Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

Dibutyltin dilaurate

EC Number: 201-039-8
CAS Number: 77-58-7

CLH-O-0000001412-86-59/F

Adopted

05 June 2015

05 June 2015

CLH-O-0000001412-86-59/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonized classification and labelling (CLH) of:

Chemicals name: Dibutyltin dilaurate

EC Number: 201-039-8

CAS Number: 77-58-7

The proposal was submitted by **Norway** and received by RAC on **30 September 2014** classifications are given in the form of CLP hazard classes and/or categories, the majority of which are consistent with the Globally Harmonized System (GHS).

PROCESS FOR ADOPTION OF THE OPINION

Norway has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation*** on **26 September 2014**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **10 November 2014**.

ADOPTION OF THE OPINION OF THE RAC

Rapporteur, appointed by RAC: **Betty Hakkert**

Co-rapporteur, appointed by RAC: **Michael Neumann**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation.

The RAC opinion on the proposed harmonized classification and labelling was reached on **05 June 2015** and the comments received are compiled in Annex 2.

The RAC opinion was adopted by **consensus**.

OPINION OF RAC

RAC adopted the opinion on Dibultyltin dilaurate that should be classified and labelled as follows

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard state-ment Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	**No current Annex VI entry**										
Dossier submitters proposal	TBD	Dibutyltin dilaurate; dibutyl[bis(dodecanoyloxy)]stannane	201-039-8	77-58-7	Muta. 2 Repr. 1B STOT RE 1	H341 H360FD H372 (immune system)	GHS08	H341 H360FD H372 (immune system)			
RAC opinion	TBD	Dibutyltin dilaurate; dibutyl[bis(dodecanoyloxy)]stannane	201-039-8	77-58-7	Muta. 2 Repr. 1B STOT RE 1	H341 H360FD H372 (immune system)	GHS08	H340 H360FD H372 (immune system)			
Resulting Annex VI entry if agreed by COM	TBD	Dibutyltin dilaurate; dibutyl[bis(dodecanoyloxy)]stannane	201-039-8	77-58-7	Muta. 2 Repr. 1B STOT RE 1	H341 H360FD H372 (immune system)	GHS08	H341 H360FD H372 (immune system)			

RAC general comment

The dossier submitter (DS) stated that experimental data indicated that dibutyltin dilaurate (abbreviated throughout this document as DBTDL) can hydrolyse into dibutyltin in the stomach producing dibutyltin dichloride (abbreviated throughout this document as DBTC). This is based on results from an *in vitro* hydrolysis study (Schilt, 2004). Furthermore, it was assumed that dibutyltin (probably as the dichloride, DBTC) is the moiety responsible for the effects when animals are exposed orally to DBTDL. When considering classification of DBTDL for hazard classes that depend on exposure through the oral route, according to the DS it is justified to take studies into account where DBTC and other rapidly acid (stomach)-hydrolysable dibutyl substances have been administered orally. Therefore, studies on DBTC were taken into consideration by the dossier submitter when proposing classification of DBTDL for the hazard classes which are the subject of the current RAC CLH opinion: specific target organ toxicity upon repeated exposure (STOT RE), mutagenicity and reproductive toxicity.

RAC assessed the DS proposal for using data on DBTC to support the classification of DBTDL following oral exposure. Data on hydrolysis in sweat or lung fluid appropriate to the dermal and inhalation routes was not available.

RAC evaluated the *in vitro* hydrolysis study of Schilt (2004), in which DBTDL was tested at a final concentration of 0.01 mg/mL under low pH (~1-2) conditions (0.07 N HCl) at 37°C in order to simulate the hydrolytic action of mammalian gastric contents. The degree of hydrolysis for the test substance was studied by determining the amount of DBTC formed after 0.5, 1, 2, and 4 hours, using GC-FPD. After 2 hours, 87.8% of the test material had hydrolysed, indicating that DBTDL is indeed converted into DBTC in sufficient amount and speed and that therefore data from DBTC can be used for the assessment of DBTDL.

Such data was previously accepted by RAC for other organotin compounds including 2-ethylhexyl 10-ethyl-4,4-dimethyl-7-oxo-8-oxa-3,5-dithia-4-stannatetradecanoate (RAC opinion, 2012a) and Dioctyltin bis(2-Ethylhexyl mercaptoacetate) (RAC opinion, 2012b).

RAC evaluation of specific target organ toxicity– repeated exposure (STOT RE)

Summary of the Dossier submitter's proposal

The CLH report summarised several repeated dose toxicity studies, conducted both with DBTDL and DBTC. As *in vitro* experimental data indicated that DBTDL can hydrolyse into DBT in the stomach producing DBTC, oral studies on DBTC were taken into consideration by the dossier submitter (DS) when proposing classification for DBTDL for STOT RE.

<u>DBTDL</u>
An oral 13-wk rat study with exposure to DBTDL via the diet at up to 2000 ppm (133 mg/kg bw/d) is included in the CLH report (Study Report, 1961). Furthermore, two oral rat studies where DBTDL was administered via gavage during exposure periods of 2 weeks (in peanut oil; up to 16 mg/kg bw/d) and 7 weeks (in corn oil; up to 20 mg/kg bw/d) were reported, with the first study focusing on immunotoxicity effects and the latter study focussing on effects in the brain

(Subramoniam *et al.*, 1994; Jin *et al.*, 2012). Finally, a 15-d rat study (only one DBTC-dose group) is included, which focussed on liver enzyme measurements (Mushtaq *et al.*, 1981).
No dermal or inhalation repeated dose studies with DBTDL are included in the CLH dossier.
Following oral DBTDL exposure effects were observed on the thymus (cell count, reduced organ weight, histological alterations and functional disturbances; LOAEL 4 mg/kg bw/d) and lymph nodes (cell count), in addition to reduced weight gain and feed intake, as well as liver and brain effects (alterations in brain antioxidant enzymes, DNA damage, apoptosis).

DBTC
An OECD 421 reproduction/developmental toxicity screening test in rats with exposure to DBTC via the diet is included (Waalkens-Berendsen, 2003), as well as a rat prenatal developmental toxicity study conducted according to OECD TG 414 with exposure to DBTC up to 10 mg/kg bw/d via oral gavage (Study Report, 1993). A rat developmental toxicity study with exposure to DBTC via drinking water (DeWitt *et al.*, 2006) and a rat/mouse sub-acute/developmental immunotoxicity feeding study (Seinen et al., 1977b) are reported in the CLH dossier. Gaunt et al. (1968) reported a rat 90-d oral feeding study, and a 28-d rat oral drinking water study was performed by DeWitt et al. (2005). Also, a 2-wk oral feeding study (Penninks and Seinen, 1982), which also included single parenteral (ip and iv) injections, and 2-wk rat and 4-wk mouse oral feeding studies by Seinen *et al*. (1977a) were reported. Finally, a rat study focused on time- and dose-dependent effects on the thymus (Snoeij *et al.*, 1988) and a study with administration of DBTC via the diet daily for 4 days or every 2 days for 12 days (focus on liver) were reported (Yermakoff *et al.*, 1979).

No dermal or inhalation repeated dose studies with DBTC were included in the CLH dossier.
The immune system was clearly the target organ, as observed after oral exposure to DBTC. Effects included reduced thymus weight, thymus atrophy with moderate to, in some studies, even very severe lymphoid depletion (LOAEL 1.7-2.4 mg/kg bw/d).

The dossier submitter concluded that the data supported classification for specific target organ toxicity following repeated exposure as STOT RE 1 with the immune system as target organ.

Comments received during public consultation

One MSCA agreed with the proposed classification for STOT RE during public consultation.
Two industry associations submitted similar comments expressing their disagreement with the proposed classification for STOT RE (and reproductive toxicity) during public consultation. Their main argument was the presence of a tributyltin (TBT) impurity, which was considered by the industry associations to be responsible for the observed immunotoxic (and reproductive) effects. In their response, the dossier submitter still considered that the available data warrant classification of DBTDL for STOT RE (and reproductive toxicity). They stated that the degree of contamination of TBT in dibutyltin (DBT) test substances is unclear, both in older and in more recent studies. Furthermore, the dossier submitter evaluated a number of mechanistic studies, which indicated that both TBT and DBT affect mechanisms underlying effects on fertility and development and immune function, although it is recognised that it is as yet not clear whether TBT and BDT act according to the same or different pathways.

Assessment and comparison with the classification criteria

The CLH report includes several animal studies with repeated oral exposure to DBTDL and DBTC. No studies are available for the dermal and inhalation route. Furthermore, no long-term carcinogenicity studies are summarised in the CLH report.

DBTDL

The following studies were evaluated:

- 13-week feeding study in rats (Study Report, 1961)
- 7-week gavage study in rats; focus on brain effects (Jin *et al.*, 2012)
- 2-week gavage study in rats; focus on immunotoxicity (Subramoniam *et al.*, 1994)
- 15-day feeding study in rats; focus on liver enzymes (Mushtaq *et al.*, 1981)

In the 13-week feeding study (non-GLP, non-guideline; Study Report (1961)), observed effects were limited to reduced body weight gain and feed intake (doses up to 2000 ppm via diet, 133 mg/kg bw/d according the CLH report). Furthermore, an enlarged bile duct was the most common gross necropsy observation. However, due to the limited number of parameters determined in this study, other effects cannot be excluded.

Results of the 7 week gavage study (non-GLP, non-guideline; 0, 5, 10 or 20 mg/kg bw/d; 5 days/week), which focussed on effects in brain, showed changes in various antioxidant enzymes in rat brain tissue, i.e. reduced superoxide dismutase and glutathione peroxidase activity and increased malondialdehyde content, nitric oxide content and nitric oxide synthase activity (Jin *et al.*, 2012). Further, dose-dependent increases in DNA-damage and apoptosis and disturbed cell cycle were observed. The highest dose of 20 mg/kg bw/d resulted in ultrastructural changes in rat brain and included neuropil cavitation and glial filaments dissolving within the axon.

A 15-d feeding study (non-GLP, non-guideline; 17.5 mg/kg bw/d) which focussed on liver enzymes was included in the CLH report as supplementary information. Effects observed included mortality (20%), reduced body weight gain, and decreased liver enzyme activities (glucose-6-phosphatase (35%), aniline hydroxylase (22%), benzo(a)pyrene hydroxylase (57%), aminopyrine-N-demethylase (32%), benzphetamine-N-demethylase (33%) and cytochrome P450 content (32%)) (Mushtaq *et al.,* 1981).

In the 2 week gavage study (non-GLP, non-guideline; 0, 2, 4, 8 or 16 mg/kg bw/d, 5 days/week) which focussed on immunotoxic effects, effects included a dose-dependent reduction in thymus weight, and reduced nucleated cell count in thymus (≥4 mg/kg bw/d), spleen (≥16 mg/kg bw/d), peripheral lymph nodes (≥8 mg/kg bw/d) and mesenteric lymph nodes (≥8 mg/kg bw/d) (Subramoniam *et al.*, 1994).

In general these studies with DBTDL were of limited value for the overall assessment of DBTDL due to the focus on specific type of effects and the limited number of parameters studied. However, the results of the 2 week gavage study (Subramoniam *et al.*, 1994) indicate that DBTDL exerts clear effects on the immune system at dose levels ≥4 mg/kg bw/d. Although this study was not conducted under GLP and only immune parameters were examined, the number of animals used is in line with the number used for standard short term toxicity studies (n=5-6) and the effects observed on the immune parameters were clearly dose related. Therefore, RAC considers these immune effects of DBTDL as relevant for the classification for STOT RE.

DBTC

The following studies were evaluated:

- OECD TG 421 reproductive/developmental toxicity screening test (diet) in rats (Waalkens-Berendsen, 2003)
- OECD TG 414 prenatal developmental toxicity study (gavage) in rats (Study Report, 1993)
- Developmental toxicity study (drinking water) in rats (DeWitt *et al.*, 2006)
- Sub-acute/Developmental toxicity study (diet) in rats and mouse - focus on immunotoxicity parameters (Seinen *et al.*, 1977b)

- 90 day feeding study in rat (Gaunt *et al.*, 1968)
- 28 day drinking water study in rat (DeWitt *et al.*, 2005)
- 2 week rat / 4 week mouse feeding study (Seinen *et al.*, 1977a)
- 2 week feeding study in rats with additional ip/iv exposure (Penninks and Seinen, 1982)
- Single oral gavage rat study with additional iv exposure (Snoeij *et al.*, 1988)
- Sub-acute oral feeding study, exposure daily for 4 days or every 2 days for 12 days (Yermakoff *et al.*, 1979)

An OECD TG 421 reproductive/developmental toxicity screening test (diet) in rats (Waalkens-Berendsen, 2003) showed, in addition to reduced body weight gain and food consumption in male and female animals, a reduced relative thymus weight and moderate to severe lymphoid depletion in dams exposed to 1.7-2.4 mg/kg bw/d (exposure 41 days for females and 28 days for males). A dose of 6.2- to 15.4 mg/kg bw/d induced a reduced absolute and relative thymus weight and a severe to very severe lymphoid depletion in dams. Lymphoid depletion was characterized by a decrease in size of thymic lobules due to an extensive loss of cortical and medullary small lymphocytes. The distinction between cortical and medullary areas was blurred. In the severe cases the cortex was very small, or partially absent. The effects on fertility and development as observed in this study are described and evaluated in the section *RAC evaluation of Reproductive Toxicity*.

An OECD TG 414 prenatal developmental toxicity study in rats (oral gavage; 0, 1, 2.5, 5, 10 mg/kg bw/d on GD 6-15) showed clear maternal toxicity (Study Report, 1993). Effects included reduced bw gain (≥5 mg/kg bw/d), reduced food consumption (≥10 mg/kg bw/d) and significantly increased number of animals with thymus atrophy (≥10 mg/kg bw/d). Maternal toxicity was not observed at a dose of 1 mg/kg bw/d. The effects on development, as observed in this study, are described and evaluated in the section *RAC evaluation of Reproductive Toxicity*.

A rat drinking water developmental toxicity study focussing on immunotoxicity (DeWitt *et al.*, 2006) was included in the section on repeated dose toxicity of the CLH-report. Maternal toxicity was, however, not evaluated. The effects on development (immunological response in mature offspring) as observed in this study are described and evaluated in the section *RAC evaluation of Reproductive Toxicity*.

A combined sub-acute/developmental toxicity study (non-GLP, non-guideline; Seinen *et al.*, 1977b) in the rat and mouse was also included in the CLH report. In the sub-acute experiment, adult rats and mice were exposed to DBTC via the diet for 4 weeks (50 and 150 ppm DBTC in the diet; corresponding to 2.5 and 7.5 mg/kg bw/d for rats, and 7.1 and 21.4 mg/kg bw/d for mice, respectively). In the developmental toxicity study, dams were exposed via the diet (50 and 150 ppm) from GD 2. Post-natally, the pups were exposed via milk and additionally via gastric intubation (0, 1, 3 mg/kg bw 3 d/wk until week 7). In both the adult rats as well as the pups (rat), a dose-dependent cellular immune response (allograft rejection) was observed. Haemagglutination and haemolysin titers, as well as the number of direct plaque-forming cells against SRBC per spleen, were decreased in a dose-related manner in rats (adults and pups). The rat antibody response to LPS (i.e. a T-cell independent antigen) was not affected upon exposure to DBTC. Secondary antibody responses were not affected by DBTC. In general, immune suppression was most pronounced in animals exposed to DBTC during the developmental phase of the lymphoid system. Effects on immune function were not observed in mice (adults or pups).

A 90-d feeding study in rats (0, 10, 20, 40, 80 ppm DBTC in diet, corresponding to 0, 0.5, 1, 2, 4 mg/kg bw/d) indicated some slight effects such as reduced food consumption and body weight

and mild anaemia at the highest dose (Gaunt *et al.*, 1968). No abnormalities were seen at autopsy or histology (including the thymus).

A 28-d rat drinking water study (0, 0.9, 1.9 mg DBTC/kg bw/d in an initial experiment; 0, 1.0, 2.8 mg DBTC/kg bw/d in the replicate experiment), which focussed on immunotoxic effects, did not reveal any treatment-related effect on organ weight (including the thymus and spleen), antibody production, delayed type hypersensitivity (DTH) response or natural killer (NK) cell activity. A slight reduction in water consumption was observed in the high dose group (DeWitt *et al.*, 2005).

A sub-acute rat/mouse immunotoxicity study (rat: 2 week, mouse: 4 week) with doses of DBTC of 0, 50 and 150 ppm in the diet (corresponding to 0, 2.5, 7.5 mg/kg bw/d for rats and 0, 7.1, 21.4 mg/kg bw/d for mice) was included in the CLH report (Seinen *et al.*, 1977a). No treatment-related effects were observed in mice. In rats, mortality was observed in the 7.5 mg/kg bw/d group (4/10 females and 2/10 males). Further, clear dose-dependent effects on the thymus were observed. Reductions in relative organ weights were noticed for the thymus (2.5 mg/kg bw/d: 53%, 7.5 mg/kg bw/d: 68-72%), but also the spleen (2.5 mg/kg bw/d: 16%, 7.5 mg/kg bw/d: 33%) and popliteal lymph nodes (2.5 mg/kg bw/d: 16%, 7.5 mg/kg bw/d: 28%). A pronounced reduction in size of the thymus was found in all DBTC-treated animals. The most important effect observed was lymphocyte depletion in lymphoid organs, which was most pronounced in the thymic cortex of DBTC-treated animals. At the 7.5 mg/kg bw/d level, the thymic cortex was almost completely depleted, although no signs of cell destruction were observed. Lymphocyte depletion was also present in the thymus-dependent areas of the spleen and popliteal lymph nodes. Also, effects on liver were observed and included thickened and dilated bile ducts accompanied by irregularly yellowish discoloured livers. These effects were found in animals that died and in 2 male and 2 female survivors of the high dose group. Microscopic analysis revealed severe proliferation of bile duct epithelial cells and bile ducts which was associated with pericholangiolitis and periportal fibrosis in livers of 4 male and 6 female rats of the high dose group. Other treatment-related histopathological changes were not observed. In addition to exposure via the diet, also iv injection was applied and results of this experiment in rats supported the findings of the oral study.

An additional 2 week rat feeding study (0, 50, 150 ppm DBTC in diet, corresponding to 0, 2.5, 7.5 mg/kg bw/d) confirmed previous findings of clear effects on thymus (Penninks and Seinen, 1982). Relative thymus weight was reduced (<30% of control group), and lymphocyte depletion was observed in thymus (mainly in the thymic cortex and in thymus-dependent lymphoid areas of the spleen). Also parenteral administration (single ip and iv injection of 2.5 mg/kg bw) was applied and caused severe thymic atrophy.

Finally, two supplementary studies are included in the CLH-report. In the first, dose- and time-effects were studied (Snoeij *et al.*, 1988). Rats were administered DBTC via oral gavage in a single dose of 15 mg/kg bw and killed after 1, 2, 3, 4, 5, 7 and 9 days and a second group of rats received doses varying between 5 and 35 mg/kg bw and were killed 4 days post-exposure. A dose-dependent reduction in thymus weight was observed, and further, thymus weights returned to normal at day 9 post-exposure. In the second supplementary study which focussed on liver, rats were exposed to DBTC via oral gavage (0, 10, 20 mg/kg bw/d) daily during 4 days or every 2 days for 12 days (Yermakoff *et al.*, 1979). Results pointed towards inflammatory reactions and included extensive inflammation in the portal tracts, biliary damage, fibrosis, necrosis, infarcted areas and granulomatous lesions.

Evaluation

The available data point towards the immune system as a clear target organ after oral exposure to DBTDL. Repeated exposure to DBTDL during 2 weeks revealed reduced thymus size and cell

depletion (reduced cell counts) with effective dose levels of ≥4 mg/kg bw/d (Subramoniam *et al.*, 1994). This is below the extrapolated guidance value for classification as STOT RE 1 (i.e. 60 mg/kg bw/d for a 2 week study). As indicated before, this study was not conducted under GLP and limited parameters were examined. However, the number of animals (n=5-6) is in line with the number used in short term toxicity studies and the effects observed on immune parameters were clearly dose related and were observed at levels of 4 mg/kg bw/d and as such these findings provide an important basis for classification for specific target organ toxicity after repeated exposure.

Given that DBTDL is, at least in part, hydrolysed in the stomach upon oral exposure producing DBTC, RAC is of the opinion that data on DBTC (as hydrolysis product of DBTDL) can be used for classification of DBTDL for repeated dose toxicity (see also under *RAC general comment*).

Studies on DBTC also revealed effects on the immune system, though more pronounced in comparison with the limited studies on DBTDL. Effects included thymus atrophy with lymphoid depletion, loss of organ-structure and reduced immune response with effective dose levels for DBTC of ≥1.7-2.4 mg/kg bw/d in a reproductive/developmental toxicity screening test (exposure period of 41 days for adult animals in this study) (Waalkens-Berendsen, 2003), ≥5 mg/kg bw/d in a rat prenatal developmental toxicity study (dams were exposed during 10 days, GD 6-15) (Study Report, 1993), ≥2.5 mg/kg bw/d in combined rat sub-acute/developmental toxicity studies (Seinen *et al.*, 1977b), and ≥7.5 mg/kg bw/d in a 2 week rat feeding study (Seinen *et al.*, 1977a). When considering differences in molecular weight between DBTC and DBTDL (DBTDL: 631.56 g/mol, DBTC: 303.84 g/mol), these effective dose levels would correspond to effective dose levels expressed as DBTDL as ≥3.5-5.0 mg/kg bw/d (in a reproductive/developmental toxicity screening test (exposure period of 41 days for adult animals in this study)), ≥10.4 mg/kg bw/d (in a rat prenatal developmental toxicity study (dams were exposed during 10 days, GD 6-15)), ≥5.2 mg/kg bw/d (in combined rat sub-acute/developmental toxicity studies), and ≥15.6 mg/kg bw/d (in a 2-week rat feeding study), respectively.

Overall, RAC considers the effects on the immune system as sufficiently severe to fulfil the classification criteria for STOT RE. The effects on the immune system include morphological changes that provide clear evidence of marked organ dysfunction and are considered as significant organ damage noted at necropsy and/or subsequently seen or confirmed at microscopic examination.

Effective dose-levels for DBTDL are below the extrapolated guidance values for classification as STOT RE 1 (i.e. 10, 30 and 60 mg/kg bw/d for a 90 day, 28 day and 14 day study, respectively). Setting of Specific Concentration Limits is not considered necessary, given the small margin between the effective dose levels and the guidance values for STOT RE.

RAC therefore supports the conclusion of the dossier submitter that DBTDL should be classified as **STOT RE 1 (H372: Causes damage to the immune system through prolonged or repeated exposure)**.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier submitter's proposal

Several mutagenicity studies conducted with both DBTDL and DBTC were included in the CLH report. As *in vitro* experimental data indicate that DBTDL can hydrolyse into DBTC in the stomach, producing DBTC upon oral exposure, studies on DBTC were therefore taken into consideration by the dossier submitter when classifying DBTDL for the hazard class germ cell mutagenicity.

DBTDL

Four *in vitro* studies and one *in vivo* study with DBTDL were presented in the CLH report. Four bacterial reverse mutation tests are all reported as negative (± S9-mix) (Bowles and Thompson, 2010; Zeiger *et al.*, 1987; Dow Corning Corp, 1981; EI du Pont de Nemours, 1977). A rat *in vivo* single cell gel electrophoresis (Comet, non- GLP, purity unknown) assay focussing on cerebral cortical cells showed a significant dose-dependent increase in DNA-damage upon repeated oral gavage exposure to DBTDL (5 d/w, for 7 weeks) (Jin *et al.*, 2012). In addition, other toxic effects such as right parietal cortex cell cycle disturbance and increased apoptosis were also observed in this *in vivo* study.

DBTC

Twelve *in vitro* studies and two *in vivo* studies with DBTC are presented in the CLH report. A GLP-compliant (similar to OECD TG 473) *in vitro* mammalian chromosome aberration test (± S9-mix) was reported with positive results (Study Report, 1990). Two bacterial reverse mutation tests were reported with one demonstrating positive results (no metabolic activation applied) (Hamasaki *et al.*, 1993) and the other presenting negative results (± S9-mix) (Schering, 1979). A CHO/HGPRT gene mutation assay (non-GLP and –guideline; Li *et al.*, 1982) showed positive results (no metabolic activation applied), whereas an OECD TG 476-compliant *in vitro* mammalian cell gene mutation test using Chinese hamster lung fibroblasts (V79) showed negative results (± S9-mix) (Lang and Schmitt, 1989).
Furthermore, a study with bacterial SOS-assay and a bacterial rec-assay (Hamasaki *et al.*, 1992) showed positive results from both assays (no metabolic activation applied). In addition, various non-guideline, non-GLP studies were included in the CLH report as well, reporting both positive and negative results: DBTC was shown to induce breakage of naked λ-DNA (Hamasaki *et al.*, 1995), to form condensates with DNA (Piro et al., 1992), and to affect spindle structure during mitosis in V79 Chinese hamster cells (Jensen *et al.*, 1991a), but did not affect chromosomal length in human peripheral lymphocytes (Jensen *et al.*, 1989), nor did DBTC induce hyperdiploid cells (aneuploidy) in human peripheral lymphocytes (Jensen *et al.*, 1991b).

Two *in vivo* mammalian erythrocyte micronucleus tests in mice (similar to OECD TG 474) were included in the CLH report. One study showed positive effects at the highest dose only (50 mg/kg bw) (Dance, 1991), whereas a similar study did not show positive effects at doses up to 200 mg/kg bw (Lang and Wedel, 1991).

Overall, for DBTC there was a mixed outcome both for *in vitro* and *in vivo* studies, but in general most studies were positive.

The dossier submitter concluded that given the absence of germ cell mutagenicity studies for both DBTDL and DBTC, classification in category 1B is not relevant. Given that DBTC distributes to several organs, distribution into testis/ovaries can, however, be expected.

The one positive well-conducted (GLP) *in vivo* somatic cell mutagenicity test as well as supportive evidence from positive results from *in vitro* mutagenicity/genotoxicity tests, indicate that DBTC is

a suspected germ cell mutagen, consistent with its current harmonized classification as Muta. 2. The dossier submitter considers that DBTDL should have the same classification as DBTC, based upon the gastric hydrolysis of DBTDL into DBTC and support from one oral *in vivo* rat study with DBTDL with evidence for genotoxicity in isolated brain cells. Therefore, the dossier submitter proposed classification as Muta. 2 (H341) for DBTDL.

Comments received during public consultation

One MSCA agreed with the proposed classification for germ cell mutagenicity during public consultation.

Assessment and comparison with the classification criteria

Sixteen *in vitro* tests and three *in vivo* tests are presented in the CLH report, with a small number of tests performed with DBTDL (4x *in vitro*, 1x *in vivo*) and the majority of the tests having been performed with DBTC (12x *in vitro* and 2x *in vivo*).

For DBTDL, all 4 bacterial reverse mutation tests were negative (with and without metabolic activation) and results of a rat *in vivo* single cell gel electrophoresis (Comet) assay focussing on rat brain showed a dose-dependent increase in DNA-damage in cerebral cortical cells (Jin *et al.*, 2012). This study suggests that DBTDL, or its metabolites, is systemically available and able to pass the blood-brain barrier and able to interact with DNA. In view of the poor quality of this non-GLP study, where no information on the purity of the test material and no positive controls were included, these data are of limited value for the overall evaluation.

Given that DBTDL is, at least in part, hydrolysed in the stomach upon oral exposure producing DBTC, RAC is of the opinion that data on DBTC (as the hydrolysis product of DBTDL) can be used for classification of DBTDL for germ cell mutagenicity (see also *RAC general comment*).

For DBTC, various *in vitro* tests are available. One bacterial reverse mutation test was negative (with and without metabolic activation) and a second bacterial reverse mutation test was positive (without metabolic activation). An OECD 476 *in vitro* mammalian cell (V79) HGPRT gene mutation assay was negative (with and without metabolic activation), and a Chinese hamster ovary cell HGPRT gene mutation assay (non-GLP and -guideline) was positive (without metabolic activation). Further, a chromosome aberration test was positive (with and without metabolic activation). Positive results were also obtained in a bacterial rec-assay and SOS chromo-assay (without metabolic activation). Additionally, induction breakage of naked λ-DNA, formation of condensates with DNA and deviated spindle structure during mitosis could be demonstrated *in vitro,* though chromosomal length was not affected, nor was induction of hyperdiploid cells observed *in vitro*. Overall, the results of the *in vitro* tests were variable with both positive and negative results.

Additionally, two *in vivo* mouse micronucleus studies with DBTC are presented in the CLH-report. In the OECD TG 474 and GLP-compliant study of Dance (1991), mice received DBTC via a single oral gavage. Dose levels of 2, 10 or 50 mg DBTC/kg bw were applied (vehicle: corn oil). A statistically significant increase in the incidence of micro-nucleated polychromatic cells was observed in bone marrow 48h and 72h after exposure of mice to DBTC at 50 mg/kg bw, with effects more clearly in female than male animals. No positive result was obtained upon DBTC-exposure at the post-treatment time-interval of 24h.

The positive mutagenic result for DBTC from the study of Dance (1991) was not confirmed in a second *in vivo* mouse micronucleus study (Lang and Wedel, 1991). Mice received a single oral gavage exposure of DBTC of 0, 50, 100 or 200 mg/kg bw (vehicle: arachis oil). The results of this second micronucleus test indicated that DBTC failed to show any evidence of mutagenic potential up to the (toxic) dose level of 200 mg/kg bw as measured at 24h, 48h and 72h post-treatment. Both mouse micronucleus studies included a sufficient number of animals. Positive as well as negative controls were included with appropriate results in both studies, and toxicity was observed in both studies. After full evaluation, no clear explanation could be found for the discrepancy in results. Without any reason to discard one of the two *in vivo* mouse micronucleus studies, the positive result of the study of Dance (1991) is taken forward for the evaluation.

In vivo mammalian germ cell mutagenicity tests are not available for DBTDL or DBTC. However, positive result(s) from *in vivo* somatic cell mutagenicity tests in mammals are available, though not in combination with adequate evidence that the substance has potential to cause mutations to germ cells. A positive result was obtained from a well-performed OECD- and GLP-compliant *in vivo* mouse micronucleus test with DBTC. The positive result is supported by indications from one *in vivo* test with DBTDL (*in vivo* Comet assay, non-GLP). Further, DBTC, as a hydrolysis product of DBTDL, is systemically available, as shown by the formation of micronuclei in the bone marrow. Although distribution into testes/ovaries can be expected, no experimental evidence is available which demonstrates a direct interaction of the substance or its metabolite with the genetic material of germ cells. Therefore, RAC considers classification in category 1B not appropriate. RAC concludes that DBTDL should be classified for germ cell mutagenicity as **Muta. 2** (**H341: Suspected of causing genetic defects**).

RAC evaluation of reproductive toxicity

Summary of the Dossier submitter's proposal

As *in vitro* experimental data indicate that DBTDL can hydrolyse into DBT in the stomach producing DBTC, studies on DBTC were therefore taken into consideration by the dossier submitter when classifying DBTDL for the hazard class reproductive toxicity.

Fertility

For DBTDL, no fertility studies are available. A fertility study with DBTC, which was used as a key study for the classification of DBTC, is included in the CLH report (Ema *et al.*, 2000). Observed effects included an increase in the number of non-pregnant females, a reduced number of implantations and an increased incidence of pre-implantation loss upon exposure to DBTC on GD 0-3, and an increased incidence of early total resorption upon exposure to DBTC on GD 4-7.
Based on 1) the increased number of non-pregnant females among successfully mated females, the reduced number of implantations, the increased pre-implantation losses and increased early total resorptions in the key fertility study with DBTC, 2) the harmonised classification of DBTC as Repr. 1B for effects on fertility and sexual function, and 3) given the stomach hydrolysis of DBTDL into DBTC, the dossier submitter considered that DBTDL should have the same classification as DBTC. The dossier submitter therefore proposed Repr. 1B for effects on fertility and sexual function for DBTDL.

Developmental toxicity

One developmental toxicity study with DBTDL is included in the CLH report (Noda *et al.*, 1993). In this study, several di-n-butyltin compounds were examined and developmental effects of DBTDL could be compared with the effects of DBTC upon exposure to a single dose on GD 8. In the

absence of maternal toxicity, significant increases in external and skeletal malformations were observed. DBTDL and DBTC showed the same type of effects, though effects were more pronounced for DBTDL.

A supportive developmental study with DBTC (exposure on GD 7-15) is presented in the CLH report (Ema *et al.*, 1991). This study also showed increased incidences of external and skeletal malformations (cleft jaw and ankyglossia as the most frequent malformations), although at the two highest dose levels clear maternal toxicity was observed (significantly higher mortality in dams with stomach haemorrhages in dead animals). In addition complete resorption of all implanted embryos was observed.

Three additional studies on potential developmental toxicity with the most sensitive window for exposure to DBTC were briefly described in the CLH report. Further, studies on DBTC published after the harmonised classification and labelling of DBTC supported previous results. Most of these studies also focussed on the mechanism, and pointed towards the potential influence of DBTC-induced reduced serum progesterone levels in pregnancy failure.

Based on the significantly increased external and skeletal malformations observed in foetuses exposed *in utero* to DBTDL, in the absence of maternal toxicity, the dossier submitter considered that classification of DBTDL for developmental toxicity is justified. Further, DBTC has a harmonised classification for developmental toxicity in category 1B and given the stomach hydrolysis of DBTDL into DBTC, the dossier submitter considered that DBTDL should have the same classification for developmental toxicity as DBTC. The dossier submitter therefore proposed Repr. 1B for developmental toxicity for DBTDL.

Comments received during public consultation

One MSCA agreed with the proposed classification for reproductive toxicity during public consultation. One MSCA commented that a guideline-study (OECD TG 421, Reproduction/developmental toxicity screening test) with DBTC is available in the CLH report (discussed in section 4.7.1.1 on oral repeated dose toxicity) which can also be used for evaluation of the endpoint reproductive toxicity. In their response, the dossier submitter agreed and summarized and evaluated this study for the endpoint reproductive toxicity (See section *Additional key elements* in the background document (BD)).

Two industry associations submitted similar comments expressing their disagreement with the proposed classification for reproductive toxicity (and STOT RE). Their main argument was the presence of a tributyltin-impurity, which was considered by the industry associations to be responsible for the observed reproductive and immunotoxic effects. In their response, the dossier submitter considered that the available data warranted classification of DBTDL for reproductive toxicity (and STOT RE) regardless. They stated that the degree of contamination of TBT in DBT test substances is unclear both in older and in more recent studies. Furthermore, the dossier submitter evaluated a number of mechanistic studies, which indicated that both TBT and DBT affect mechanisms underlying effects on fertility and development and immune function, although it is recognised that it is as yet not clear whether TBT and DBT act according to the same or different pathways.

Assessment and comparison with the classification criteria

Fertility

No substance-specific fertility studies are available for DBTDL and a rat fertility study (non-guideline, non-GLP) with DBTC is presented (Ema *et al.*, 2000). Successfully mated female rats were exposed via gastric intubation to DBTC in olive oil (0, 3.8, 7.6, 15.2 mg/kg bw/d) on GD 0-3 or GD 4-7. In addition to a control group (olive oil), also a pair-fed group (feed restricted to same amounts as high dose DBTC-group) was included. A significantly higher number of non-pregnant dams was observed upon exposure to the mid and high dose of DBTC on GD 0-3 (high dose: 87%, mid dose: 31.3%, low dose: 0%, control: 0%, pair-fed: 5.9%). Further, a reduced number of implantations (high dose: 1.8, mid dose: 10.1, low dose: 15, control: 15, pair-fed: 13.4) and increased incidences of preimplantation loss (high dose: 87.9%, mid dose: 35.6%, low dose: 4.1%, control: 2.7%, pair-fed: 16.4%) was observed as well in these DBTC-exposed groups as evidence for effects on fertility. The fertility effects for the high dose group were statistically significantly different from the control group as well as from the pair-fed group, whereas effects for the mid dose groups were statistically significantly different from the control group. Slight general toxicity was observed and included reduced bw and feed consumption in the high dose group (not specified in CLH report; see for details on BW changes *Supplemental information – in depth analyses*). Upon DBTC-exposure during GD 4-7, increased early total resorptions were observed in the high dose group (87.5%; statistically significantly different from the control (0%) and from the pair-fed groups (11.8%)). Also in these animals, some slight general toxicity was observed and included reduced adjusted bw gain (i.e. excluding the uterus) and reduced feed consumption (not specified in CLH report; for details on BW changes see *Supplemental information – in depth analyses* in the BD). RAC evaluated the general toxicity effects and concluded that the reproductive effects are not due to a secondary non-specific consequence of parental toxicity.

The reprotoxic effects of DBTC observed in the OECD TG 421 reproduction/developmental toxicity screening study (Waalkens-Berendsen, 2003) were summarized and provided by the dossier submitter in response to the comments (RCOM) during public consultation (see the RCOM and *Additional key elements in the BD*). Effects observed were a significant increase in the incidence of ovarian cysts in nine of the twelve high-dosed females (200 mg/kg diet; corresponding to 6.2-15.4 mg/kg bw/d). Furthermore, an increase in the number of dams with post implantation loss, a reduction in the number of live pups and a reduction in the gestation index were observed. In line with the conclusion of the dossier submitter, RAC considers these effects as relevant for classification of DBTC for fertility, although it is recognized that some of these effects (e.g. reduced number of live pups) could also be due to developmental toxicity.

Finally, no effects on sexual function were noticed in the repeated dose studies as presented in the CLH report, although no full analyses of these parameters were included in these repeated dose studies. However, information on an additional study (Ananie *et al.*, 2001) as presented by the dossier submitter during public consultation indicated that upon daily i-p injection of DBTC for seven days effects on testis weight, sperm density, rate of survival of sperm and sperm abnormalities were observed. In view of the route of administration (i-p), RAC considers these findings as supportive evidence.

Given that DBTDL is, at least in part, hydrolysed in the stomach upon oral exposure, producing DBTC, RAC is of the opinion that data on DBTC (as a hydrolysis product of DBTDL) can be used for classification of DBTDL effects on sexual function and fertility (see also under *RAC general comment*).

Given that 1) the observed effects in the key rat fertility toxicity study with DBTC (increased number of non-pregnant dams, reduced implantations/increased pre-implantation losses) can be considered treatment-related, 2) although these fertility effects were observed to occur together with other general toxic effects (i.e. slight reduced bw gain and feed consumption), the adverse effects on reproduction were considered not to be a secondary non-specific consequence of the reduced bw gain and feed consumption as confirmed in a pair-fed control group, and 3) there are no indications that the observed effects are not relevant to humans, RAC therefore supports the conclusion of the dossier submitter that DBTDL should be classified as toxic to reproduction for effects on sexual function and fertility as **Repr. 1B** (**H360F: May damage fertility**).

Setting of Specific Concentration Limits is not considered necessary for reproductive toxicity (effects on sexual function and fertility), given that ED_{10}-values for DBTDL fall within the range of a medium potency group (i.e. 4 mg/kg bw/d < ED_{10} < 400 mg/kg bw/d) and modifying factors which might change the potency group are considered not needed, resulting in a GCL of 0.3% (cf. section 3.7.2.5 of the CLP-guidance).

Developmental toxicity

A rat developmental toxicity study (non-guideline, non-GLP) with DBTDL is presented (Noda *et al.*, 1993). Also other di-n-butyl compounds including DBTC (tested at equimolar levels) were evaluated in this study. Female rats were exposed to DBTDL (80 µmol/kg bw, corresponding to 50.5 mg/kg bw) in a single dose on GD 8. Significantly higher incidences of external and skeletal malformations compared to control group were observed in foetuses exposed *in utero* to DBTDL (30.6% and 28.1%, respectively, vs. 0% and 0% in the control group). A significant increase in the number of mandible malformations (cleft mandible, cleft lower lip, ankyloglossia or schistoglossia, and exencephaly), anomalies of mandibular fixation, fused mandibula, cranial hypoplasia, fused ribs, fused vertebral arches, and cleft maxilla were observed upon exposure to DBTDL. Skeletal variations were predominately a supernumerary cervical rib. Higher incidences of external malformations were observed in *in utero* DBTDL-exposed foetuses compared with *in utero* DBTC-exposed foetuses, while similar incidences of skeletal malformations were found in this study. The effects of DBTDL and DBTC (also tested at 80 µmol/kg bw, corresponding to 24.3 mg DBTC/kg bw) were similar, but more pronounced for the DBTDL group. Maternal toxicity was not observed upon exposure to DBTDL or DBTC.

In a supportive rat developmental toxicity study, rats were exposed during the gestation period (GD 7-15) via oral gavage to DBTC in olive oil (0, 2.5, 5, 7.5, 10 mg/kg bw/d) (Ema *et al.*, 1991). Clear maternal toxicity was observed at the two highest dose levels and effects included significantly higher mortality in dams (5/10 and 9/10 dams died in the 7.5 and 10 mg/kg bw/d dose groups, respectively) with stomach haemorrhages observed in dead animals. In the 7.5 and 10 mg/kg bw/d dose-groups, total resorptions were observed in the remaining 5/10 and 1/10 pregnant rats, respectively. *In utero* exposure of foetuses resulted in developmental effects such as increased incidences of external and skeletal malformations, with cleft jaw and ankyglossia being the most frequently observed type of malformations. Although observed at the two highest dose levels in the presence of clear maternal toxicity, these developmental effects were also observed at the dose-level of 5 mg/kg bw/d (i.e. without the presence of maternal toxicity).

Furthermore, the reproduction/developmental toxicity screening study (OECD TG 421) with DBTC, as summarized by the dossier submitter during public consultation (see *Additional key elements* in the BD), demonstrated clear reprotoxic effects (Waalkens-Berendsen, 2003). For a description of the study and the effects see the section on fertility (above). As noted, for some of the effects observed it is difficult to assess whether these are due to effects on fertility or development and

as such this study contributed to the overall picture of the adverse effects of DBTC on reproduction.

Studies on DBTC published after the harmonised classification and labelling of DBTC support previous results with DBTC. Three additional studies on potential developmental toxicity in relation to the most sensitive window for exposure to DBTC indicated that DBTC-induced teratogenic effects were observed following exposure on GD 7-8 and were most pronounced when dams were exposed on GD 8 (Ema *et al.*, 1992, 1995, 1996). Embryo-lethality was observed at all tested time-points for exposure during gestation (GD 6-15).

Finally, in the section on repeated dose toxicity of the CLH report, some additional studies relevant for the endpoint reproductive toxicity were included. These studies also revealed effects on reproductive toxicity and these can be considered as supportive evidence.

According to the industry comments provided during the public consultation, tributyltin-impurities have driven the adverse effects or at least contributed to them, and the validity of the 'older' studies for classification of DBTDL was questioned by industry.

For the studies included in the CLH-report, minimal information on the purity of the test substance (either DBTDL or DBTC) was provided and information on the presence of impurities is lacking for almost all studies. Exceptions are the studies of Waalkens-Berendsen (2003) (OECD TG 421 reproduction/developmental toxicity screening study) with reported DBTC purity of 98.57% and the 90-d study of Gaunt *et al.* (1968), which reported DBTC with a purity of 99.7% with 0.25% tributyltin chloride impurity. For all the other toxicity studies, qualitative and quantitative information on impurities is lacking.

In 2002 industry agreed to reduce the tributyltin content to concentrations of 0.67% (w/w) or less. Industry stated in their comments that studies performed after this agreement did not show malformations any longer and referred to the OECD TG 421 reproductive/developmental toxicity screening study of Waalkens-Berendsen (2003) and the study of Ema *et al.* (2000). However, the study of Ema *et al.* (2000), apart from being performed before the 2002 agreement, included in their analysis of the foetuses inspections for external malformations and malformations within the oral cavity, but no full evaluation of skeletal malformations. Furthermore, an OECD TG 421 reproductive/developmental toxicity screening study is not considered an appropriate test for demonstrating or negating malformations (a too low number of animals is included and no full evaluation of malformations). Waalkens-Berendsen (2003) did not include skeletal examination of the foetuses in their analysis.

With respect to the mechanism, various mechanistic studies were presented both by industry and dossier submitter. RAC acknowledges that tributyltin compounds exert reprotoxic effects (RAC opinion, 2013). However, RAC considers that the available (mechanistic) data do not exclude dibutyltin-compounds exerting those kind effects as well.

RAC compared the effective dose-levels of the dibutyltin compounds DBTDL and DBTC vs. the effective dose-levels of the tributyltin compounds for the human health endpoints repeated dose toxicity, effects of fertility and developmental effects (based on RAC's ODD on tributyltin compounds (2013) and the disseminated REACH registration dossiers on ECHAs website for tributyltin compounds, though the latter not evaluated in-depth by RAC) in order to conclude on the potential contribution of tributyltin impurities to the observed adverse effect. RAC noted that the observed adverse effects are comparable. Moreover, given that the effective dose levels of the tributyltin compounds are quite similar to the effective dose levels for DBTDL and DBTC, this

indicates that a very high percentage tributyltin impurity would be needed to result in clear adverse effects.

All in all, RAC is of the opinion there is no robust information that demonstrates that the observed effects are due to an impurity and therefore there is no reason to discard the results of the developmental toxicity studies in the CLH report for the assessment of DBTC and DBTDL. The same applies for fertility and repeated dose toxicity.

Given that 1) the observed foetal effects in the key rat developmental toxicity study (Noda *et al.*, 1993) with DBTDL (skeletal and external malformations) can be considered treatment-related, 2) the foetal effects were observed in the absence of maternal toxicity, and 3) there is no reason to question the human relevance of the observed foetal effects, RAC considers that there is clear evidence of an adverse effect on development upon exposure to DBTDL.

In addition, the studies with DBTC of Ema *et al.* (1991) and Waalkens-Berendsen (2003) also provide evidence of adverse effects on development. Given that DBTDL is, at least in part, hydrolysed in the stomach upon oral exposure, producing DBTC, RAC is of the opinion that data on DBTC (as a hydrolysis product of DBTDL) can be used for classification of DBTDL for effects on development (see also under *RAC general comment*).

Altogether, RAC supports the conclusion of the dossier submitter that DBTDL should be classified as toxic to reproduction for developmental toxicity as **Repr. 1B** (**H360D; May damage the unborn child**).

Specific Concentration Limits (SCL)

Setting of SCL is not considered necessary for reproductive toxicity (effects on development), given that the ED_{10}-values for DBTDL fall within the ranges of a medium potency group (i.e. 4 mg/kg bw/d < ED_{10} < 400 mg/kg bw/d) and modifying factors which might change the potency group are considered not needed, resulting in the GCL of 0.3% (cf. section 3.7.2.5 of the CLP-guidance).

Additional references

RAC opinion (2012a). Committee for Risk Assessment (RAC). Opinion proposing harmonized classification and labelling at Community level of *2-ethylhexyl 10-ethyl-4,4-dimethyl-7-oxo-8-oxa-3,5-dithia-4-stannatetradecanoate*. Adopted on 30 November 2012. http://echa.europa.eu/documents/10162/13579/dmt_ehma_opinion_adopted_en.pdf

RAC opinion (2012b). Committee for Risk Assessment (RAC). Opinion proposing harmonized classification and labelling at Community level of *Dioctyltin bis(2-Ethylhexyl mercaptoacetate)*. Adopted on 8 June 2012. http://echa.europa.eu/documents/10162/5266b444-9e22-4051-86ec-e0c59a95649b

RAC opinion (2013). Committee for Risk Assessment (RAC). Opinion proposing harmonized classification and labelling at Community level of *Tributyltin compounds, with the exception of those specified elsewhere in Annex VI*. Adopted on 5 December 2013. http://echa.europa.eu/documents/10162/1f9d68cc-add6-4da6-a68d-bc540079b785

ANNEXES:

Annex 1 Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in RAC boxes.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and rapporteurs' comments (excl. confidential information).