg \) for TBT, DBT, TPT and DOT.

• The Panel noted that intake for the general population is below the TDI. However, in few cases where seafood contamination with OTC is high, the TDI might be exceeded for example in the case of consumption of contaminated fish, mussels and other marine animals from the vicinity of harbours and heavily used shipping routes.

• In addition to the OTC dietary exposure from fishery products, consumers are also exposed to OTC from other sources, e.g. pesticides, additives used in plastics, other food contact materials and consumer products. Therefore an integrated risk assessment of OTC is needed, taking into account human exposure from all possible sources.

• Epidemiological studies incorporating biomarkers of exposure and health effects are recommended, particularly in high exposed populations.
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**SCIENTIFIC PANEL ON CONTAMINANTS IN THE FOOD CHAIN**

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ANNEX 1. STATISTICAL ASSESSMENT OF ORGANOTIN COMPOUNDS (OTC) IN EUROPEAN FOODSTUFFS

In October 2003, the final report of Scientific Co-operation Task 3.2.13, entitled Assessment of dietary exposure to organotin compounds of the population of the EU Member States (EC, 2003b) was released. Eight countries - Belgium, Denmark, France, Germany, Greece, Italy, Norway, and The Netherlands, further referred to as “participating countries” - delivered the available data on the occurrence of the aforesaid chemicals in food products, i.e. fish and seafood products. Country-specific samples appear to have been collected from various sites in the years 1993 – 2002. However, the analytical data selected for statistical assessment cover the period 1995 – 2002. Along with the data, relevant supporting information was collected on their quality together with an evaluation of whether they were suitable to estimate national dietary intakes.

There appear to be large differences in the amount of data submitted by the participating countries. Although not directly relevant to this assessment, it may however be observed that results were obtained from monitoring plans/activities not specifically planned for evaluating human intake. The data submitted concern primarily tributyl- (TBT), dibutyl- (DBT), monobutyl- (MBT), triphenyl- (TPT), diphenylin (DPT), and monophenylin (MPT) compounds, altogether forming the bulk of the SCOOP report. Data on other OTC are available, but they do not provide an adequate statistical base.

The SCOOP report database contains a large set of OTC concentrations (raw data) in fresh, semi-preserved, and fully preserved fish and seafood products - “seafood other than fish” - the latter including molluscs, crustaceans, cephalopods, and echinoderms. As to the occurrence study, the database may be outlined as follows: Table 1a provides a country-based breakdown of the TBT, DBT, MBT, TPT, DPT, and MPT data sets; Table 1b shows the numerousness (N) of each set after removing the non-representative data as identified by country delegates; data numerousness of two coarse food aggregations (“seafood other than fish” and “fish”) derived from the selected database is given in Table 2. From Tables 1a and 1b, the predominant contribution of Germany to all data sets can be noticed.

In the database, there are large differences in the amount, detail, and quality of the data from the participating countries. In particular, analytical results appear to have been obtained largely without adequate harmonization of analytical procedures and/or inter-calibrating processes of the different laboratories, this ultimately influencing result comparability. For the analysis of the raw data, the “medium bound” approach was used, i.e. undetermined results were entered in calculations as “0.5 × LOD” (WHO/GEMS/Food-EURO, 1995) where LOD stands for “limit of determination”.

Due to the characteristics of representative data set frequency distributions, these were subjected to non-parametric statistical analysis. Descriptors were obtained suitable to describe
the distributions of the OTC of interest in the bulk of fish and seafood products considered (Table 3) or in the two coarse food aggregations selected (Tables 4 and 5). All these tables are largely self-explanatory.

In the SCOOP report database, many concentration values reflect averaging of analytical results at the origin, so that the number of data (or “true observations”, N in Table 1b) is actually less than the number of physical samples they represent (N\text{WEIGHTED} in Tables 3 – 5). The N\text{WEIGHTED} values were used to obtain the weighted estimates summarized in Tables 3 – 5. In the notes of these tables, the “frequencies” reported indicate how many times a given value occurs in its distribution: this may be due to an individual entry (e.g., a LOD) repeated at the origin and/or to a mean estimate from multiple determinations thereby entered several times (“weighting”). The “≥” sign appearing in some confidence intervals of the 99th percentiles (Q\text{.99}) indicates that their upper confidence limit (UCL) values fall beyond the (high) end of the distributions, where no experimental data are available.

For each OTC, cut-off values (95th and 99th percentiles) for risk management may be derived from Tables 3 – 5. Figures 1 – 6 exhibit the contamination level frequency distributions for the fish and seafood products analyzed and for the coarse food aggregations thereof, after log-transformation (natural logarithms) of chemical concentrations: due to the large concentration ranges of the OTC dealt with, log-transformation was required for clarity of representation and to make some details visible. In addition to spreading over several orders of magnitude, OTC concentration distribution patterns are also skewed toward high values. This is explained by the variety of organisms taken into account, including farmed as well as wild fish, fish from various regions of the world, fish belonging to different levels of the trophic web, molluscs, crustaceans, cephalopods, and echinoderms. In several cases, a remarkable difference exists between medians and arithmetic means: the greater values of the latter are by-and-large associated with distribution tailing towards high values.

To improve exposure evaluation and the identification of risk management cut-off values, the aforesaid two coarse food aggregations (“seafood other than fish” and “fish”) were considered. By comparing the data in the tables and figures, it may be observed how the OTC levels in seafood other than fish are in general higher than those detected in fish.
Tables to Annex 1

Table 1a. Breakdown of total country-based organotin data contributions as derived from the EU SCOOP Task 3.2.13 database (EC, 2003b).

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Table 1b. Breakdown of total country-based organotin data contributions (see Table 1a) after selection.

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TOTAL  | 100 | 1229 | 100 | 1207 | 100 | 1196 | 100 | 1071 | 100 | 1080 | 100 | 432
Table 2. Breakdown of selected organotin data available from European countries (see Table 1b) by coarse fish and fishery product aggregations.

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<td>N</td>
<td>%</td>
<td>N</td>
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<td>N</td>
<td>%</td>
<td>N</td>
</tr>
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<td>77.8</td>
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<td>8</td>
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<td>1196</td>
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<td>1071</td>
<td>100</td>
<td>1080</td>
<td>100</td>
<td>432</td>
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</tbody>
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http://www.efsa.eu.int
Table 3. Statistical descriptors of selected organotin concentrations in European fish and fishery products (see Table 1b); original data from the EU Task 3.2.13 database (EC, 2003b). Concentrations are expressed in µg/kg, whole weight basis. Except for N and N_{WEIGHTED}, in general an effective three-figure format is used.

<table>
<thead>
<tr>
<th>DESCRIPTOR</th>
<th>TBT</th>
<th>DBT</th>
<th>MBT</th>
<th>TPT</th>
<th>DPT</th>
<th>MPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>1229</td>
<td>1207</td>
<td>1196</td>
<td>1071</td>
<td>1080</td>
<td>432</td>
</tr>
<tr>
<td>N_{WEIGHTED}</td>
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<td>1902</td>
<td>1777</td>
<td>1754</td>
<td>664</td>
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<td>0.150</td>
<td>0.0800</td>
<td>0.100</td>
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<tr>
<td>X_{MEAN}</td>
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<td>16.8</td>
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<td>17.1</td>
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<td>7.04</td>
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<td>13.5–20.2</td>
<td>7.65–12.6</td>
<td>13.0–21.3</td>
<td>2.20–2.94</td>
<td>5.73–8.35</td>
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<td>2.50 (b)</td>
<td>2.50 (c)</td>
<td>4.00 (d)</td>
<td>1.50 (e)</td>
<td>2.50 (f)</td>
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<td>2.50–3.00</td>
<td>2.50–2.50</td>
<td>3.50–4.10</td>
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<td>2.50–2.50</td>
</tr>
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<td>X_{MAX}</td>
<td>1830</td>
<td>1400</td>
<td>1920</td>
<td>2330</td>
<td>166</td>
<td>198</td>
</tr>
<tr>
<td>Q_{95}^{(3)}</td>
<td>107 (g)</td>
<td>34.8 (h)</td>
<td>25.0 (i)</td>
<td>63.4 (j)</td>
<td>4.00 (k)</td>
<td>23.0 (l)</td>
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<tr>
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<td>29.0–50.0</td>
<td>21.0–35.0</td>
<td>46.4–90.0</td>
<td>4.00–7.00</td>
<td>15.0–73.0</td>
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<tr>
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<td>421 (n)</td>
<td>220 (o)</td>
<td>200 (p)</td>
<td>26.0 (q)</td>
<td>82.9 (r)</td>
</tr>
<tr>
<td>CI_{Q_{99}}^{(1)}</td>
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<td>370–650</td>
<td>205–250</td>
<td>120–260</td>
<td>23.0–46.0</td>
<td>80.0–107</td>
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Countries: BE, DE, DK, GR, IT, NL, NO

(1) CI, confidence interval (P = 95%).
(2) Frequencies: (a) 38/2110; (b) 386/2064; (c) 630/1902; (d) 57/1777; (e) 232/1754; (f) 240/664.
(3) Frequencies: (g) 1/2110; (h) 0/2064 (interpolated); (i) 12/1902; (j) 0/1777 (interpolated); (k) 44/1754; (l) 3/664.
(4) Frequencies: (m) 6/2110; (n) 0/2064 (interpolated); (o) 5/1902; (p) 0/1777 (interpolated); (q) 5/1754; (r) 0/664 (interpolated).
Table 4. Statistical descriptors of selected organotin concentrations in European seafood other than fish (see Table 2); original data from the EU SCOOP Task 3.2.13 database (EC, 2003b). Concentrations are expressed in µg/kg, whole weight basis. Except for N and N_{WEIGHTED}, in general an effective three-figure format is used.

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<th>DESCRIPTOR</th>
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<th>DBT (µg/kg)</th>
<th>MBT (µg/kg)</th>
<th>TPT (µg/kg)</th>
<th>DPT (µg/kg)</th>
<th>MPT (µg/kg)</th>
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<td>225</td>
<td>222</td>
<td>156</td>
<td>177</td>
<td>92</td>
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<td>391</td>
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<td>204</td>
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<td>16.1–26.4</td>
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<td>260</td>
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<td>86.0</td>
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<td>210 (g)</td>
<td>370 (h)</td>
<td>215 (i)</td>
<td>120 (j)</td>
<td>2.50 (k)</td>
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<td>260 (p)</td>
<td>7.00 (q)</td>
<td>86.0 (r)</td>
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<td>250–260</td>
<td>120–260</td>
<td>7.00–14.0</td>
<td>81.0–86.0</td>
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Countries

(1) CI, confidence interval (P = 95 %).
(2) Frequencies: (a) 5/553; (b) 127/507; (c) 13/391; (d) 55/325; (e) 125/314; (f) 39/204.
(3) Frequencies: (g) 5/553; (h) 5/507; (i) 0/391 (interpolated); (j) 35/325; (k) 120/314; (l) 5/204.
(4) Frequencies: (m) 0/553 (interpolated); (n) 5/507; (o) 5/391; (p) 5/325; (q) 10/314; (r) 5/204.
Table 5. Statistical descriptors of selected organotin concentrations in European fish (see Table 2); original data from the EU SCOOP Task 3.2.13 database (EC, 2003b). Concentrations are expressed in µg/kg, whole weight basis. Except for N and NWEIGHTED, in general an effective three-figure format is used.

<table>
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<th>DESCRIPTION</th>
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<th>MBT</th>
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<th>DPT</th>
<th>MPT</th>
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<td>966</td>
<td>910</td>
<td>895</td>
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<td>1530</td>
<td>1503</td>
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<td>DE, NL</td>
<td>DE, NL</td>
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(1) CI, confidence interval (P = 95 %).
(2) Frequencies: (a) 151/1530; (b) 301/1530; (c) 31/1503; (d) 3/1447; (e) 107/1432; (f) 201/456.
(3) Frequencies: (g) 5/1530; (h) 25/1530; (i) 3/1503; (j) 10/1447; (k) 44/1432; (l) 10/456.
(4) Frequencies: (m) 2/1530; (n) 5/1530; (o) 5/1503; (p) 0/1447 (interpolated); (q) 0/1432 (interpolated); (r) 0/456 (interpolated).
Figure 1. Distribution of TBT levels in fish and fishery products.
Figure 2. Distribution of DBT levels in fish and fishery products.
Figure 3. Distribution of MBT levels in fish and fishery products.
Figure 4. Distribution of TPT levels in fish and fishery products.
Figure 5. Distribution of DPT levels in fish and fishery products.
**Figure 6.** Distribution of MPT levels in fish and fishery products.
## Annex 2. Examples of country-based total intakes of fish and seafood products

Indicative country-based “consumers only” total intakes (g day\(^{-1}\) person\(^{-1}\), in adults) of fish and seafood products primarily derived from EU reports, respectively on heavy metals (Task 3.2.11) and OTC (Task 3.2.13) (EC, 2003b, 2004b).

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<th>Reference</th>
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</tr>
<tr>
<td>Denmark</td>
<td>23(^b)</td>
<td>—</td>
<td>Task 3.2.11</td>
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<tr>
<td>Finland</td>
<td>53(^b)</td>
<td>—</td>
<td>Task 3.2.11</td>
</tr>
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<td>France</td>
<td>41(^b)</td>
<td>103(^b)</td>
<td>Task 3.2.13</td>
</tr>
<tr>
<td>Germany</td>
<td>30</td>
<td>80</td>
<td>—(^d)</td>
</tr>
<tr>
<td>Greece</td>
<td>37(^c)</td>
<td>95(^c)</td>
<td>Task 3.2.13</td>
</tr>
<tr>
<td>Ireland</td>
<td>23</td>
<td>75</td>
<td>Task 3.2.11</td>
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<td>Task 3.2.13</td>
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<tr>
<td>Norway</td>
<td>80</td>
<td>165</td>
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<tr>
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<tr>
<td>The Netherlands</td>
<td>10</td>
<td>—</td>
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<tr>
<td>UK</td>
<td>14</td>
<td>—</td>
<td>Task 3.2.11</td>
</tr>
</tbody>
</table>

\(^a\) In general, the 95\(^{th}\) percentile.

\(^b\) Fish only.

\(^c\) Seafood other than fish only.

\(^d\) National Consumption Survey (1985-1988)

\(^e\) Bergsten C. (2004), personal communication.
Annex 3. Genotoxicity


**Tributyltin oxide (TBTO)**

The genotoxic potential, as reported by US EPA (1997) and WHO-IPCS (1999b) was evaluated in multiple *in vitro* and *in vivo* short-term tests by Davis *et al.*, (1987): TBTO was negative in the following tests: “Rec Assay” in *Bacillus subtilis*, reverse mutations in *Salmonella typhimurium* strains TA1530, TA1535, TA1538, TA97, TA98 and TA100 with and without metabolic activation (rat liver S9), gene mutations in *Schizosaccharomyces pombe*, gene mutations in cultured V79 Chinese hamster cells or in mouse lymphoma cells tk assay in the presence or absence of metabolic activation, mitotic gene conversion in *Saccharomyces cerevisiae*, sister chromatid exchanges (SCEs) in cultured Chinese hamster ovary (CHO) cells in the presence or in the absence of metabolic activation, recessive lethal mutations in adult males of Drosophila melanogaster by either feeding or injection and did not induce X-linked recessive mutations.

TBTO was mutagenic in *S. typhimurium* strain TA100 in a fluctuation test at cytotoxic concentrations only in the presence of rat liver S9 and induced structural chromosome aberrations, endoreduplicated and polyploid cells in cultured CHO cells. The aberrations were observed only after 8 hours treatment (not after 15 or 24 hours) and only at the highest, cytotoxic concentrations in the presence of metabolic activation (S9 Mix). TBTO increased the number of micronuclei in bone marrow polychromatic erythrocytes of male BALB/C mice 48 hours after a single oral dose (60 mg/kg b.w.). However a re-analysis of the slides failed to confirm the increase in micronuclei (WHO-IPCS, 1990).

It has been shown that TBTO and (TPTCl) produced a 50 % increase of the frequency of micronuclei induced by Mitomycin C in mouse peripheral reticulocytes, when 50 mg TBTO/kg b.w. and 100 mg TBTCl/kg b.w. were given orally to mice. No effect was observed when the chemicals were administered alone (Yamada and Sasaki, 1993).

In conclusion, the weight of evidence suggests that TBTO is devoid of significant genotoxic potential.

**Dibutyltin dichloride (DBTCl₂)**

DBTCl₂ was found able to induce gene mutations in the CHO/HGPRT mutation assay up to 0.2 μg/ml (Li *et al.*, 1982). DBTCl₂ was not mutagenic in the Ames test (Clark, personal communication).
Triphenyl Compounds

In vitro

TPTOH (purity not stated) and TPTAc (97.1 % pure) did not induce gene mutations in five strains of Salmonella typhimurium and one strain of Escherichia coli with and without S9 metabolic activation up to mildly cytotoxic concentrations (5 µg/plate) (TPTOH) or clearly cytotoxic concentrations (20 µg/plate) (TPTAc) (Richold et al., 1981; Jung and Weigand, 1986).

TPTOH (97.2 % pure) and TPTAc (97.4 % pure) did not induce gene mutations in the yeast Schizosaccharomyces pombe (Milone and Hirsch, 1985; Milone, 1986).

TPTOH (97.2 % pure) did not induce mitotic gene conversion in the yeast Saccharomyces cerevisiae with and without metabolic activation (Milone and Hirsch, 1985). An almost identical gene conversion study in S. cerevisiae with TPTAc (97.1% pure) produced also negative results (Milone, 1986).

TPTAc (97.1 % pure) did not induce gene mutations at the hprt locus in Chinese hamster V79 cells up to cytotoxic concentrations with and without metabolic activation (Heidemann, 1986).

TPTOH (purity not given) was weakly positive in the mouse lymphoma tk assay in the absence of metabolic activation and negative in the presence of metabolic activation (N.N., 1984).

In another study with the mouse lymphoma tk assay TPTOH was weakly positive only in the presence of metabolic activation (DenBoer and Hoorn, 1985).

TPTOH (96.2 % pure) induced chromosome aberrations in cultured human lymphocytes at the highest concentration (1.47 µg/ml) in the presence of metabolic activation (Nunziata, 1988).

TPTOH (97.2 % pure) induced chromosome aberrations in cultured human lymphocytes in the absence of metabolic activation with a clear dose-response up to 1 µg/ml (Kirkland, 1985).

Chao et al., (1999) have shown that TPTAc induced a dose-related increase of micronuclei in CHO cells in the absence of S9 metabolic activation. In the presence of metabolic activation it induced a significant increase of micronuclei only at the highest dose tested (150 ng/ml). In the same type of cells it induced sister chromatid exchanges (SCEs) only in the presence of metabolic activation. TPTOH induced a significant increase of micronuclei in CHO cells at the highest concentration (150 ng/ml) both in the presence and in the absence of S9 and SCE only in the absence of S9.
TPTOH (97.2 % pure) did not induce unscheduled DNA synthesis (UDS) in rat hepatocytes cultures up to cytotoxic concentrations (≤ 0.5 µg/ml) (Cifone and Mihr, 1985).

**In vivo**

TPTOH (97.2 % pure) was tested for induction of micronuclei in the bone marrow cells of NMRI mice (6 animals per sex). The animals were dosed by gavage in carboxymethyl cellulose at 0, 35, 70 or 140 mg/kg b.w., with sampling times at 24, 48 and 72 hours after dosing. 1000 PCE per animal were analysed. The top dose was 80 % of the LD₅₀ and produced initial signs of toxicity (sedation and ataxia). The PCE:NCE ratio was not altered. Only slight increases of micronuclei were observed at the top dose. Based on this study there was no clear evidence of genotoxicity (Banduhn et al., 1985).

In a very similar bone marrow micronucleus assay TPTAc (97.4 % pure) was tested in CD-1 mice dosed by gavage in sesame seed oil at 0, 15, 50, or 150 mg/kg b.w.. The PCE: NCE ratio was clearly altered at the top dose. A dose-related increase in micronuclei/1000 PCE was observed only after 24 hours from dosing and none of the mean values was statistically significant. In conclusion, there was no convincing evidence of genotoxicity of TPTAc in this study (Loquet, 1985).

TPTOH (96.2 % pure) was tested for induction of chromosome aberrations in bone marrow cells of Chinese hamster treated by gavage in starch mucilage at 0, 20, 50 or 80 mg/kg b.w.. The mitotic index was reduced by 30 - 50 % in the top dose. There was evidence of a weak clastogenic effect only in the females and only at 12 and 24 hours, of equivocal biological significance. Based on this study there was no clear evidence of *in vivo* genotoxicity (Mueller, 1987).

Oral gavage of TPTAc in single (LED: 12.50 mg/kg b.w.) or triple treatment (at 2.0, 10.0 or 20.0 mg/kg b.w.) resulted in a significant increase of micronuclei in peripheral erythrocytes in BALB/C mice. Ip administration of TPTAc at 18.75 or 25 mg/kg b.w., and 15 or 20 mg/kg b.w. for single or triple treatment respectively, resulted in serious lethal response, with only random increases possibly due to toxic effects. On the basis of this study TPTOH induced micronuclei *in vivo* only after a single oral treatment (LED: 2.50 mg/kg b.w.) (Chao et al., 1999).

TPTOH (94.8 % pure) was tested for induction of dominant lethal mutations in Sprague-Dawley rats administered at 0, 3, 20, 38 or 150 mg/kg b.w./day by gavage in corn oil for 5 consecutive days. At 150 mg/kg b.w. pre-implantation loss was increased, but the ratio of total dead implants: total implants were unaffected. On the basis of this study TPTOH did not induce dominant lethal mutations in germ cells of SD rats at sub lethal doses (Ravert et al., 1978).

Sasaki et al., (1993) showed that three TPT compounds and three TBT compounds enhance the frequency of chromosome aberrations (of breakage-chromatid type) induced in Chinese
hamster CHO K1 cells during the G2 phase by five S-phase dependent well-known clastogens (Mitomycin C, cisplatin, 4-nitroquinoline-1-oxide, methyl methanesulfonate, Actinomycin D). Similarly, the frequency of micronuclei induction by Mitomycin C in mouse peripheral reticulocytes was enhanced by treatment with TPTCl, although TPTCl itself did not induce micronuclei (Yamada and Sasaki, 1993).

In conclusion, TPTOH is not genotoxic in several studies in vitro for genetic end-points like gene mutations, gene conversion and UDS. It is a weak in vitro clastogenic agent in human lymphocytes. So far, its capacity to induce chromosome aberrations has not been clearly shown in vivo. Similarly, TPTAc is not genotoxic in vitro for genetic end-points like gene mutations in bacterial or mammalian cells and gene conversion in yeast. In vitro it induces micronuclei and SCEs in CHO cells. Its capacity to induce in vivo micronuclei in bone marrow cells of mice is equivocal. However, it is able to induce micronuclei in peripheral erythrocytes in mice.

**Dioctyltin**

Octyltin stabilizers have been assessed by SCF for their genotoxic potential in September 1999. Di-n-octyltindichloride was not mutagenic in bacterial and fungal cells. It induced gene mutations in two tests with mammalian cells in vitro in the absence of metabolic activation. It was negative in an in vitro test of UDS in rat hepatocytes and in an in vivo host-mediated assay with mouse lymphoma cells in mice. It was also negative in an in vivo micronucleus and SCE assays. Based on the negative results in two other in vitro mammalian gene mutation assays and on the lack of covalent binding to rat hepatic or thymic DNA in vivo and to calf thymus DNA or to DNA of CHO cells in vitro, it was assumed, as an overall evaluation, that di-n-octyltindichloride does not possess genotoxic properties.
Annex 4. Reproductive and developmental toxicity

Tributyltin compounds

One and two generation studies

In a two-generation reproduction study, groups of 30 male and 30 female Crl:CD(SD)BR rats were fed 0, 0.5, 5.0 or 50 mg TBTO/kg diet (purity 97.1 %) for 10 weeks prior to mating and during cohabitation (7 days), with exposure of females continuing during gestation and lactation (F0 generation). Groups of 30 male and 30 female offsprings (F1 rats) were fed the parental diets for 15 weeks and mated to produce the F2 generation. Based on food consumption and body weight data, the respective mean compound intakes during the pre-mating period were estimated as: 0, 0.02, 0.29 and 2.95 mg/kg b.w./day for F0 males; 0.03, 0.34 and 3.43 mg/kg b.w./day for F0 females; 0, 0.03, 0.36 and 3.98 mg/kg b.w./day for F1 males; and 0.04, 0.44 and 4.42 mg/kg/b.w./day for F1 females.

Body weight gain was significantly reduced in high-dose F1 males and females (approximately 19 and 15 % lower than controls, respectively) at the beginning of the pre-mating growth period and remained reduced in males throughout the entire (15-week) pre-mating period (> 8 %). No significant changes in body weight gain occurred in F1 males during the post-mating period, although body weight was significantly lower than controls at week 38 (> 8 %) at the high dose. No treatment-related effects on food consumption or gross examination or histopathology were found in either sex or generation. Relative thymus weights were slightly but not significantly lower than control values in F0 males and females at the high dose (8 and 17 %, respectively) and significantly lower than controls in F1 males and females at the high dose (31 % and 26 %, respectively). No histological changes in the thymus were found. Compound-related reproductive and developmental effects were limited to decreased pup body weight during lactation in both generations at the high dose (> 17 % and 20 %, in F1 and F2 pups on day 21, respectively).

The NOAEL for adult toxicity was 0.29 mg/kg/day with decreased thymus weight at higher doses and the NOAEL for reproductive and developmental toxicity was 0.34 mg/kg with decreased pup body weight during lactation at higher doses.

In a two-generation study, Wistar rats (P0 = 10/sex/group; F1 = 13 - 18/sex/group; were exposed to dietary concentrations of 5, 25, and 125 mg TBTCI/kg diet (Ogata et al., 2001, Omura et al., 2001). Treatment of P0 (both sexes) started from pregnancy day 0 (sperm positive vaginal smear) and animals were euthanized at the weaning of F1. Treatment of F1 continued throughout mating (postnatal day 92), a 14-day cohabitation period, pregnancy, lactation and up to weaning of the F2. F2 males were killed on postnatal day 119 and F2 females were killed on postnatal day 91; F2 animals were not mated.
Male effects

A dose-related decrease of the testis weights were seen in all dose groups of F1, (6 %, 7 %, 18 % respectively) and at the top dose of F2 (20 %). A significant and dose-related decrease of the epidydimal weight was observed at the top dose only (14 % and 12 % in F1 and F2, respectively). The weight of the ventral prostate was decreased at the top dose in F1 (16 %) and at the two highest doses in F2 (16 % and 31 %, respectively). Homogenization-resistant spermatid counts were significantly reduced at the top dose (F1 20 %, F2 25 %) and in F2 males at 25 mg/kg (10 %). Sperm count was reduced in F2 males at 125 mg/kg (25 %). Slight histopathologic changes were also observed in the testis at 125 mg/kg and included vacuolization of the seminiferous epithelium, spermatid retention, and delayed spermiation.

While the serum concentration of luteinizing hormone was unaltered, the serum 17beta-estradiol concentration was decreased to 55 % of the control value in the 125 mg/kg diet group in the F1 generation and in the F2 generation decreased to 57 % and 78 of the control value in the 25 mg/kg diet and 125 mg/kg groups, respectively. Testosterone was concurrently increased at top dose in both F1 (90 %) and F2 males (40 %).

Effects on female reproduction and litters.

At the top dose, decreased number of live pups (18 % and 25 % in F1 and F2, respectively), livebirth index (8 % in both generations), body weight of pups on day 1 (18 % in both F1 and F2) and the pup weight gain up to postnatal day 14 (20 - 30 %) were seen. A dose-related increase in anogenital distance was found in all treated groups on postnatal day 1 in the F1 (6 %, 7 % and 9 %, respectively), suggesting an antiestrogenic effect on female neonates while in F2 pups the increase was present at the top dose only on postnatal day 1. At postnatal day 4 in F1 and F2 pups, only at 125 mg/kg a significant increase was present. Eye opening showed a one-day delay in both F1 and F2 pups at top dose. A delay in vaginal opening of about one week was observed in both F1 and F2 at top dose, accompanied by a significantly lower growth in F2. The percentage of animals with normal estrous cyclicity was significantly reduced in the 125 mg/kg group in both F1 and F2 females (60.5 % and 63.2 %, respectively, vs. 88.9 % and 85.3 % in controls). Ovarian relative weight was significantly reduced (17 %) in F1 top dose animals and uterine weight was significantly increased in F2 top dose females (27 %). No histopathological changes were present in these organs and no changes in serum estradiol or testosterone concentrations were detected.

The altered oestrus cyclicity, the reduced oestradiol and increased testosterone levels suggest that TBTCI might be an aromatase inhibitor. Although marked reproductive effects were observed only at high dose level, a NOAEL could be established because of the dose-related decrease of testis weight and increase of ano-genital distance observed in F1 at all dose levels. Therefore, the dietary concentration of 5 mg/kg diet, equivalent to 0.25 mg/kg b.w., should be considered as a marginal LOAEL.
Developmental toxicity studies

Groups of 24 mated female CD Sprague-Dawley rats were treated with TBTO (purity 96.9 %) in corn oil by gavage at doses of 0, 5, 9 or 18 mg/kg/day on days 6-19 of gestation (Schroeder et al., 1981). The dams were sacrificed on gestation day 20.

Clinical signs (staining of the fur in the anogenital area) and decreased body weight gain during days 6 - 20 occurred in maternal rats at the mid- and high-dose (> 1.8 % and 26 %, respectively). Adjusted weight gain (excluding uterus) was 5.5, 22.2 and 69.4 % lower than controls at the low-, mid- and high-dose, respectively. Based on decreased adjusted weight gain, the LOAEL for maternal toxicity was 5 mg/kg b.w./day, and a NOAEL could not be established.

Indications of developmental toxicity were observed in all dose groups. Effects included dose-related increased incidences of minor anomalies of the axial skeleton, particularly asymmetric sternebrae, rudimentary ribs, and 14th rib pair. Increased incidences of other skeletal anomalies (cervical unilateral and bilateral ribs, unossified caudal vertebrae) and some cases of malformations (cleft palate) were observed at the high dose. Evaluation of these data is complicated by lack of statistical analysis and litter incidences, however, percentages of foetuses with at least one skeletal anomaly was significantly increased at the mid- and high-dose. Other effects occurred at the high-dose, including a significantly decreased percentage of foetuses to implants (86.8 % compared with 94.7 % in controls), increased percentage of resorptions (13.2 % compared with 5.3 % in controls) and decreased fetal weight (16 % lower than controls in both sexes). The LOAEL for developmental toxicity was 5 mg/kg/day.

Groups of 118, 12, 10, 22, 20, 12 and 6 mated NMRI mice were treated with 0, 1.2, 3.5, 5.8, 11.7, 23.4 or 35.0 mg/kg/day TBTO in olive oil by gavage on gestation days 6 - 15 (Davis et al., 1987). Animals were sacrificed on gestation day 18.

Slight maternal toxicity, indicated by reduced body weight gain, was observed at 11.7 and 23.4 mg/kg/day and one case of maternal death was recorded at the highest dosage. A dose-related increased frequency of cleft palate were found in foetuses (0.7, 0.8, 3.0, 2.0, 7.0, 24.0 and 48.0 %).

Effects observed at 23.4 and 35.0 mg/kg/day included reduced average foetal body weight (8 and 20 %, respectively), increased number of foetuses with minor skeletal abnormalities (28 and 29 %, respectively, compared with 0.5 % in controls) and skeletal variations (43 and 43 %, respectively, compared with 10 % in controls). Resorption rate was increased at 35 mg/kg/day (58.8 % vs. 8.3 - 15.7 % in the control and other groups). The number of resorptions/litter and percentage of litters with resorptions also were increased.

In an accompanying experiment by Davis et al., (1987), no embryonic damage (assessed using electron microscopy) was found in mice 26 and 48 hours after treatment with a single 30 or 110 mg/kg dose of TBTO on gestation day 10. Based on reduced body weight gain in
dams and increased cleft palate in foetuses, the NOAEL for maternal and developmental toxicity was 5.8 mg/kg/day.

Developmental toxicity was evaluated in Long-Evans rats exposed pre- or postnataally to TBTO (purity 97 %) in corn oil by gavage (Crofton et al., 1989). Rats were administered doses of 0, 2.5, 5.0, 10, 12 or 16 mg/kg/day (15 - 18 rats/group) on days 6 - 20 of gestation.

Effects observed included vaginal bleeding in 60 and 75 % of the rats administered 12 and 16 mg/kg/day, respectively. Maternal body weight gain was significantly reduced at 10 and 12 mg/kg/day, and body weight was decreased at 16 mg/kg/day. One dam in each of the 10-, 12- and 16 mg/kg/day groups died during the study. Litter size and pup body weight (at postnatal days 1 and 3) were significantly reduced at 10, 12 and 16 mg/kg/day. Litter sizes on postnatal day 1 were 50, 73 and 96 % lower than control values at 10, 12 and 16 mg/kg/day, respectively. Pup survival on days 1 - 3 also was also decreased in these groups. There were no significant changes in litter size or neonatal pup weight in the groups treated with 2.5 or 5 mg/kg/day. Postnatal mortality was increased (14 %) on day 21 at 10 mg/kg/day, and body weight gain was decreased up to postnatal day 19 at 10 mg/kg/day. There was a significant delay in age of vaginal opening in 10 mg/kg/day offspring, the sexual maturity in males was not altered. Motor activity was significantly reduced on postnatal days 47 and 62 at 10 mg/kg/day. No effects on acoustic startle response were observed in the prenatally exposed rats. Whole brain, cerebellum, and hippocampus weights were reduced significantly following exposure to 10 mg/kg/day (measured on postnatal day 110). The NOAEL for maternal and developmental toxicity was 5.8 mg/kg /day.

Rats were treated with a single oral dose of 0, 40, 50, or 60 mg/kg TBTO in corn oil on postnatal day 5 and sacrificed on day 64 (Crofton et al., 1989). Mortality was increased in rats treated with 50 or 60 mg/kg (32 %), and body weight was 25 % lower than controls at all dosages (40 - 60 mg/kg) by day 10. Body weight remained reduced on postnatal day 30, but recovered by postnatal day 62 at 40 and 50 mg/kg (still decreased at 60 mg/kg). No changes in motor activity were observed. Amplitude of response in the acoustic startle test was decreased in all groups (40 - 60 mg/kg) on day 22, but this effect did not persist to day 47 or 62 and was not accompanied by significant alterations in latency to onset or number of responses. Whole brain and cerebellum weights were significantly reduced at 60 mg/kg (measured on postnatal day 64).

Inseminated female Wistar rats were orally administered TBTCl, dissolved in olive oil, at 8.1, 16.3, or 32.5 mg/kg on days 0-3 of pregnancy (12 - 16 dams/group), or at 8.1, 16.3, 32.5, or 65.1 mg/kg on days 4 - 7 of pregnancy (11 - 13 dams/group). Pregnancy outcome was determined on day 20 (Harazono et al., 1998). There was no apparent maternal toxicity in any treated group. TBTCl on days 0 - 3 of pregnancy at 8.1 mg/kg and higher produced a dose-related increase in pregnancy failure (0 %, 15.4 %, 62.5 % and 87.5 %, respectively). There was also a dose-related increase of the incidence of post-implantation losses at 16.3 mg/kg and higher (7.0, 7.2, 10.1 and 24.8 % per litter, respectively). TBTCl on days 4 - 7 of pregnancy caused a significant increase of pregnancy failure at top dose only (38.5 % vs. 0 in
control). There was a marked and dose-related increase in the incidence of post-implantation loss at 16.3 mg/kg and higher (6.0, 5.6, 26.5, 32.4 and 52.5 % per litter, respectively), accompanied by a concurrent, dose-related reduction in the number of live foetuses per litter (average litter size 14.2, 15.3, 11.2, 10.2 and 7.1, respectively). No increase in the incidence of fetal malformations was found in any TBTCl-treated groups. Mean fetal weight was significantly reduced at 8.1 mg/kg and higher (days 0 - 3 of pregnancy) and at 16.3 mg/kg and higher (days 4 - 7 of pregnancy). The early pre-implantation period appears as a phase especially sensitive to the effects of TBTCl. No NOAEL could be established in this study.

TBTCl in olive oil was given orally to pseudopregnant rats at doses of 4.1, 8.1, 16.3 and 32.5 mg/kg on pseudopregnant day (PPD) 0 to PPD 3 or 8.1, 16.3, 32.5 and 65.1 mg/kg on PPD 4 to PPD 7. The decidual cell response was induced by bilateral scratch trauma on PPD 4. The uterine weight on PPD 9 served as an index of uterine decidualization. Uterine weight and serum progesterone levels on PPD 9 were significantly decreased after administration of TBTCl at doses of 16.3 mg/kg and above on PPD 0 to PPD 3 or PPD 4 to PPD 7. Administration of TBTCl at doses of 8.1 mg/kg and above on PPD 0 to 3 also significantly decreased serum progesterone levels on PPD 4. TBTCl had no effect on ovarian weight and number of corpora lutea. Overall these studies indicate that TBT impairs uterine decidual cell response and decreases progesterone levels in rats eliciting early pregnancy loss. The overall NOAEL was 4.1 mg/kg b.w./day

Dibutyltin compounds

Pregnant Wistar rats (10 - 12/group) of 3, 7.5 or 12 months were treated orally with DBTAc dissolved in olive oil at 0, 7.5, 10, 15 or 22 mg/kg on day 8 of gestation (Noda et al., 2001). Caesarean sections were performed on day 20 of gestation. Reduced body weight gain was observed at all dose levels in dams at 7.5 and 12 months, whereas no significant effects were observed at 3 months of age. Live litter size was significantly affected in the 7.5-month dams at all dose levels, whereas no effects were present in the 3-month dams. The death of most of the foetuses of the 12-month dams made it difficult to evaluate the teratogenic potential. In the 3-month groups, foetuses with external malformation, such as cleft mandible, cleft lower lip, ankyloglossia and/or schistoglossia were observed at 15 and 22 mg/kg (overall incidence of 28.4 % and 61.8 %, respectively), while similar malformations were observed in 7.5-month groups at doses of 10 mg/kg and above (overall incidence 7.9 %, 34.8 % and 64 % at 10, 15 or 22 mg/kg, respectively. The results suggest that DBTAc possess a teratogenic and embryotoxic potential, and that in older dams effects may be more marked. A NOAEL could not be established.

Pregnant Wistar rats (n = 19 - 23/group) were administered 1, 2.5, 5 or 10 mg DBTCl2/kg b.w. orally in olive oil on day 6 - 15 of pregnancy (Farr et al., 2001). Signs of maternal toxicity, including significantly decreased food consumption (8 %), body weight gain (18 %),
and thymus weight (25 %), were observed at 10 mg/kg body weight. At this dose, no evidence of embryotoxicity, including resorptions, viable foetuses, or fetal weights, was noted in any litter data. There was a slightly increased frequency of total malformations at the 10 mg/kg dose level (1.5 % in treated vs. 0.25 % control foetuses; 15 % of treated litters vs. 0.5 % in controls). Malformations included ankyloglossia, internal hydrocephalus, agnathia without a clear pattern. The overall NOAEL for both maternal and developmental toxicity was 5 mg/kg b.w./day.

**Triphenyltin**

**Reproductive toxicity**

No additional data are available to those evaluated by ECCO and WHO/IPCS mentioned earlier. To summarize, TPT impairs spermatogenesis in adult rats at 20 mg/kg b.w./day and no NOEL could be established. In a two-generation study, reduced litter size, weight gain and survival, as well as reduced spleen and thymus weight were observed in rat pups at exposure levels not inducing toxicity in adults. The NOEL was 0.4 mg/kg b.w./day.

**Developmental toxicity**

In a series of studies evaluated by ECCO and WHO/IPCS, prenatal toxicity and concurrent maternal toxicity were observed in rats and rabbits. The lowest NOELs for maternal and prenatal toxicity were 0.1 and 0.3 mg/kg b.w./day, respectively, in rabbits. In the rat, the NOEL for maternal toxicity and prenatal toxicity were 1.0 and 3.2 mg/kg b.w./day, respectively.

Female Wistar KY rats (10 - 12/group) were given TPTC1 by gastric intubation (3.1, 4.7, and 6.3 mg/kg dissolved in olive oil) on pseudopregnant day (PPD) 0 to 3 and the decidual cell response was induced on PPD 4 (Ema *et al.*, 1999). The uterine weight on PPD 9 served as an index of uterine decidualization. There was no sign of general toxicity in TPT-treated animals. A significant decrease in the uterine weight, which indicates suppression of the uterine decidualization, was detected at 4.7 and 6.3 mg/kg (– 50 % and – 75 %, respectively). The ovarian weight and number of corpora lutea in the TPTC1-treated groups were comparable to that of the controls. A significant decrease in serum progesterone levels after administration of TPTC1 was found at 4.7 and 6.3 mg/kg, which was roughly parallel to the effect on uterine weight. Overall TPT induced effects on implantation in rats analogous to TBT, although it appears somewhat more potent. The NOAEL was 3.1 mg/kg.

**Supportive in vitro studies**
TBT was found to be a partial competitive inhibitor of aromatase activity with an IC50 value of 6.2 µM with 0.1 µM androstenedione as substrate (Heidrich et al., 2001). TBT impaired the affinity of the aromatase to androstenedione but did not affect electron transfer from NADPH to aromatase via inhibiting the NADPH reductase. DBT acted as a partial but less potent inhibitor of human aromatase activity (65 % residual activity), whereas TeBT and MBT had no effect. The residual activity of TBT-saturated aromatase was 37 %. In contrast, human 3beta-HSD type I activity was only moderately inhibited by TBT (80 % residual activity). Moreover, neither TeBT or DBT nor MBT inhibited the 3beta-HSD type I activity.

5alpha-reductase type 1 was completely inhibited by TBT chloride (IC50 = 19.9 µM) and DBT dichloride (IC50 = 32.9 µM), whereas 5alpha-reductase type 2 was only inhibited by TBT chloride (IC50 = 10.8 µM) (Doering et al., 2002). Both isoenzymes were not affected by TeBT or MBT indicating that at least two butyl groups bound to the positively charged Sn are required for the interaction of butyltins with the enzymes. TBT inhibited 5alpha-reductase type 1 competitively whereas an irreversible inhibition was evident for the type 2 isoenzyme. In contrast to the distinct effects on 5alpha-reductases, reductive brain 17beta-hydroxysteroid dehydrogenase activity was not inhibited by any butyltin.

TBT and TPT were assayed on a LA16 clone that stably expresses androgen-responsive luciferase reporter gene and proliferates in response to androgen, established from human prostate cancer cell line LNCaP (Yamabe et al., 2000). Stimulation of LA16 cells with 100 nM TBT or 1 nM TPT enhanced both AR-dependent transcription of luciferase gene and cell growth to the same extent as those by 1 nM dihydrotestosterone (DHT). TBT or TPT also enhanced the DNA synthesis and expression of endogenous AR target genes such as prostate specific antigen, but not the expression of AR itself. However, an androgen antagonist, flutamide, did not inhibit the TBT- or TPT-induced AR activation. On the other hand, simultaneous treatment of LA16 cells with DHT and TBT or TPT caused highly enhanced effects on AR activation. Therefore, TPT can activate AR-mediated transcription in mammalian cells without a direct interaction with the ligand-binding site of androgen receptor.

Overall these results indicate that TBT and, to a lesser extent, DBT may disrupt sex steroid metabolism by affecting both androgen activation and aromatase-mediated synthesis of estrogens. Moreover, TBT can activate AR-mediated transcription in mammalian cells without a direct interaction with the ligand-binding site of androgen receptor.

Octyltins

Octyltin compounds have been assessed by the EC Scientific Committee on Food in 1999 (EC, 1999) based on the following studies.
In a two-generation reproduction toxicity study, Sprague-Dawley rats were fed diets containing 0, 20, 60 or 200 mg/kg mixture of 80 % di-n-octyltin di-isoctylthioglycolate (DOTTG) and 20 % mono-n-octyltin di-isoctylthioglycolate (MOTTG), corresponding to about 0, 1.9, 5.4 or 17.1 mg/kg b.w./day (Mitterer, 1997). In the highest dose feed group (F0-parental generation) a statistically significant reduction of the thymus weight and an increased thymus involution in both sexes were observed. In the F1-pups the incidence of thymic involution was increased at the highest dose in the males. The relative thymus weight was decreased (dose-related) in both sexes from 60 mg/kg b.w./day onwards. The only observed reproduction effects were a statistically significant increased number of stillbirths including foetal residues and a statistically significant decrease in F1-pup weight (both males and females) at the highest dose. No effects were observed in the F2-pups. For the mixture DOTTG/MOTTG, the dose level without adverse effects NOAEL was 20 mg/kg feed, equal to 1.9 mg/kg b.w./day (1.5 mg/kg b.w./day for DOTTG), based on thymus effects.

Two teratogenicity studies, one in rats and one in rabbits were also evaluated by SCF (EC, 1999).

A formulation of 80 % DOTTG and 20 % MOTTG was administered by gavage from day 6 - 15 of gestation, to 4 groups of wistar rats, at dose levels of 0, 1, 5 or 25 mg/kg b.w./day (Pharma Research Report, 1993, unpublished). At the highest dose 7 foetuses died, leading to an increase in the % of dead foetuses. No irreversible structural changes were observed. The dose level without maternal and/or embryo-foetotoxicity was 5 mg/kg b.w./day (equivalent to 0.77 mg Sn/kg b.w./day), based on decreased body weight in dams and on pup mortality.

The same formulation of 80 % DOTTG and 20 % MOTTG was administered by gavage from day 6 - 18 of gestation to four groups of NZW rabbits, at dose levels of 0, 1, 10 or 100 mg/kg b.w./day (Pharma Research Report, 1992, unpublished). An increased rate of abortions and of early resorptions as well as a statistically significant reduction of the mean foetal weight were observed at the highest dose tested. In the highest dose group a statistically significant increased incidence of minor visceral anomalies of the thoracic and abdominal cavities in foetuses was observed. Also an increased incidence of minor skeletal head anomalies in foetus from 10 mg/kg b.w./day onwards, was observed. In the 100 mg/kg group an increased incidence in the number of foetuses with variations of feet bones was observed. The abortions, resorptions and minor anomalies were considered as indicative of an embryo-foetal toxicity. No irreversible structural changes were observed. The dose level without maternal toxicity was 100 mg/kg b.w./day (highest dose tested) and that without embryo-foetotoxicity was 1 mg/kg b.w./day (equivalent to 0.15 mg Sn/kg b.w./day), based on minor visceral and skeletal anomalies.

After the SCF evaluation a new developmental toxicity study in mice was made available on octyltin stabilizers (Faqi et al., 2001). A mixture of 80 % DOTTG and 20 % MOTTG was used to treat orally NMRI mice with doses of 20, 30, 45, 67, or 100 mg/kg b.w./day from gestation day 6 through 17. Resorption rates were significantly increased and foetal weights significantly reduced at the two highest doses. External anomalies, such as bent forelimbs,
cleft palate and exencephaly were observed at 100 mg/kg b.w./day, with the 67 mg/kg b.w./day dose also producing a significant increase in cleft palate. Skeletal anomalies were increased in foetuses exposed to 100 mg/kg b.w./day. The doses of 20, 30 and 45 mg/kg b.w./day elicited a significant increase in supernumerary lumbar ribs. The study showed that the mixture DOTTG/MOTTG produces embryo-foetotoxicity and birth defects in mice, with a NOAEL of 45 mg/kg b.w./day for malformations. The maternal NOAEL was 30 mg/kg b.w./day, whereas no NOAEL was identified for skeletal anomalies (supernumerary lumbar ribs).

In conclusion TBT and, with lesser evidence, DBT and TPT affect the metabolic pathways of steroids. No such evidence is yet supported for TeBT and MBT.

A NOAEL for short-term exposure to TPT or TBT was established at 4 mg/kg based on early pregnancy loss in rats. This NOAEL may be consistent with the LOAEL of 5 mg/kg for skeletal anomalies in rats induced by TBT, as reported by the US EPA. I.R.I.S.

DBT elicits teratogenicity and embryotoxicity in rats at dose levels inducing also maternal toxicity. The higher impact on older dams and dependency on chemical structure (DBTAc being more potent than DBTCI) need further clarification. A NOAEL for both maternal and developmental toxicity of DBTCI was established at 5 mg/kg b.w./day.

A NOAEL for long-term (two-generation) exposure is not available since a dose-related increase of ano-genital distance observed in female pups at all dose levels in the most recent two-generation study. Therefore, the dietary concentration of 5 mg/kg diet, equivalent to 0.25 mg/kg b.w./day, has to be considered as a LOAEL. Overall, the studies on TPT already evaluated by ECCO and WHO/IPCS indicate effects consistent with those induced by TBT, from both a qualitative and a quantitative point of view.
Annex 5. Neurotoxicity

The neurotoxic potency of OTC differs substantially and for some OTC it is still partially unexplored. Trimethyltin (TMT) and triethyltin (TET) are well-known potent neurotoxic compounds that induce a consistent picture of damage in humans and animals. There is a consistent, but differential, target for the neurotoxic effects of TMT and TET. TET primarily targets myelin sheaths. It causes interstitial oedema throughout the white matter of the central nervous system, particularly the brain; less marked damage occurs in the peripheral nervous system. TET is primarily associated with a diffuse leukencephalopathy which is histopathologically characterized by a vacuolar myelopathy. Intoxications in humans and in animals are associated with seizures, visual disturbances and parapareses. TMT also causes severe damage to the central nervous system; however, the effect is neuronal necrosis, rather than oedema. It targets preferentially neurons, in particular of the limbic system. TMT is mainly associated with limbic seizures and memory deficits (Krinke 2000a,b).

Other OTC, especially the intermediate trialkyltins tripropyltin (TPrT) and TBT, as well as TPT and the higher homologues trihexyltin (THT) and trioctyltin (TOT) are less or not neurotoxic at all. Clear-cut evidence for human neurotoxicity is not reported with the exception of TMT, TET and TPT. Also systematic experimental dose-response studies on their comparative in vivo neurotoxicity are rare and there is a substantial lack of in vivo animal data on neurotoxic effects of these compounds. Also, not much is known on the mechanistic level, e.g. the pathogenesis leading to cell damage by TET and TMT. However, in vitro data show that all toxic OTC seem to share the mechanism of disrupting chemical energy generation.

Cellular and biochemical mechanisms

It is well established that the nervous system in general and neurons in particular are critically dependent from a continuous supply of chemical energy to stabilize their membrane potentials. A common theme of the biochemical effects of various OTC seems to be mitochondrial toxicity (Snoeij et al., 1986a,b, 1987). TET decouples oxidative phosphorylation and inhibits the mitochondrial ATPase (Jakobs et al., 1983; Stockdale et al., 1970). It has also been shown that the mitochondrial ATPase is inhibited by TMT (Aldridge, 1977b). Furthermore Aldridge (1976) showed in liver mitochondria that the order of effectiveness in causing 50 % inhibition of ATP production was found to be TET > TPrT > TBT > THT > TMT. The fact that TET was the most effective compound and that the other established human neurotoxin TMT was the „weakest“ compound, leads to the conclusion that all OTC are potentially potent neurotoxins. Also the dialkyltin compounds, such as DMT, DET, DBT and DOT have similar biochemical effects in vitro (Snoeij et al., 1987; Boyer, 1989). Consistent with this view are the results of the studies on calcium load and apoptosis in rat pheochromocytoma PC 12 cells after exposure to TBT and TPT. Both compounds
elicited a sustained increase in intracellular Ca\(^{2+}\) concentration and induced apoptotic cell death. However TET modified only slightly the Ca\(^{2+}\) concentration and TMT had no effect. As there is no apparent correlation with the \textit{in vivo} situation, it was concluded that cell specific mechanisms and/or different tissue levels achieved in the nervous system may be responsible for the selective \textit{in vivo} neurotoxicity of these compounds (Viviani \textit{et al.}, 1995). Various immortalized cell lines and primary neuronal cultures were used to characterize the selective toxicity of TMT, TET and TBT. Primary neuronal cell cultures were very sensitive to these OTC and showed pattern of selective toxicity with respect to neuronal and glial cells. In addition it was shown that a protein - stannin - was expressed in those cell lines and primary neurons in culture which are sensitive for TMT. Further data suggest that stannin expression may play a role in apoptosis and cellular death caused by TMT (Thompson \textit{et al.}, 1996). It seems also possible that the toxic effects on mitochondria affecting energy production and in consequence membrane potentials will make neurons more vulnerable to excitatory amino acids. Therefore excitotoxic principles may play an important role in the induction of cell damage by TMT.

**Tributyltin (TBT) and dibutyltin (DBT)**

Some experiments indicate that the blood brain barrier is not preventing transfer of TBT or DBT into the central nervous system. After a single oral dose of TBTCl (15 mg/kg b.w.), initially high concentrations were found in the cerebellum but also in the frontal and temporal lobe of rabbits (cited in WHO-IPCS, 1990). Also after a single oral dose of 40 mg TBTCl/kg b.w. to male Wistar rats, TBT initially increased in the brain. It was dealkylated to DBT, MBT and inorganic tin. Mainly MBT and inorganic tin persisted in the brain during the observed period of 8 days (Iwai \textit{et al.}, 1981). After i.p. administration of 4 mg DBTCl\(_2\)/kg b.w. to male Wistar rats the parent compound and small amounts of its metabolites 3-OH-DBT and MBT were detected in the brain over a period of 7 days (Ishizaka \textit{et al.}, 1989).

There have been a number of experimental attempts to demonstrate neurotoxic effects of TBT. In most studies, TBT was applied in dosages between 0.5 and 10 mg/kg b.w.. However, there is no experimental study available which would convincingly show that TBT is toxic to the nervous system and valid conclusions can not be drawn (Benya, 1997; Poitou \textit{et al.}, 1978). The most noticeable result was reported by Barnes and Stoner (1958, 1959). Their observations seem to resemble the effects of TET since they reported the induction of interstitial oedema of the white matter of the central nervous system in a subchronic feeding study (three months) of TBTOAc (100 mg/kg feed) to male rats or after administration of a lethal dose to female rats. This result is in principle consistent with the results seen in TET toxicity, but also with the \textit{in vitro} evidence of the interference of TBT with chemical energy production by the mitochondrial chain. There are parallels to studies of ocular toxicity in which the induction of corneal oedema was reported (Yoshizuka \textit{et al.}, 1991). However, this result has never been reproduced. Although Krajnc \textit{et al.}, (1984) observed ataxia (which is in
principle consistent with a disease of white matter) after feeding a dietary concentration of 320 mg/kg TBTO to Wistar rats (equivalent to 30 mg/kg b.w./day) over four weeks. No evidence of brain oedema was seen. TBTO was given orally to male Sprague-Dawley rats in dosages of either 37.5 or 75 mg/kg b.w. for three consecutive days. Mortality rates were 12 and 30 %, respectively; in the remaining animals aggression and seizures were seen. Although some histopathological features remained non-specific, a complete absence of Purkinje cells in the cerebellum was detected 7 days post-treatment. The activity of total brain ATPase, Na\(^+/\)K\(^+\) ATPase, and Mg\(^2+\) ATPase was found to be suppressed (Elsabbagh et al., 2002).

I.p. injection of 10 mg TBTO/kg b.w. or 80 mg DBTO/kg b.w. induced a reduction of cerebral 5-hydroxytryptamine and noradrenaline concentrations in the rat (cited in Benya, 1997). Judgement on the importance of the observed changes of monoamine levels is not trivial since in this particular experiment, neither behavioural alterations of the animals nor morphological changes were described. In a similar study exposure of female Wistar rats to DBT dilaureate (20, 40 or 80 mg/kg b.w.) for three days caused a decrease in levels of noradrenaline, dopamine and serotonin at all treatment levels. Hypothalamus and frontal cortex appeared to be most affected. Maximum decrease of dopamine was found in corpus striatum, nordrenaline in pons medulla and of serotonin in frontal cortex. The animals also showed a decrease in spontaneous locomotor activity and learning at all doses. A NOAEL was not established (Alam et al., 1988). The behavioural effects of a single oral exposure to nonlethal doses of TBTCI (6.3, 12.5, 25.0 or 50.0 mg/kg b.w.) were studied in male Wistar rats. Spontaneous motor activity (SMA) and acquisition of conditioned avoidance responses were monitored. TBT caused a dose-related decrease in SMA during the dark phase. The 24-hour total daily and 12 hours nocturnal activity was decreased at doses of 12.5 mg/kg and above. The acquisition of shock avoidance responses was inhibited in all TBTCI-treated groups in a dose-dependent manner. The difference was significant at 25 mg/kg b.w.. The authors conclude that TBT can cause significant changes in rat behaviour (Ema et al., 1991).

Dietary exposure of male mice (BALB/c) to TBT (1 - 125 mg/kg feed) for 30 days produced inhibition of ligand binding to the NMDA receptor (Konno et al., 2001).

Gardlund et al., (1991) administered 1 or 5 mg/kg b.w. TBTCI orally during the 6th to the 20th day of pregnancy to Sprague-Dawley rats. As adults the TBT treated offsprings showed clear evidence of alterations of behaviour such as a general hyperactivity, dysfunction of spatial learning performance and a drastic potentiation of the stimulating effects of d-amphetamine. These alterations were induced at doses that did not induce overt maternotoxicity or decreased viability of the offspring. However, no neurochemical correlate for these observations were found as neurohistological changes were not investigated. A NOAEL was not established. O'Callaghan and Miller (1988) reported that administration of a single dose of TBTO (2, 3 or 4 mg/kg i.p.) to the neonatal rat (Long-Evans, PND 5) causes dose- and region-dependent decreases in brain weight with the cerebellum being most affected. Reductions of p38 (synaptic vesicle associated protein) and myelin basic protein (oligodendroglia and myelin-sheath associated protein) were seen in the cerebellum and forebrain, but not in the hippocampus. These effects, determined on postnatal day 13, 22 and 60, were seen at dosages that did not affect brain, thymus, or body weight; if dosages did not
influence body weight, reductions in brain weight, p38 and myelin basic protein were reversible.

**In vitro neurotoxicity**

TBT was toxic in slice cultures of immature hippocampus dissected from postnatal 10 day-old Wistar rats. It induced neuronal death in a concentration- (0.1 - 10 µM) and time-dependent manner. The morphology of the cell death had apoptosis-like features and free radical scavengers or antagonists to the AMPA glutamate receptor subtype were shown to be protective (Mizuhashi et al., 2000). Interestingly, in this system the concentrations necessary to disrupt cell integrity were tenfold lower than those reported for the established potent neurotoxin TMT (Noraberg et al., 1998). TBT was a potent inhibitor of GABA uptake into mouse (male Swiss-Webster) forebrain synaptosomes with an IC_{50} value tenfold lower than that of TMT. This effect appears to be due in part to inhibition of Na^+/K^+ ATPases (Costa, 1985). TBT has been shown to induce apoptosis in immortalized cell lines as well as primary neuronal cells (Thompson et al., 1996) and in pheochromocytoma PC 12 cells (Viviani et al., 1995). These results are consistent with those obtained by others in non-neuronal cells (Aldridge, 1976; Snoeij et al., 1986a,b) and are also consistent with disruptive effects on chemical energy synthesis in neurons. Kobayashi et al., (1996) showed in mouse (male ICR) cortex that the butyl tins (1 - 100 µM) inhibited various parameters of cholinergic activity with TBT being more potent than DBT and MBT. Eskes and collaborators showed in brain cell cultures of the 16 day old foetal rat that DBT appeared to be more toxic than TMT (Eskes et al., 1999).

**Triphenyltin (TPT)**

In neonatal Long-Evans rats dosed orally with 30 mg TPTAc /kg b.w. and day from day 3 to day 30, no light microscopic or electron microscopic changes were observed in the hippocampus or pyriform cortex/lobe, which are susceptible to neuronal necrosis with TMT. In addition TPT did not cause oedema in the myelin sheath, as was usually induced by TET (Bouldin et al., 1981). In animals treated with > 20 mg/kg b.w. diarrhoea, anorexia, weakness, a staggering gait, paralysis of hindlimbs, tremor, convulsions, coma and death were described (Bock, 1981); a picture reminiscent of TET neurotoxicity. A number of biochemical changes in experimental animals have been observed, most importantly including inhibition of mitochondrial oxidative phosphorylation and inhibition of Na-K-ATPase, but also reduced glucose utilisation, catecholamine depletion, inhibition of microsomal adenosine triphosphatase, and reduction of aldehyde dehydrogenase activity (Bock, 1981). Also, increased expression of the glial fibrillary acidic protein (GFAP) was found in various brain regions e.g. cerebellum after oral feeding of 10.06 mg/kg TPTAc to Wistar rats for five days; behavioural-motor alterations were not investigated (Malkiewicz et al., 2000). The authors
conclude that TPT is responsible for time-progressive glial damage and that increases in GFAP would follow the same pattern as described for TET and TMT by O’Callaghan (1991). In the maze learning test, male CFY rats orally given Tinstan (containing 60% TPTAc) at doses of 0.6 (corresponding to 0.36 mg TPTAc/kg b.w./day) or 6 mg/kg b.w./day, 6 days/week for 6 weeks, apparently made many mistakes and showed slow reaction speed. In the conditioned avoidance response test, no difference was observed between the dose groups and the control group. However, extinction of behaviour was delayed in the high-dose group after discontinuation of the stimulus. Tin levels in the brain tissues were increased. The authors conclude that Tinstan (TPTAc) crosses the blood-brain barrier, and causes performance deficits in the learning ability as an early sign of neurotoxic action. There was no evidence on detectable neurohistological signs. An effect level was at 0.36 mg TPTAc/kg b.w./day and a NOAEL could not be established (Lehotzky et al., 1982).

In vitro neurotoxicity

TPT disrupts chemical energy production by inhibition of oxidative phosphorylation (Stockdale et al., 1970; Aldridge 1976). It has also been shown that TPT was a potent inhibitor of GABA uptake into mouse (male Swiss-Webster) forebrain synaptosomes. The IC₅₀ was 100 fold lower than that of TMT demonstrating no apparent correlation with the in vivo neurotoxicity of both OTC. This effect appears to be due to inhibition of Na⁺/K⁺ ATPase, but also to other mechanisms (Costa, 1985).

In conclusion reliable reports on organotin neurotoxicity in humans exist for TET, TMT, and more recently TPT. There is no evidence for human neurotoxicity of TBT. Animal experiments seem to suggest that other OTC are less neurotoxic than TET and TMT. There is at present no satisfactory mechanistic explanation for that. In vitro studies have shown that TMT, TET, TBT and TPT seem to share the mechanism of interruption of chemical energy supply; a supply critical for excitable tissue, in particular neurons. With regard to risk assessment, NOAELs could be derived from the studies presently available. The lowest effect levels reported for rats were 1 mg TBTCI/kg b.w./day in a neurodevelopmental study and 0.36 mg TPTAc/kg b.w./day in the maze learning test.
Annex 6. Observations in humans

Tributyltin (TBT)

Human neurotoxicity

There are no reliable and consistent reports on TBT neurotoxicity in humans. Toxic effects are unequivocally reported in maximally 10 patients (Shelton et al., 1992; Wax and Dockstader, 1995; Benya, 1997). The reported symptomatology after acute exposure includes only non-specific effects like nausea, vomiting, headache, anorexia and pain syndromes. There is no convincing evidence that these symptoms are due to damage to structures of the nervous system. The lack of knowledge of TBT effects is demonstrated by the report (cited in Benya, 1997) on a syndrome of „musculotonic dystrophy“ which does not even exist in the neurological nomenclature, in particular not as a syndrome known to be induced by neurotoxins. There are no reports on neurotoxicity after chronic human exposure to TBT.

Exposure by inhalation

A woman reported that she and her two children had become ill after the walls and ceilings of two rooms of her apartment had been painted. Reported symptoms included a burning sensation in the nose and forehead, headache, nose bleed, cough, loss of appetite, nausea, and vomiting. The woman, who was in the third trimester of pregnancy, also complained of a persistent odor from the paint. The family vacated the unit 1 week after the apartment was painted. A paint additive was used for mildew control. This product contained 25 % TBTO as its only active ingredient. Two days after the move, an air sample in the painted apartment was collected. The air sample contained 0.002 mg/m³ of TBTO measured as tin (Knobeloch et al., 1991).

All five members of a family (two adults and three children) complained of nausea, vomiting, headache, sore throat, burning nose, watery eyes and wheezing. A hallway of their home had been painted with an interior latex paint. They first noticed a paint fume odor followed by the onset of the above symptoms. Prior to the application, TBTO had been mixed into the paint for mildew control. A week prior to this event, family members had been exposed to the same latex paint when other rooms were painted. This painting was performed without the addition of the mildew control agent. No symptoms developed during or after this earlier painting. It is concluded that inhalational exposure against vapours of the latex paint containing TBTO induces mucous membrane irritations (Wax and Dockstader, 1995).

Unpublished data were reported by the Centers for Disease Control involving a series of six cases of illness attributed to the use of TBTO in interior paint. In these cases, symptoms consisted of headache, sore throat, respiratory complaints, adenopathy and weakness. It is
assumed that these cases are consistent with the previously reported cases associating mucous membrane irritative symptoms with the use of TBTO in interior housepaints. It was suggested that this additive was the causative agent (Wax and Dockstader, 1995).

A 52 year old previously healthy woman had acute retrosternal chest pain, nausea, and lethargy within a few hours of arriving at work. Thirty-six hours earlier the carpet had been sprayed with Ultrafresh (25 % TBTO in water with 2 % alcohol) after a flood. Her symptoms cleared over 2 days at home but recurred on return to work, with chest tightness and soreness, dry cough, and wheeze, but no upper respiratory complaints. Subsequent absences from work resulted in similar alleviation of symptoms, followed by exacerbations with work exposure on at least four occasions within 14 weeks, each lasting for 2 to 3 days, despite removal of the carpet. Three other workers in the same area had no symptoms. The patient had a 32-pack year smoking history, but she denied previous lower respiratory symptoms. She had a history of hayfever, which began at the age of 27 years and lasted for 2 years only. Skin testing by prick test with 12 common aeroallergens revealed various positive responses. Testing with TBTO at a non-irritant concentration was negative. The authors concluded that TBTO was likely the etiologic factor for this patient’s asthma. The mechanism for this response however is unknown (Shelton et al., 1992).

Dermal exposure

A Chinese welder and painter contaminated his right shoulder and knee with a TBTO containing liquid. 5 hours later, he felt itchiness over his right shoulder. 10 hours later, the redness, itching and burning intensified with blister formation involving his right shoulder and knee. He had no history of dermatitis and no personal or family history of atopy. The lesions resolved after 1 week of treatment with amoxicillin and antihistamines. On resuming work, he put on the freshly laundered work clothes previously contaminated with TBTO. Redness, itching and burning and blisters reappeared on the same areas 4 hours later. There were eczematous lesions, with vesicles and erosions, on the right shoulder and right knee respectively, which resolved after treatment with potassium permanganate washes, oral antibiotics and antihistamines. The lesions healed, with post-inflammatory hyperpigmentation, after 2 weeks. It was concluded that in this patient, the contaminated clothing caused a severe irritant contact dermatitis (Grace et al., 1991).

After starting painting, 2 Chinese males noted itchy redness, swelling and vesiculation over their limbs at sites of skin contact with the paint. The rash appeared 8 to 10 hours after contact, and disappeared after a few days. The rash was not photosensitive and covered contaminated skin was also affected. Two months later during painting again, paint dripped onto face, neck and trunk. They developed again severe itching, redness, swelling and blistering over the trunk, face and limbs after the first day of painting. Examination showed extensive vesiculo-bulous lesions with redness and oedema of the face, neck, trunk, arms and thighs. The lesions cleared completely after 10 days of symptomatic treatment. From the clinical findings and the patch tests performed, the authors concluded that TBTO was most
likely the responsible irritant. Unless other biocides used in paints, TBTO does not appear to be an allergen (Goh, 1985).

A 39-year old shipwright developed pruritus, erythema and vesiculation on both wrists and forearms with a few lesions on the abdomen after using an antifouling paint for wooden blocks. The paint contained 10 - 11.7 % TBTO. Other major constituents were xylene, cuprous oxide and copper thiocyanate. During the painting operation, his skin was contacted by overspray. The patient gave a history of dermatitis on both wrists 2 months previously while using a TBTO containing paint. At least 4 other workers performing similar tasks developed dermatitis on that occasion. Although there were no immediate symptoms on contact with the paint, some irritation was noted within about an hour. Erythema and ulceration were noted on the second day. In addition, he had noted some mild pustular lesions on the mucous membrane of the lips, apparently related to wiping his lips with his paint-contaminated arm. He gave no other history of dermatitis and there was no personal or family history of atopy. He was in good general health except for essential hypertension. The authors concluded that the dermatitis observed was induced by the TBTO-containing paint. The strong irritant potential of TBTO was displayed by apparent reactions to merely brushing a contaminated arm across the mouth and by wearing contaminated shoes. TBTO was corrosive to the skin at 0.1 % aq., indicating the extreme care which must be taken in patch testing (Lewis et al., 1987).

Human data summarized by Boyer (1989) also suggest that TBTO is a potent dermal non-allergic irritant.

**Triphenyltin (TPT)**

**Human neurotoxicity**

In a case report (N = 3) on the acute nephrotoxicity of TPTAc the „development of neurological disorders“ is mentioned (Lin and Hsueh, 1993). However, a detailed description is missing and the symptomatology seemed to be completely reversible. In a second report on a single patient cutaneously exposed to an unknown amount of TPTAc some electroencephalographic abnormalities (paroxysms) but no specific neurotoxic effects, in particular seizures, were reported (Colosio et al., 1991). A third report also did not mention specific neurotoxic effects (Manzo et al., 1981). In a detailed report on a 23 year old male with an acute severe TPTAc intoxication in a suicide attempt, the authors observed unspecific symptoms such as abdominal pain, vomiting, headache, and visual disturbances which are in principle consistent with the reversible symptomatology in the milder cases (Wu et al., 1990). In addition, this observation gives reliable information on a reversible (months) cerebellar syndrome, hearing impairment, loss of consciousness (weeks) associated with paroxysmal activity in the EEG, and an apparent late onset sensorimotor axonal polyneuropathy which could not be distinguished from critical care neuropathy. Another report on a single patient by
Lin et al., (1998) is consistent with the presence of a cerebellar syndrome and seizures. This patient consumed about 1/3 of a 100 g pack containing 45% TPTAc.

Exposure by inhalation

A patient who inhaled 5 days before hospitalisation a fungicide powder containing 60% TPTAc complained about dizziness, nausea and photophobia. He had an episode of sudden malaise with dizziness, and temporary loss of consciousness 1 day before his visit. On admission, general appearance and physical examination showed no abnormality except for a mild impairment of body balance. In spite of treatment with various antiemetics, nausea and photophobia persisted until 4th day. Complete recovery was seen 10 days after hospitalization. Another patient noted general malaise, weakness and dryness of the mouth. Severe headache, weakness and photophobia appeared on the day following hospitalization. Higher concentration of tin was found in blood and urine. At the time of admission, the subjective symptoms had totally disappeared. There were no abnormal neurological findings (Manzo et al., 1981).

Dermal exposure

A man spilt an unknown amount of pesticide powder containing 18.95% TPTAc (Brestan) on the exposed cutaneous area of his arms. He washed immediately but about 12 hours later experienced bilateral plantar pain and, after around 24 hours severe genital oedema. Some hours later an erythematous eruption appeared on his trunk. In the past he had had similar accidents with TPTAc without reporting any adverse effects. 2 days after the accident, the patient had general malaise, dizziness, nausea and abdominal pain. Temperature, pulse rate and blood pressure were normal; physical examination showed slight genital oedema, urticarial eruptions on his trunk, and pronounced hepatomegaly. Despite steroid treatment, the patient showed recrudescences of urticarial eruption on his trunk and arms. Allergological investigation, performed by patch tests with entire Brestan and with each single component in its formulation, did not produce any positive results. Ten days after the accident, an electroencephalogram (EEG) showed alterations. On the eleventh day serum IgE was raised. Twelve days after poisoning a hepatic echotomography showed a generalised enlargement of the liver. Needle biopsy specimen showed slight and non-specific inflammatory abnormalities. Three weeks after poisoning, the patient apparently completely recovered. Somewhat high ALT and gamma-GT activities with slight hepatomegaly persisted. During the next months the patient suffered from period urticaria on his trunk and arms. He developed angioedema which persisted for several days. Four months after the poisoning, the EEG showed slight anomalies during hyperpnea (Colosio et al., 1991).

Hypersensitivity reaction to a series of 36 TPT containing pesticide formulations was surveyed among 652 subjects in Italy. Patch tests were performed and irritant and allergic reactions were evaluated. With a patch of 1% TPT in 45 of 350 subjects irritant reactions
were seen and in 1 out of 350 allergic reactions. With a patch of 0.5 % TPT only irritant reactions were seen. Therefore TPT is a moderately strong irritant and not an allergen (Lisi et al., 1987).

Oral exposure

Three male patients developed acute nephropathy following ingestion of TPTAc. The amounts of the ingested dosages were 9, 15 and 22.5 g. All of the patients had significant proteinuria, azotemia and polyuria. Hematuria and pyuria were noted in 1 severely poisoned patient. Evidence for hepatitis was present in 2 patients and for pancreatitis in 1. The time course of the nephropathy indicated maximal damage approximately during 2 - 10 days after ingestion. Renal biopsy showed focal fusion of glomerular cell processes and proximal tubular damage with cellular necrosis. Two patients survived with complete recovery of renal functions. One old patient died of aspiration pneumonia. The acute nephropathy appears to result mainly from proximal renal tubular damage with a benign and reversible clinical course (Lin et al., 1993).

In conclusion no information was found regarding the toxicity of OTC following long-term exposure. However there are several case reports claiming various health effects following acute inhalation, dermal or oral exposure to OTC. However, causality to OTC was not established and none of these reports contain sufficient information to characterize a dose-response relationship for the reported effects.
Annex 7. Mechanistic data of immunotoxic OTC

Organic tin compounds (OTC) show various adverse effects in vitro as well as in vivo targeting e.g. immune and blood cells (Hennighausen and Lange, 1980; Hennighausen et al., 1980a,b; Lange et al., 1980; O’Brien, 1963; Penninks et al., 1985; Snoeij et al., 1985; 1988), the nervous system (Alam et al., 1988; Bondy and Hall, 1986; Costa, 1985; Reuhl et al., 1985), cell membranes (Ali et al., 1987; Gray et al., 1987a,b; Srivastava, 1990; Zucker et al., 1988a,b,c), calcium homeostasis (Chambers et al., 1987; Kodavanti et al., 1991; Komulainen and Bondy, 1987; Miura and Matsui, 1991; Reader et al., 1993; 1994; Yallapragada et al., 1991) and mitochondrial function (Aldridge et al., 1977a,b; 1981; Kauppinen et al., 1988; Skilleter, 1975). In vivo most studies indicate strong effects of DBT, TBT, TPT and DOT on immune functions. All tested mammalian species treated with one of these four different OTC showed finally the same effect: loss of thymic weight by thymus atrophy.

During the last decade intense investigations have been carried out to gain more insight into the molecular mechanisms of OTC. Herewith the most important findings on the biochemical mechanisms with respect to the immunological impairment by relevant OTC will be discussed.

Cytotoxicity

OTC, especially trialkylated tin, are taken up by cells in vitro fast and effectively, e.g. a toxic dose of tripropyltin chloride (TPrT) or TBT within 30 seconds to 2 minutes (Krug et al., 1998). The so-treated cells could not be rescued by immediate removing of the medium and intense washing. Cells treated with an acute cytotoxic concentration of TPrT, TBT or tripentyltin chloride (TPeT) died within the treatment period of 2 hours. The toxicity of all tested compounds is a function of both concentration and duration of exposure (Ade et al., 1996; Zucker et al., 1992). Within the range of a 2 – 24 hours treatment the LC50 values for the most toxic compounds TBT and TPeT are as low as 3.5 µM down to 0.5 µM. In animal as well as in plant cellular model systems the OTC have been shown to be cytotoxic in a strong structure-dependent manner. Parabolic quantitative structure-activity relationship (QSAR) models using the molecular weight of trialkylated tin compounds describe their effects with a maximum toxicity for TBT (Ade et al., 1996; Huang et al., 1997; Schüürmann and Marsmann, 1991; Schüürmann and Röderer, 1988; Schüürmann and Segner, 1994) and decreasing toxicity for shorter or longer chain lengths. The same seems to be true for dialkylated tin compounds although the dependency on the chain length is not as obvious as for the trialkylated compounds (Hennighausen et al., 1980a). Comparing the LC50 of the homologous series of trialkylated compounds (TMT, TET, TPrT, TBT and TPeT) in vitro in human cell culture systems the LC50 values range from the millimolar (TMT) to the low micromolar level (TBT) after a 2 hours period of treatment. Another study demonstrates the cytotoxicity of 21 different OTC in a fish cell culture system. It reveals that the trialkylated tin compounds are the most toxic, and the length of the carbon side chains determines the
toxicity with butyl- and cyclohexyl-groups as the most potent congeners (Brüschweiler et al., 1995). Levels of toxic concentrations were found to be in the same order of magnitude as shown for other cellular systems between $4 \times 10^{-7}$ M and $10^{-5}$ M for the trisubstituted compounds whereas disubstituted and tetrasubstituted are less toxic ($10^{-5}$ M – $10^{-3}$ M).

**Dibutyltin (DBT)**

Disubstituted OTC bind to a high affinity site at the plasma membrane of cells (Ali et al., 1987). Such an interaction of DBT with plasma membranes and its constituents might cause derangement of their structural and functional organisation, thus leading to cytotoxicity (Srivastava, 1990). Although *in vitro* QSAR studies revealed a higher toxicity for trialkylated OTC, several biological systems show a similar or even higher sensitivity for DBT compared to TBT indicating a higher affinity of DBT to distinct elements of the cell (Cima et al., 1998; Merkord et al. 2000) or a better bioavailability in the organism (Gennari et al., 1997; Snoeij et al., 1988). This is further corroborated by the study of Ueno et al. (2003). They found a lower IC$_{50}$ value for TBT in isolated mitochondria from mice differing more than one order of magnitude to that of DBT. *In vivo* the dose of DBT was only one third of the dose of TBT to induce comparable effects. They discuss possible differences in the bioavailability dependent on the high affinity of DBT to dithiol structures (Merkord et al., 2000) and the higher content of sulfhydryl groups in the mitochondrial fractions of those species that are affected by lower concentrations of the OTC (Ueno et al., 2003).

**Tributyltin (TBT)**

TBT induces massive cell death in a variety of different cell types at low concentrations (Ade et al., 1996; Käfer and Krug, 1997; Umebayashi et al., 2004; Zaucke et al., 1998). This potent cytotoxic effect seems to be partially influenced by proteins or thiol-containing molecules. In the presence of serum the toxic effect of TBT is attenuated indicating a possible binding of TBT to sulfhydryl groups of proteins and nonproteins (Umebayashi et al., 2004). After 3 hours in the presence of serum 1 µM TBT induces a slight increase of apoptotic cell death (2 – 5 %) in freshly prepared rat thymocytes whereas in the absence of serum the population undergoing apoptosis increases up to 50 %. Nevertheless, when incubation time was prolonged to 24 hours only nanomolar concentrations were needed to induce such an effect regardless the serum was present or absent. Moreover, at micromolar concentrations the fast and irreversible uptake within seconds could not be prevented in the presence of serum (Krug et al., 1998) and adding glutathione (GSH) to the culture media did not protect the cells. Also depletion of intracellular GSH with buthionine-[S,R] sulfoximine did not alter the cytotoxicity of TET or TMT (Cookson et al., 1998). The cytotoxicity of TBT might be the result of a massive alteration of the intracellular calcium concentration that normally is very low in resting cells. Various investigations demonstrated an increase of intracellular Ca$^{2+}$ concentration after exposure to a variety of OTC (see below), and this effect is discussed to be
responsible for their cytotoxicity, immunotoxicity and neurotoxicity in mammalian (Ade et al., 1996; Aw et al., 1990; Chow et al., 1992; Gennari et al., 2000; Komulainen and Bondy, 1987; Mizuhashi et al., 2000; Orrenius et al., 1992; Viviani et al., 1995) but also in nonmammalian systems (Reader et al., 1993; 1994; 1999).

Triphenyltin (TPT)

On the cellular level TPT induces cell death and cell cycle alterations after 48 hours and 72 hours of treatment at 1 µM in human MCF-7 breast cancer cells or with higher concentrations in mouse thymocytes (Bollo et al., 1996; Lin and Garry, 2000). Another study could demonstrate a similar potency of TPT on rat thymocytes in vitro showing an increased [Ca²⁺], a decreased glutathione content and a dramatic rise in apoptotic cells after treatment with 1 – 3 µM TPT for 3 hours (Arata et al., 2002). Moreover, it has been discussed that aneugenic effects are responsible for the observed aneuploidy in cultured cells when sub lethal concentrations were used. TPT acts as a potent spindle poison and exhibits a strong synergistic activity with PCBs in vitro. Concentrations as low as 50 nM for TPT and 10 nM for 2,3,3',4,4'-pentachlorobiphenyl caused no biological effects when applied separately but induced abnormal configuration during mitosis when combined (Jensen et al., 1991; 2000).

Cytoskeleton and calcium homeostasis

Adverse effects and acute cytotoxicity may be the result of a perturbation of the ion homeostasis or the cytoskeleton. Organometals can bind to sulfhydryl-containing proteins of the cellular cytoskeleton (Tan et al., 1978; Röderer and Doenges, 1983; Zimmermann et al., 1985; 1986; 1987; 1988) and this has been shown for TET that interferes with the aggregation of neurotubules (Bondy and Hall, 1986). However a comparable disassembly of microtubules induced by DMT, TMT, DBT, TBT and TPT via an interaction with tubulin with no involvement of the sulfhydryls of the protein has also been observed (Jensen et al., 1991). Orrenius et al. (1992) discussed the role of calcium as a critical element in both toxic cell killing and programmed cell death (apoptosis). An unphysiological increase of intracellular Ca²⁺ concentration may lead to the activation of a variety of proteases disrupting cytoskeletal organisation and leads to the formation of surface protrusions (blebs). The additional activation of calcium-dependent phospholipases can result in an impairment of mitochondrial function with a collapse of membrane potential (Orrenius et al., 1992; Stridh et al., 1999a). The alterations of cytoskeleton structures by different alkylated tin compounds have been observed at micromolar concentrations in embryos of invertebrates (Cima et al., 1996) as well as in human cells (Chow and Orrenius, 1994; Käfer and Krug, 1997; Krug et al., 1993; Marinovich et al., 1990; 1996). It becomes more and more evident that the disruption of cytoskeletal structures is a result of the activation of caspases and happens during apoptosis (see chapter below). Caspases exhibit a tightly regulated pattern for the cleavage of specific
proteins, of which several filamentous and cytoskeletal proteins are individual candidates, which are cleaved after TBT-treatment (Lavastre and Girard, 2002).

The role of calcium in the mechanism of OTC toxicity is demonstrated by a multitude of different studies. Calcium seems to be involved in protease and phospholipase activation, membrane blebbing, cytoskeleton breakdown and, finally, cell death. Dependent on the biological system investigated and on the OTC congener used for treatment of organisms and cells, different effects on the calcium homeostasis are described. Kauppinen et al. (1988) discussed the alterations of the mitochondrial membrane potential as responsible for the increase in intracellular Ca\textsuperscript{2+} concentration and this observation was corroborated by Stridh et al. (1999b) stating a rapid and sustained elevation of intracellular calcium levels after treatment of human T cells (Jurkat) with 2 µM TBT. This effect was preceded by mitochondrial hyperpolarisation and the subsequent loss of membrane potential ΔΨ. But different concepts exist for the explanation of such an effect of OTC on the alteration of the intracellular calcium concentration. For human neutrophils it has been shown that in the presence of extracellular calcium TPT and diphenyltin dichloride (DPT) increased [Ca\textsuperscript{2+}]i at concentrations from 1 to 10 µM or from 2.5 to 10 µM, respectively (Miura and Matsui, 1991). In isolated rainbow trout hepatocytes 1 to 5 µM TBT induced a sustained elevation of cytosolic free calcium and experiments carried out in calcium-free medium suggest that TBT mainly mobilizes Ca\textsuperscript{2+} from intracellular stores (Reader et al., 1993; 1994; 1999). The same result was published for rat PC12 cells when treated with micromolar concentrations of TPT or TBT (Viviani et al., 1995). On the contrary, experiments with primary cultured rat hepatocytes (Kawanishi et al., 1999), human granulocytes (Ade et al., 1996) or human thymocytes (Krug et al., 2003) demonstrated that TBT at micromolar concentrations leads to increased intracellular Ca\textsuperscript{2+} concentration only in the presence of extracellular calcium. These data were further confirmed by an earlier study with primary mouse thymocytes, which exhibit an increase in the intracellular Ca\textsuperscript{2+} concentration after treatment with TBT (100 nM – 1 µM). This effect is greatly reduced under nominal external Ca\textsuperscript{2+}-free conditions (Chikahisa and Oyama, 1992). Treatment of cells in vitro under calcium-free conditions or intracellular calcium chelating with BAPTA completely prevents the calcium rise but not membrane blebbing or cell death. Moreover, after inhibition of the TBT-induced increase in [Ca\textsuperscript{2+}]i achieved by incubation of the cells in calcium-free medium and additional buffering of the intracellular calcium by BAPTA, the initiation of several apoptotic events including cell shrinkage, phosphatidylserine externalisation, caspase activation and Poly (ADP-Ribose) Polymerase-cleavage persisted. These data support the concept that the rise in [Ca\textsuperscript{2+}]i is not a necessary component for the early signal transduction pathways in OTC-induced apoptosis in human immunocompetent cells (Krug et al., 2003).

Signalling pathways and proliferation of immunocompetent cells

It is well known that alterations of the calcium homeostasis can affect various enzyme- and kinase-systems as well as different signalling pathways. As described above, the involvement
of calcium in triggering apoptosis has been discussed and there are several hints for additional signalling pathways that are influenced by alkyltin compounds in a calcium-dependent or – independent manner.

First evidence came from experiments with DBT and DOT that exert immunosuppressive properties in rats via a severe decrease of lymphocyte number within the thymus, a strongly reduced graft-versus-host response and a diminished response to the T-cell mitogens phytohemagglutinin and concanavalin A (Seinen and Willems, 1976; Seinen et al., 1977a; 1979). These data were confirmed by animal studies with DOT that suppresses thymocyte proliferation after oral administration to rats (Miller et al., 1986b) and comparable effects observed for DBT that impaired the interaction between immature thymocytes and thymic epithelial cells (Pieters et al., 1995). To compare these findings with human systems, OTC were tested with B cells freshly isolated from human tonsils. 100 nM of the tested OTC killed non-stimulated B cells after 8 hours of treatment and the proliferation of stimulated tonsillar B lymphocytes was reduced (De Santiago and Aguilar-Santelises, 1999). As demonstrated by phosphatidylserine externalisation, DBT and TPT induced B cells to die by apoptosis and the authors suggest that human B cells are diminished in their capacity to survive, proliferate and differentiate in the presence of OTC in vitro.

Chemical effects on proliferation and survival are often mediated via kinase cascades that regulate cellular growth as well as stress responses. Besides the decrease in proliferation, more evidence was found that points to a direct effect of OTC on signalling cascades connected to cell proliferation and stress responses. Rat PC12 cells were found to react instantaneously to TBT-treatment with the expression of immediate early genes (Matsuoka and Igisu, 1996). At concentrations of 0.4 and 1 µM TBT the expression of c-fos and c-jun was induced within 15 to 30 min. This expression was considered to be due to transcriptional activation because it was completely abolished by actinomycin D. By selective dimerization these proteins form transcription factor variants of the AP-1 family (Jun/Jun or Fos/Jun). AP-1 is ubiquitous and occupies a central role in the control of cell homeostasis as well as in the control of functions as important as cell division, differentiation, response to stress and apoptosis.

Prolonged exposure to TBT decreased viability, and the cells died exhibiting signs of apoptosis. Moreover, in this study it was shown that an increase of intracellular calcium is a prerequisite for the enhanced expression of immediate early genes. Another investigation reports on the induction of heat shock proteins by 0.5 µM DBT in a variety of lymphocytes, thymocytes or splenocytes, while overall protein synthesis was diminished by 50 % (Albers et al., 1996). The specific inhibition of an important signalling pathway could be responsible for the growth retardation of thymocytes as observed for the reduced expression of interleukin-2 after the treatment of rats with DOT (Volsen et al., 1989). The regulation of transcription factors, heat shock proteins or cytokines is often or mostly dependent on kinase signalling pathways, such as the activation of MAP-kinases that was found to be switched on by TBT in human leukaemia cells (Zaucke and Krug, 1996). This was confirmed by the work of Yu et al. (2000) which indicates that in response to the incubation with 0.25 – 2 µM TBT for 1
hour, the levels of the phosphorylated forms of extracellular signal-regulated protein kinase (ERK), c-Jun NH(2)-terminal kinase (JNK), and p38 MAPK increased in a dose-dependent manner. The comparison of different OTC resulted in TBT with the highest potency of phosphorylation followed by TPT and DBT. MBT, TMT and TET showed no such activity in the same concentration range. Chelating with BAPTA suppresses the induction of phosphorylation by OTC, proving that activation of the kinase cascades is dependent on intracellular calcium concentration (Yu et al., 2000). The simultaneous activation of all known MAP-kinase cascades leads to an integration of all three signalling pathways that normally are responsible for proliferation (ERK), cell death or stress responses (JNK and p38) thereby leading to cell damage. It was clarified that this activation of the phosphorylation cascades is the result of an inhibition of negatively regulating elements within these signalling pathways, namely the responsible phosphatases (Gulati et al., 2001).

The combination of increased calcium and activation of the MAP-kinase cascade further leads to activation of enzyme systems that were dependent on both signals, e.g. the cytosolic phospholipase A2 (cPLA2). It has been described earlier that OTC treatment induces the liberation of arachidonic acid from membrane phospholipids in human granulocytes (Käfer et al., 1992; Käfer and Krug, 1994; Krug, 1992; Krug et al., 1994). This unsaturated fatty acid is the precursor of a multitude of lipid mediators, e.g. prostaglandins and leukotrienes, involved in inflammatory, allergic and other immunologic processes. The key enzyme cPLA2 has been shown to be induced via a dual stimulatory mechanism: the activation of this enzyme requires the phosphorylation by ERK kinases and the elevation of the intracellular calcium that provokes the translocation of the enzyme to the plasma membrane. cPLA2 finds its substrates phosphatidylcholine and phosphatidylethanolamine at the membrane, finally leading to a dramatic release of arachidonic acid out of these membrane phospholipids (Zaucke et al., 1998). Furthermore, it has been demonstrated that the re-incorporation of arachidonic acid into lyso-phospholipids is inhibited by organic metal compounds (Krug, 1992). Therefore, the permanent degradation of phospholipids could contribute to the observed perforation of the cell membrane ending in necrotic cell death.

Whereas the stimulation of arachidonic acid liberation only happens at relatively high concentrations of TBT (5 – 20 µM), in vitro experiments with primary lymphocytes prepared from marine mammals and humans demonstrated a strong anti-proliferative effect of TBT and DBT, even at considerably lower concentrations. Both compounds affected the in vitro proliferation of peripheral blood mononuclear cells at concentrations below 10^{-6} M (≈ 300 nM) (Nakata et al., 2002). Such low concentrations of TBT provoke alterations within the lipid metabolism of immunocompetent cells (HL-60, mouse macrophages P388D1, human mast cell line HMC-1) when combined with miscellaneous food additives, e.g. erythrosin (E 127), 3,5-di-t-butyl-4-hydroxytoluol (BHT, E 321) and 2-t-butyl-4-hydroxyanisol (BHA, E 320) (Krug et al., 1996). Another study points to a reduced immunological function after DBT-treatment. It was demonstrated that human natural killer (NK) cells were influenced dramatically by low concentrations of DBT. The authors detected an altered pattern of cell surface marker proteins involved in NK cell interactions with possible target cells and
conclude from these results a loss of NK cell cytotoxicity via a measurable loss of NK cell tumour binding capacity (Odman-Ghazi et al., 2003, Whalen et al., 1999; 2002).

Mitochondrial effects

Mitochondria are highly sensitive to the treatment with OTC (Snoeij et al., 1987). TET and TBT inhibited substrate uptake by mitochondria (Skilleter, 1975) and various OTC induce their swelling (Wulf and Byington, 1975). Trialkylated tin compounds perturb mitochondrial functions (Aldridge et al., 1977a,b), and cause enhanced Cl/OH exchange across mitochondrial membranes influencing the membrane potential (Aldridge et al., 1981). TPT acts as a powerful inhibitor of the proton conductivity of the H+-ATPase (Papa et al., 1982). The skin irritant activity of TBT was explained by the production of reactive oxygen species (ROS) within the respiratory chain in mitochondria at the ubiquinone site. ROS activate transcription factors and promote IL-1α synthesis (Corsini et al., 1996), enhance intracellular calcium levels and release second messenger molecules from mitochondria that induce the transcription factor NF-κB expression pattern (Corsini et al., 1997a,b). All of these studies indicated a significant role of mitochondria in the concert of OTC induced toxic effects. The most important effect was described by the group of Orrenius, who demonstrate that mitochondria play a central role in the mechanism of OTC-induced apoptosis (Stridh et al., 1998). Hitherto the main focus in apoptosis provoked by OTC was directed on alterations of the intracellular Ca²⁺ concentration (Aw et al., 1990; Chow et al., 1992; Orrenius et al., 1992; Raffray et al., 1993; Viviani et al., 1995). But it has become more and more evident that other players coming from mitochondria overtake the key role in the game of cytotoxic effects. The function of small proteins that were released from the mitochondria after TBT-treatment and induce apoptosis was elucidated. The most important pro-apoptotic protein released by mitochondria is cytochrome c (Gogvadze et al., 2002; Ott et al., 2002; Robertson and Orrenius, 2002; Stridh et al., 1998; 1999b) which is released from the intermembrane space of the mitochondria but the exact mechanism remains unclear. Several models have been proposed, including the mitochondrial permeability transition followed by the opening of the permeability transition pore and a loss of membrane potential ΔΨ. Another model claims a central role for calcium and the regulation of pro- and anti-apoptotic proteins involved in the permeabilisation of the outer mitochondrial membrane. However, in their review on the role of mitochondria in toxic cell death Robertson and Orrenius (2002) neither presented facts that support a model nor clarify which element is affected by TBT or other OTC. One possible target of TBT-induced release of cytochrome c is described by Nishikimi et al. (2001) who examined the effects of TBT on mitochondrial ΔΨ, swelling and cytochrome c localisation. They demonstrated that TBT binds to critical cysteine residues of the adenine nucleotide translocator (ANT), thereby opening the permeability transition pore, decreasing ΔΨ and releasing cytochrome c. Investigations of isolated mitochondria revealed a more specific picture of the TBT-induced mechanism. On the one hand, an increase of the intracellular Ca²⁺ concentration is still an initial insult that will facilitate the collapse of ΔΨ by mitochondrial permeability transition. On the other hand, the direct inhibition of ANT and the respiratory

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chain and the alteration of the anion exchange by TBT lead to mitochondrial swelling and thereby to the opening of membrane pores by which cytochrome c is released (Gogvadze et al., 2002). The release of pro-apoptotic proteins from mitochondria is the result of a variety of chemical stresses, e.g. OTC exposure, and leads to the intrinsic onset of the apoptotic machinery (Robertson and Orrenius, 2002). The intrinsic pathway is not the only mechanism by which cells can be activated to undergo apoptosis. Mainly immunocompetent cells, e.g. lymphocytes, dendritic cells, macrophages, and granulocytes, exhibit so-called death-receptors on their surface that were activated by ligand-binding and induce the extrinsic apoptotic machinery. In this connection, data exist that describe an apoptosis-inducing effect for TBT without the direct involvement of mitochondria (Krug et al., 2001; Nopp et al., 2002).

**Apoptosis and thymus atrophy**

The literature of the last 15 to 20 years on OTC-induced effects on cell death, especially apoptosis, reveals that mostly immunocompetent cells were affected. This might be due to the fact that cells were chosen which exert a high sensitivity thereby focusing mainly on lymphocytes or other blood cells. There is reason to assume that immunocompetent cells are extremely sensitive to OTC because of the existence of various death receptors on their surface and TBT and other congeners have a direct effect on these proteins inducing the extrinsic machinery of apoptosis (Krug, 2002a,b). Recently published data focus further on classical pathways of alkyltin induced cell death, the intrinsic apoptotic signalling pathway. DBT and TBT are described to interfere with calcium homeostasis and mitochondrial parameters in rat thymocytes resulting in the generation of ROS, release of cytochrome c, activation of caspases and fragmentation of DNA (Gennari et al., 2000). Other publications, however, demonstrate induction of apoptosis by OTC without a direct or immediate participation of the mitochondria. For human peripheral blood lymphocytes it is shown that TBT does not induce activation of caspases and mitochondrial alterations in all subclasses. In CD8\(^+\) T-cells no caspase activation could be detected, and they were directed towards necrotic deletion (Stridh et al., 2001). In isolated rat thymocytes that were treated with 3 \(\mu\)M DBT, as well as in rats after administration of 60 mg DBT/kg b.w., a rapid transcription of *nur77* was observed (Gennari et al., 2002a). This product encodes a number of ligand-dependent transcription factors, belongs to the steroid/thyroid hormone receptor superfamily and is considered to be a required transcription factor for the initiation of apoptosis in thymocytes. In addition DBT and TBT at low doses inhibit immature thymocyte proliferation, whereas higher doses, especially of TBT, induce apoptotic cell death. Also, these effects seem to be protein synthesis dependent (Gennari et al., 2002b).

As mentioned above, thymocytes are not the only cell type responding to OTC treatment by undergoing apoptosis. Also human granulocytes exhibit all signs of apoptosis when 2 \(\mu\)M TBT was applied. This incubation activates caspase 3 downstream of the mitochondria without the preceding mitochondrial permeability transition induction, loss of mitochondrial
membrane potential ($\Delta \Psi$) or release of cytochrome c (Nopp et al., 2002). A comparable ultra-rapid caspase activation independent from the apoptosome formation downstream of the mitochondria was demonstrated in human blood platelets after addition of 2 $\mu$M TBT (Berg et al., 2003).

Slight effects of TPT on the rat immune system were observed after 25 mg/kg b.w. (Vos et al. 1984b) and strong immunotoxicity was observed at doses of 1 and 10 mg/kg i.p. in mice (Nishida et al. 1990) and in clams (Cima et al. 1998). This is associated with thymus atrophy in mice and rats (Nishida et al., 1990; Snoeij et al. 1985) comparable to that after treatment with other OTC.

**Di-n-octyltin (DOT)**

No extended studies on the molecular mechanism of DOT with respect to apoptosis have been done so far. Indirect evidences suggest a similar mode of action to TBT or TPT but this has to be evaluated. For instance, studies dealing with the influence of DOT on thymus function and tissue growth (Seinen and Willems, 1976; Seinen et al., 1977a,b) demonstrate the suppression of thymus-dependent immunity in rats. DOT was shown to reduce the number and viability of cells that can be isolated from the thymus of treated rats and strong immunosuppression was detected (Seinen et al., 1979). Miller et al. (1986b) described the decrease of thymocyte proliferation when rats were administered with 75 mg DOT/kg b.w. and several authors reported on the thymic injury after oral administration of DOT (Boyd and Jones, 1986; Miller et al., 1984; Smialowicz et al., 1988; Smialowicz, 2002).

To gain insight into the molecular mechanism of OTC-induced apoptosis, the intrinsic and extrinsic apoptotic pathways were investigated in more detail. The release of pro-apoptotic proteins from mitochondria is under the control of the Bcl-2 protein family. These proteins are potent regulators of apoptosis that can influence the permeability of the outer mitochondrial membrane. Family members Bax and Bak, for instance, promote apoptosis while Bcl-2 or Bcl-xL inhibit cell death (for review: Sharpe et al., 2004). The regulation of this protein family can be achieved by a direct influence on the mitochondria, but also via a death receptor activation. Experiments with type I cells (apoptosis is independent from mitochondrial activation, e.g. B lymphocytes) and type II cells (activation of mitochondria is mandatory for apoptosis, e.g. T cells) (Scaffidi et al., 1998) that are genetically manipulated to overexpress the anti-apoptotic protein Bcl-2 provide further evidence for the induction of apoptosis by OTC independent from mitochondria. Using parental type I cells (SKW) and type II cells (Jurkat) as well as their Bcl-2 overexpressing variants, it could be demonstrated that only Bcl-2 overexpressing type II cells can be rescued from TBT-induced apoptosis. Type I cells, however, undergo apoptosis in spite of their high level of anti-apoptotic protein and no loss in membrane potential ($\Delta \Psi$) could be detected (Krug, unpublished results).
Tributyltin (TBT)

TBT induces massive apoptosis of thymocytes in vivo as well as of cells in culture (Aw et al., 1990; Fent, 1996), and it causes rapid and sustained elevation of intracellular calcium, mitochondrial dysfunction and activation of caspases (Kass and Orrenius, 1999; Stridh et al., 1999a). In human T lymphoblastoid cells apoptosis induced by 1 µM TBT seems to be dependent on an increase in the intracellular Ca^{2+} concentration (Yu et al., 2000) whereas studies with human Jurkat T cells and treatment with 1 µM TBT demonstrate that these cells undergo apoptosis totally independent of calcium (Krug et al. 2003). Furthermore, numerous studies with thymocytes (Aw et al., 1990; Chikahisa and Oyama, 1992; Chow et al., 1992; Chow and Orrenius, 1994; Gennari et al., 2000; 2002b; Grundler et al., 2001; Pieters et al., 1992; Raffray and Cohen, 1991; Raffray et al., 1993; Stridh et al., 1998; 1999a,b; Umebayashi et al., 2004; Zaucke et al., 1998; Zucker et al., 1994), leukocytes or granulocytes (Ade et al., 1996; Lavastre and Girard, 2002; Nopp et al., 2002; Tiano et al., 2003; Zaucke et al., 1998), peripheral blood cells (Nakata et al., 2002; Stridh et al., 2001), blood platelets (Berg et al., 2003), neuronal cells (Kelvest et al., 1994; Mizuhashi et al., 2000; Viviani et al., 1995; Yamanoshita et al., 2000) and hepatocytes (Reader et al., 1999) provide substantial evidence for TBT-induced apoptotic cell death. Recently an additional pathway induced by TBT was described in human T- and B-lymphocytes. The results point to a direct activation of the "death-inducing signalling complex" (DISC) showing that active initiator caspases are coupled to their receptor after TBT treatment and that this effect was totally independent of an increase in intracellular Ca^{2+} concentration and alterations in the membrane potential of mitochondria (Krug et al., 2001). As early as 2 hours after starting the exposure with 1 µM TBT, the active DISC was found. In contrast, mitochondria remain unaffected at this time point. The hypothesis includes the binding of TBT to thiol-groups of receptor molecules thereby inducing their aggregation and activation, which could be confirmed by immunohistochemistry (Krug, unpublished results).

Besides mammalian systems cells from other species were affected by TBT as well. In trout blood cells 1 – 5 µM TBT induces apoptosis within 1 hour (Tiano et al., 2003) and in gill tissue of the mussel Mytilus galloprovincialis treated with 1 µg/g b.w. TBT (= 3 µM) apoptosis could be detected after 24 hours incubation (Micic et al., 2001). It has been intensely discussed that TBT exerts strong immunosuppressive properties and this effect might be the consequence of specific induction of cell death in immunocompetent cells. Killing lymphocytes by TBT can be observed as a loss in thymus weight. Thymus atrophy (Albers et al., 1996; Gennari et al., 1997; 2000; 2002b) debilitates the immune function of animals, making them vulnerable to infectious diseases (Anderson et al., 1996; Bryan et al., 1989; Cooper et al., 1995; Krajnc et al., 1984; Miller et al., 1986a; Schüürman et al., 1992; Vos et al., 1984a; 1990; Whalen et al., 1999; 2002).
Dibutyltin (DBT)

DBT-treated lymphocytes (100 nM) exhibited reduced proliferation and undergo apoptosis (De Santiago and Aguilar-Santelises, 1999). An earlier study discussed a cytocstatic effect of the two compounds DBT and DOT on thymus lymphocytes (Penninks et al., 1985). Electron microscopy indicated no overt destruction of thymocytes. But in the middle of the 1980s apoptotic cells could not be detected very easily in tissues and the reduction in the number of small cortical lymphocytes from the actual point of view was definitely due to apoptosis.

Oral administration of DBT and TBT caused a 50 % reduction of thymus weight, and DBT exerts this effect at substantially lower doses than TBT (Snoeij et al., 1988). Furthermore, the number of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes was reduced by DBT treatment (Pieters et al., 1992), possibly via the disturbance of the binding between thymocytes and thymic epithelial cells (Pieters et al., 1995). In many studies thymus atrophy could be observed after DBT treatment (Gennari et al., 1997; 2000; 2002b; Merkord et al., 2001; Pieters et al., 1989; 1992; 1994; 1995), and this effect might be the consequence of the above described mechanisms.

Triphenyltin (TPT)

TPT was shown to induce an elevation of intracellular calcium concentration in rat thymocytes less than TBT (Aw et al., 1990) but to the same extent in PC12 cells (Viviani et al., 1995). In freshly prepared mouse thymocytes, 1 – 12 µM TPT is cytotoxic and the cells exhibit all morphological signs of apoptosis (Bollo et al., 1996). A closer look on the subpopulations of mice thymic primary culture provides evidence for a depletion of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes after TPT exposure (Bollo et al., 1997). In human HL-60 cells, TPT induces a rapid increase in the intracellular Ca<sup>2+</sup> concentration, breakdown of cytoskeleton, DNA fragmentation and NF<sub>kB</sub> activation at concentration of 2.5 and 5 µM (Marinovich et al., 1996); whereas in other studies, TPT applied at higher concentrations than 1 µM induces necrotic cell death (Krug, unpublished). Human T-cell lines (Hut-78 and Jurkat) are shown to be more sensitive against TPT than to TBT. Between 10 nM and 1 µM caspase activity was induced whereas TBT has to be used at concentrations of 0.5 to 5 µM to exert the same effect (Stridh et al., 1999c). At higher concentrations (10 µM) it induces apoptosis in human MCF-7 breast cancer cells (Lin and Garry, 2000) but only 200 ng/ml (∼0.5 µM) induce apoptosis in human granulosa-like tumour cells (Saitoh et al., 2001). In comparison to other organometal compounds such as triphenylbismuth, TPT was considerably more toxic to rat thymocytes and induces apoptosis at concentration between 1 to 3 µM (Arata et al., 2002).

Slight effects of TPT on the rat immune system were observed after 25 mg/kg b.w. (Vos et al., 1984b) and strong immunotoxicity was observed at doses of 1 and 10 mg/kg i.p. in mice (Nishida et al., 1990) and in clams (Cima et al., 1998). This is associated with thymus atrophy in mice and rats (Nishida et al., 1990; Snoeij et al., 1985) comparable to that after treatment with other OTC.
Dioctyltin (DOT)

No extended studies on the molecular mechanism of DOT with respect to apoptosis have been done so far. Indirect evidences suggest a similar mode of action to TBT or TPT but this has to be evaluated. For instance, studies dealing with the influence of DOT on thymus function and tissue growth (Seinen and Willems, 1976; Seinen et al., 1977ab) demonstrate the suppression of thymus-dependent immunity in rats. DOT was shown to reduce the number and viability of cells that can be isolated from the thymus of treated rats and strong immunosuppression was detected (Seinen et al., 1979). Miller et al., (1986b) described the decrease of thymocyte proliferation when rats were administered with 75 mg DOT/kg b.w. and several authors reported on the thymic injury after oral administration of DOT (Boyd and Jones, 1986; Miller et al., 1984; Smialowicz et al., 1988; Smialowicz, 2002).

Inbred rats fed diets containing 75 mg/kg DOT for 8 or 12 weeks demonstrated marked reduction in thymic weight. In other studies DOT administration resulted in addition in a selective immunodeficiency (Evans et al., 1986; Miller and Scott, 1985; Miller et al., 1984; 1986a,b; Nicklin et al., 1985). Oral administration of 75 mg DOT/kg b.w. to rats induces the production or release of factors which appear to prevent thymocyte maturation at an early stage. In vitro uptake of tritiated thymidine by thymocytes obtained from DOT-gavaged rats was markedly reduced. These events occurred within 24 to 72 hours of DOT treatment and preceded the thymic weight loss (Miller et al., 1986b; Penninks et al., 1985). These effects may be a consequence of blocking the intrathymic differentiation of T cell precursors or cell number reduction via apoptosis. But the restricted development or growth of thymocytes can additionally be the result of severe dysregulation of important signalling pathways such as interleukin-2 expression that is suppressed by DOT in treated rats (Volsen et al., 1989).

Further observations were made earlier by Hennighausen and Lange (1979; 1980) who described a reduction in cell number of thymus and a loss of thymus weight after single administration of DOT to mice. Additionally, in rats thymus atrophy was observed to be sex and strain dependent (Boyd and Jones, 1986).
## Annex 8 – Summary table of the most relevant toxicological data for TBT, DBT, TPT and DOT

<table>
<thead>
<tr>
<th>Type of test/ Compound</th>
<th>Species (Group size, route of Exposure, duration)</th>
<th>Results and Comments</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repeated dose TBTOT</td>
<td>Wistar rats (diet, 4 wk, 12 months)</td>
<td>NOAEL 0.025 mg/kg b.w./day, based on depleted iron stores in spleen and sinus erythrocytosis in mesenteric lymph nodes.</td>
<td>Krajnc et al., 1984; Wester et al., 1988; 1990; Bressa et al., 1991</td>
</tr>
<tr>
<td>Repeated dose TPTOT</td>
<td>NMRI mice (diet, 3 months)</td>
<td>NOAEL: 3.44-4.12 mg/kg b.w./day, based on aematological and biochemical parameters.</td>
<td>Suter and Horst, 1986a</td>
</tr>
<tr>
<td>Repeated dose TPTOT</td>
<td>Wistar rats (diet, 13 weeks)</td>
<td>NOAEL 1.56-1.72 mg/kg b.w./day, based on haematological and biochemical parameters.</td>
<td>Suter and Horst, 1986b</td>
</tr>
<tr>
<td>Repeated dose TPTOT</td>
<td>Beagle dogs (diet, 1 year)</td>
<td>NOAEL 0.593 mg/kg b.w./day, based on haematological and biochemical parameters</td>
<td>Sachose et al. 1987</td>
</tr>
<tr>
<td>Repeated dose DOT</td>
<td>Wistar rats (diet, 90 days)</td>
<td>NOAEL ~ 15 mg/kg b.w./day, based on dose-related decrease in thymus weight.</td>
<td>EC, 1999</td>
</tr>
<tr>
<td>Chronic toxicity/Carcinogenicity TBTO</td>
<td>Wistar rats (n=60; diet; 2 years, n=10 interim kill at 12 months)</td>
<td>NOAEL 0.025 mg/kg b.w./day based on changes in haematological parameters and immunoglobulin levels Increased incidence of benign pituitary tumours in both sexes at low 0.025 mg/kg b.w./day) and high (2.5 mg/kg b.w./day), but not intermediate, doses. Other endocrine-related tumours at high dose.</td>
<td>Wester et al., 1988; 1990</td>
</tr>
<tr>
<td>Carcinogenicity TBTO</td>
<td>CD1 mice (n=50, diet, 18 months)</td>
<td>No increased incidence of tumours up to the top dose of 7.7-9.2 mg/kg b.w./day.</td>
<td>Daly, 1992</td>
</tr>
<tr>
<td>Chronic toxicity/ Carcinogenicity DBTAc</td>
<td>F344 rats (n=50, diet, 2 years) and B6C3F1 mice (n=50, diet, 18 months)</td>
<td>No increased incidence of tumours up to the top dose (133 ppm in the diet) in male rats; inconclusive evaluation for female rats for accidental loss of tissues. No increased incidence of tumours in mice up to the top dose (152 ppm in the diet).</td>
<td>NCI, 1979</td>
</tr>
<tr>
<td>Carcinogenicity TPTOT</td>
<td>KFM-Han NMRI mice (n=50, diet, 80 weeks)</td>
<td>NOAEL 1.3 mg/kg b.w./day, based on reduced body weight gain. Increased incidence of hepatocellular adenomas in both sexes and hepatocellular carcinomas in females at top dose (21.76 mg/kg b.w./day).</td>
<td>Tennekes et al., 1989a</td>
</tr>
<tr>
<td>Type of test/ Compound</td>
<td>Species (Group size, route of Exposure, duration)</td>
<td>Results and Comments</td>
<td>Reference(s)</td>
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</tr>
<tr>
<td>Chronic toxicity/ Carcinogenicity TPTOH</td>
<td>KFM-Han Wistar rats (n=70, diet, 2 years)</td>
<td>LOAEL 0.3 (males) 0.4 (females) mg/kg b.w./day based on reduction in immunoglobulin and in white blood cells. Increased incidence of Leydig cell tumours and pituitary tumours at &gt;1.6 mg/kg b.w./day.</td>
<td>Tennekes et al., 1989b</td>
</tr>
<tr>
<td>Carcinogenicity DOT/MOT (2:1 mixture)</td>
<td>F3-hybrid of RII 1/Tif x RII 2/Tif rats (n=60, diet, 2 years)</td>
<td>Increased incidence of thymus lymphomas in the females at the top dose (6.0 mg/kg b.w./day) and increase of generalized malignant lymphomas in the females at the top dose and in the males at the two highest doses (2.2 and 5.5 mg/kg b.w./day).</td>
<td>Ciba-Geigy Ltd, 1982, EC, 1999</td>
</tr>
<tr>
<td>Genotoxicity DBTC</td>
<td>In vitro</td>
<td>Mutagenic in the CHO/HGPRT assay.</td>
<td>Li et al., 1982</td>
</tr>
<tr>
<td>Genotoxicity TPTOH</td>
<td>In vitro/in vivo</td>
<td>Weakly clastogenic in vitro; not convincing evidence in vivo.</td>
<td>EC, 1997</td>
</tr>
<tr>
<td>Genotoxicity TPTAc</td>
<td>In vitro/in vivo</td>
<td>Weakly clastogenic in vitro; weakly positive in in vivo micronucleus assay.</td>
<td>EC, 1997; Chao et al., 1999</td>
</tr>
<tr>
<td>Genotoxicity DOTC</td>
<td>In vitro/in vivo</td>
<td>Essentially negative.</td>
<td>EC, 1999</td>
</tr>
<tr>
<td>Immunotoxicity TBTO</td>
<td>Wistar rats (2 weeks)</td>
<td>LOAEL 1 mg/kg b.w./day, based on reduced thymus-dependent parameters and resistance to <em>T.spiralis</em> infection.</td>
<td>Vos et al., 1984a; 1985</td>
</tr>
<tr>
<td>Immunotoxicity TBTO</td>
<td>Wistar rats (diet 4-6 or 15-17 months)</td>
<td>NOAEL 0.025 mg/kg b.w./day based on reduced resistance to <em>T.spiralis</em> infection.</td>
<td>Vos et al., 1990</td>
</tr>
<tr>
<td>Immunotoxicity DBTC</td>
<td>Wistar rats (2 weeks)</td>
<td>Reduction in thymus weight and various parameters for humoral and cellular response: LOAEL 2.5 mg/kg b.w./day.</td>
<td>Seinen et al. 1977 a,b</td>
</tr>
<tr>
<td>Immunotoxicity TPTCl</td>
<td>Male Wistar rats (diet, 2 weeks)</td>
<td>LOAEL 0.75 mg/kg b.w./day, based on reduced thymus and spleen weight.</td>
<td>Snoeij et al., 1985</td>
</tr>
<tr>
<td>Immunotoxicity TPTOH</td>
<td>Mice (strain unspecified)</td>
<td>NOAEL 1 mg/kg b.w./day, based on reduced liver and thymus weight and decreased IgM.</td>
<td>McCormick and Thomas, 1990</td>
</tr>
<tr>
<td>Immunotoxicity DOTC</td>
<td>Wistar rats (2 weeks)</td>
<td>Reduction in thymus weight and various parameters for humoral and cellular response: LOAEL 2.5 mg/kg b.w./day</td>
<td>Seinen et al. 1977 a,b</td>
</tr>
<tr>
<td>Type of test/ Compound</td>
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</tr>
<tr>
<td>Reproduction (two generation) TBTO</td>
<td>SD rats (diet)</td>
<td>NOAEL 0.29-0.34 mg/kg b.w./day, based on decreased thymus weight in adults and decreased pup weight during lactation.</td>
<td>Schroeder, 1990</td>
</tr>
<tr>
<td>Reproduction (two-generation) TBTCI</td>
<td>Wistar rats (diet)</td>
<td>LOAEL 0.25 mg/kg b.w./day, based on decreased testis weight in F1 adults and increased anogenital distance in pups.</td>
<td>Ogata et al., 2001; Omura et al., 2001</td>
</tr>
<tr>
<td>Female reproduction TBTCI</td>
<td>Female Wistar rats (gavage, 0-3 or 4-7 day of gestation)</td>
<td>NOAEL 4:1 mg/kg b.w./day, based on increased implantation failure, reduced progesterone, and reduced foetal weight (treatment on day 0-3 of gestation).</td>
<td>Harazono et al., 1998; 2000</td>
</tr>
<tr>
<td>Reproduction (two-generation) TPTOH</td>
<td>Wistar rats (diet)</td>
<td>NOAEL 0.4 mg/kg b.w./day, based on decreased litter size, pup weight and relative spleen/thymus weight in weanlings.</td>
<td>Young, 1986</td>
</tr>
<tr>
<td>Prenatal developmental toxicity TBTO</td>
<td>NMRI mice (gavage, 6-15 day of gestation)</td>
<td>NOAEL (maternal toxicity) 5.8 mg/kg b.w./day based on reduced weight gain; NOAEL for prenatal toxicity 5.8 mg/kg b.w./day, based on increased incidence of cleft palate.</td>
<td>Davis et al., 1987</td>
</tr>
<tr>
<td>Prenatal developmental toxicity TBTO</td>
<td>SD rats (gavage, 6-19 day of gestation)</td>
<td>LOAEL (maternal toxicity) 5 mg/kg b.w./day based on reduced weight gain; LOAEL for prenatal toxicity 5 mg/kg b.w./day, based on increased incidence of minor skeletal anomalies.</td>
<td>Schroeder, 1981</td>
</tr>
<tr>
<td>Developmental toxicity (prenatal exposure, postnatal follow-up) TBTO</td>
<td>Long-Evans rats (gavage, 6-20 day of gestation)</td>
<td>NOAEL (maternal toxicity) 5 mg/kg b.w./day, based on reduced weight gain; NOAEL for developmental toxicity 5 mg/kg b.w./day, based on reduced litter size, pup weight and survival, delayed vaginal opening and reduced brain weight.</td>
<td>Crofton et al., 1989</td>
</tr>
<tr>
<td>Prenatal developmental toxicity DBTAc</td>
<td>Wistar rats mice (gavage, 6-15 day of gestation)</td>
<td>NOAEL (maternal toxicity) 5 mg/kg b.w./day based on reduced weight gain, food consumption and thymus weight; NOAEL for prenatal toxicity 5 mg/kg b.w./day, based on increased incidence of total malformations.</td>
<td>Farr et al. 2001</td>
</tr>
<tr>
<td>Prenatal developmental toxicity TPTOH</td>
<td>NZW Rabbit (gavage, 6-18 of gestation)</td>
<td>NOAEL (maternal toxicity) 0.1 mg/kg b.w./day, based on reduced weight gain; NOAEL for foetotoxicity (lower foetal weight) 0.3 mg/kg b.w./day</td>
<td>Rodwell, 1987</td>
</tr>
<tr>
<td>Type of test/ Compound</td>
<td>Species (Group size, route of Exposure, duration)</td>
<td>Results and Comments</td>
<td>Reference(s)</td>
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<tr>
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<tr>
<td>Prenatal developmental toxicity TPTOH</td>
<td>SD Rats (gavage, 6-15 day of gestation)</td>
<td>NOAEL (maternal toxicity) 1 mg/kg b.w./day, based on reduced weight gain and food consumption; NOAEL for embryotoxicity (increased resorptions, reduced foetal weight) 2.8 mg/kg b.w./day.</td>
<td>Rodwell, 1985</td>
</tr>
<tr>
<td>Prenatal developmental toxicity. Mixture DOTTG (80%)- MOTTG (20%)</td>
<td>NMRI mice (gavage)</td>
<td>NOAEL 45 mg/kg b.w./day for malformations.</td>
<td>Faqi et al., 2001</td>
</tr>
<tr>
<td>Teratogenicity Mixture DOTTG (80%)- MOTTG (20%)</td>
<td>NZW rabbits (gavage)</td>
<td>NOAEL 1 mg/kg b.w./day for embryo-foetotoxicity</td>
<td>Pharma Research, 1992; EC, 1999</td>
</tr>
<tr>
<td>Teratogenicity Mixture DOTTG (80%)- MOTTG (20%)</td>
<td>Wistar rats (gavage)</td>
<td>NOAEL 5 mg/kg b.w./day for embryo-foetotoxicity</td>
<td>Pharma Research, 1993, EC, 1999</td>
</tr>
<tr>
<td>Neurotoxicity TPTAc</td>
<td>Male CFY rats (gavage, 6 weeks, 6 day/week)</td>
<td>LOAEL 0.36 mg/kg b.w./day based on performance deficits in maze learning test</td>
<td>Lehotzky et al., 1982</td>
</tr>
<tr>
<td>Neurotoxicity (developmental) TBTCI</td>
<td>SD rats (gavage, 60-20 day of gestation)</td>
<td>LOAEL 1.0 mg/kg b.w./day, based on impaired spatial performance and hyperactivity in adult offspring</td>
<td>Gardlund et al., 1991</td>
</tr>
<tr>
<td>Neurotoxicity TBTCI</td>
<td>Male Balb/c mice (diet, 30 days)</td>
<td>LOAEL 0.1 mg/kg b.w./day, based on impaired N-methyl-D-aspartate receptor binding</td>
<td>Konno et al., 2001</td>
</tr>
</tbody>
</table>