Centre on Endocrine Disrupters

# Evaluation of 22 SIN List 2.0 substances according to the Danish proposal on criteria for endocrine disrupters

DANISH CENTRE ON ENDOCRINE DISRUPTERS

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## Terms of reference and scope

This report has been prepared by the Danish Centre on Endocrine Disrupters (CEHOS) as a project contracted by the Danish Environmental Protection Agency. The Danish Centre on Endocrine Disrupters is an interdisciplinary scientific network without walls. The main purpose of the Centre is to build and gather new knowledge on endocrine disrupters with the focus on providing information requested for the preventive work of the regulatory authorities. The Centre is financed by the Ministry of the Environment and the scientific work programme is followed by an international scientific advisory board.

The overall scope of this project is to provide a science based evaluation of the endocrine disrupting properties of the 22 substances on the SIN list version 2.0.

## 1. Background and aim

During the last years Denmark has been focusing the work under the national strategy on endocrine disruptors on regulatory measures with the aim to reduce human and environmental exposure to endocrine disruptors. As a first step towards more systematic regulation of endocrine disruptors the Danish Environmental Protection Agency contributed to the ongoing EU-process on criteria setting for endocrine disruptors by submitting the report: Establishment of Criteria for Endocrine Disruptors and Options for Regulation in May 2011 (Danish EPA, 2011). In relation to this work and the REACH process the DK-EPA has asked the Danish Centre for Endocrine Disrupters to conduct a project which is to assess the 22 substances on the SIN[1] List 2.0. These substances have been identified by the NGO ChemSec as Substances of Very High Concern (SVHC) according to the criteria in REACH, solely due to their endocrine disrupting properties. The overall aim is to categorize the 22 substances on the basis of the Danish proposal for criteria for endocrine disrupters which is scientifically justified by a report from the Danish Centre on Endocrine Disrupters (Hass et al., 2011). This means that the evaluation is based on results from both human health, *in vitro/vivo* studies and studies in the environment. Furthermore, all 22 substances are evaluated according to the Joint British-German Position Paper: Regulatory Definition of an Endocrine Disrupter in relation to Potential Threat to Human Health that is based on a potency cut-off criteria (DE-UK, 2011).

The substances and their main use(s) are shown in table 1.

[1] Substitute It Now

**Table 1. The evaluated substances and their main use(s) according to SIN list document**

| **Substance** | **Use(s)** |
|---|---|
| **The 22 SIN List substances** | |
| 3-benzylidene camphor | UV-filter |
| 4-methylbenzylidene camphor | UV-filter |
| 4-nitrophenol | In dyes and to darken leather. It is also used as a building block for the manufacture of drugs (e.g., acetaminophen/paracetamol), and pesticides (fungicides and methyl and ethyl parathion insecticides), and is released from diesel combustion |
| 4,4´-dihydroxybenzophenone | UV-filter |
| Benzophenone-1 | UV-filter |
| Benzophenone-2 | UV-filter |
| Benzophenone-3 | UV-filter |
| Butylparaben | A preservative used in personal care products |
| Dicyclohexyl phthalate (DCHP) | A plasticiser with numerous uses. It is found in cellulose and PVC products, in paints, inks and in food packaging. |
| Diethyl phthalate (DEP) | Numerous uses, including in plastic products and in cosmetics as a fragrance carrier. |
| Dihexyl phthalate (DHP) | A plasticiser and can be found in many different products including tool handles and PVC flooring |
| Ethylhexyl methoxycinnamate (OMC) | UV-filter |
| Metam natrium | In paints, leather tanning as a preservative and a broad-spectrum micro biocide |
| Methyl tertiary butyl ether (MTBE) | An extraction solvent and as a fuel component in gasoline to raise the octane number |
| Pentachlorophenol (PCP) | A wood preservative but is also used in glues and starches |
| Perchloroethylene | The main use is in dry-cleaning |
| Propylparaben | A preservative used in personal care products |
| Quadrosilan | Unclear. May be used as bearing grease and in breast implants and/or medical products. |
| Resorcinol | Numerous uses, including rubber and resins, in cosmetics, pharmaceuticals and hair dye |
| Tert-butylhydroxyanisole | The primary use is as an antioxidant and preservative in food, food packaging, animal feed, cosmetics, rubber, and petroleum products. BHA is also commonly used in medicines. |
| Thiram | In industry, for example in rubber products manufacturing. It is also a crop fungicide and an animal repellent, applied to protect e.g. fruit trees from grazing |
| Zineb | In paints, in surface treatments and as a pesticide, in use as biocide in EU. |

### 1.1 Danish Criteria for identification of ED

The Danish proposal for criteria for EDs are described in detail in a previous report from CEHOS (Hass et al 2011) and will only be briefly described here.

The criteria include 3 categories, i.e. ED (category 1), suspected ED (category 2a) and indicated ED (category 2b). The definitions of the categories are:

***An endocrine disrupter*** *is an exogenous substance or mixture that alters function(s) of the endocrine system and causes adverse health effects in an intact organism, or its progeny, or (sub)populations."*

***Potential endocrine disrupter:***

***A suspected endocrine disrupter*** *is an exogenous substance or mixture that may alter function(s) of the endocrine system and consequently may cause adverse health effects in an intact organism, or its progeny, or (sub)populations."*

*A substance with* ***indication of endocrine disrupting properties (called indicated ED)*** *is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations.*

The definition of EDs and suspected EDs both include the term "adverse". The WHO/IPCS definition of the term "adversity" is used:
"*A change in morphology, physiology, growth, reproduction, development or lifespan of an organism which results in impairment of functional capacity or impairment of capacity to compensate for additional stress or increased susceptibility to the harmful effects of other environmental influences*." (WHO/IPCS 2004)

In table 2 the criteria for placing substances in each of the three Categories are presented**.**

**Table 2 Proposed criteria for EDs**

***Category 1 - Endocrine disrupter***

Substances are placed in category 1 when they are known to have produced ED adverse effects in humans or animal species living in the environment or when there is evidence from animal studies, possibly supplemented with other information, to provide a strong presumption that the substance has the capacity to cause ED effects in humans or animals living in the environment. The animal studies shall provide clear evidence of ED effect in the absence of other toxic effects, or if occurring together with other toxic effects, the ED effects should be considered not to be a secondary non-specific consequence of other toxic effects. However, when there is e.g. mechanistic information that raises doubt about the relevance of the adverse effect for humans or the environment, category 2a may be more appropriate.

Substances can be allocated to this category based on:

- Adverse *in vivo* effects where an ED mode of action is highly plausible
- ED mode of action *in vivo* that is clearly linked to adverse *in vivo* effects (by e.g. read-across)

***Category 2a - Suspected ED***

Substances are placed in category 2a when there is some evidence from humans or experimental animals, and where the evidence is not sufficiently convincing to place the substance in category 1. If for example limitations in the study (or studies) make the quality of evidence less convincing, category 2a could be more appropriate. Such effects should be observed in the absence of other toxic effects, or if occurring together with other toxic effects, the ED effect should be considered not to be a secondary non-specific consequence of other toxic effects.

Substances can be allocated to this category based on:

- Adverse effects *in vivo* where an ED mode of action is suspected
- ED mode of action *in vivo* that is suspected to be linked to adverse effects *in vivo*
- ED mode of action *in vitro* combined with toxicokinetic *in vivo* data (and relevant non test information such as read across, chemical categorisation and QSAR predictions)

***Category 2b – Substances with indications of ED properties (indicated ED)***

Substances are placed in category 2b when there is *in vitro*/*in silico* evidence indicating potential for endocrine disruption in intact organisms. Evidence could also be observed effects *in vivo* that could be ED-mediated.

A substance can be considered an ***ED (category 1)*** based on data from:

- *In vivo* assays providing data on effects clearly linked to endocrine mechanisms (OECD, conceptual Framework (CF) level 5)
- On a case-by-case basis, *in vivo* assays providing data about single or multiple endocrine mechanisms and effects (OECD, CF level 3 & 4) combined with other relevant information

- In special cases, categorisation or QSAR approaches may provide the necessary data in combination with *in vivo* ADME information and *in vitro* data
- Reliable and good quality evidence from human cases or epidemiological studies.

A substance can be considered a ***suspected ED (category 2a)*** based on data from:

- *In vivo* assays providing data on effects linked to endocrine or other mechanisms (OECD, CF level 5), but where ED mode of action is suspected
- *In vivo* assays providing data about single or multiple endocrine mechanisms and effects (OECD, CF level 3 & 4)
- In some cases, read across, chemical categorisation and/or QSAR approaches may provide the necessary data in combination with *in vivo* ADME information and *in vitro* data
- Good quality epidemiological studies showing associations between exposure and adverse human health effects related to endocrine systems.

A substance can be considered an ***indicated ED (category 2b)*** based on data from:

- *In vitro* assays providing mechanistic data (OECD, CF level 2)
- QSAR, read-across, chemical categorization, ADME information (OECD, CF level 2)
- System biology methods indicating associations between the substance and adverse human health effects related to endocrine systems.

### 1.2 DE/UK Criteria for identification of EDs of very high regulatory concern

A joint DE – UK position paper was launched in May 2011 (DE-UK, 2011). It addressed many issues in the discussion of criteria for endocrine disrupters in relation to adverse effects on human health. The following will focus on the proposal in the position paper in relation to potency and cut-off to identify ED of very high regulatory concern.

The paper states that: "In general terms, toxic effects are only of regulatory relevance when they occur at relevant dose levels. Toxic effects that occur at excessively high dose levels (above the Maximum Tolerated Dose) tend to represent the unspecific and generalised response of the body to the chemical insult e.g. arising from the saturation of kinetic processes. Mostly, these effects are not realistically relevant to humans and are not used to drive regulatory action. This concept is applied in various regulatory approaches, such as hazard classification and labelling". Moreover, the paper proposes to use the dose thresholds for STOT[2] Repeated Exposure (RE) to determine whether or not the hazardous property of "endocrine disruption" should be identified for regulatory purposes.

[2] Specific Target Organ Toxicity

There are two categories (Categories 1 and 2) of classification for STOT-RE, covering substances of relatively higher and lower potency. The guidance values ("cut-offs") for both categories are defined in CLP[3] and GHS[4] and shown in table 3.

**Table 3 Guidance values for STOT-RE**

| For sub-acute and other short-term studies (e.g. prenatal developmental toxicity studies): | | |
|---|---|---|
| | STOT-RE Cat 2 | STOT-RE Cat 1 |
| Oral | 300 mg/kg bw/day | 30 mg/kg bw/day |
| Dermal | 600 mg/kg bw/day | 60 mg/kg bw/day |
| Inhalation (vapour) | 3 mg/l/6h/day | 0.6 mg/l/6h/day |
| Inhalation (dust/mist/fume) | 0.6 mg/l/6h/day | 0.06 mg/l/6h/day |
| For sub chronic and other medium-term studies (e.g. 2-generation studies): | | |
| | STOT-RE Cat 2 | STOT-RE Cat 1 |
| Oral | 100 mg/kg bw/day | 10 mg/kg bw/day |
| Dermal | 200 mg/kg bw/day | 20 mg/kg bw/day |
| Inhalation (vapour) | 1 mg/l/6h/day | 0.2 mg/l/6h/day |
| Inhalation (dust/mist/fume) | 0.2 mg/l/6h/day | 0.02 mg/l/6h/day |
| There are no guidance values in the CLP Regulation for chronic studies, but it is proposed here that they should be half the subchronic study values (by applying the subchronic to chronic extrapolation assessment factor of 2 recommended in the REACH guidance on information requirements and chemical safety assessment, chapter R8), i.e.: | | |
| | STOT-RE Cat 2 | STOT-RE Cat 1 |
| Oral | 50 mg/kg bw/day | 5 mg/kg bw/day |
| Dermal | 100 mg/kg bw/day | 10 mg/kg bw/day |
| Inhalation (vapour) | 0.5 mg/l/6h/day | 0.1 mg/l/6h/day |
| Inhalation (dust/mist/fume) | 0.1 mg/l/6h/day | 0.01 mg/l/6h/day |

In relation to potential human health concerns, it is proposed by DE-UK that a substance is regarded as an ED of very high regulatory concern based on the following definition and the associated criteria: It should be an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or (sub)populations. And in doing so fulfil the following criteria (each of which is expanded on in the position paper):

[3] Classification, Labelling and Packaging
REGULATION (EC) No 1272/2008 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006

[4] Globally Harmonised System of Classification and Labelling of Chemicals
Globally Harmonised System of Classification and Labelling of Chemicals (GHS), Second revised edition, United Nations New York and Geneva, 2007

- Adverse effects have been seen in one or more toxicity studies of acceptable quality, in which the substance was administered by a route relevant for human exposure.
- There is a plausible mode-of-action/mechanistic link between the toxic effects of concern and endocrine disruption.
- The effects seen in experimental animals are judged to be of potential relevance to human health.
- Serious adverse effect(s) related to endocrine disruption to have been produced at a dose at or below the relevant guidance value for the application of Category 1 "Specific Target Organ Toxicity-Repeated Exposure, STOT-RE" classification and labelling.

## 2. Methods

### 2.1 Literature

Generally, the literature used for evaluation of the substances aimed to comprise all relevant publicly available scientific papers.

The main literature search was done by experts at Institute of Biology, University of Southern Denmark (SDU).

The search was done in MEDLINE and Web of Knowledge using relevant and similar search criteria for each substance – apart from substance identification. For example, for propylparaben the following search criteria were used:
((propylparaben or propyl and paraben*) and (endocrine*))
((propylparaben or propyl and paraben*) and (estrogen* or oestrogen*))
((propylparaben or propyl and paraben*) and (androgen*))
((propylparaben or propyl and paraben*) and (aromatase*))
((propylparaben or propyl and paraben*) and (thyroid*))

Papers included in the database developed by Chemsec in relation to their evaluations of the SINList substances, but not identified in our MEDLINE/Web of Knowledge literature search, were added to the list of papers to evaluate. Subsequently, the papers on the resulting list were downloaded and distributed to the relevant experts in the project group.

If needed, follow-up literature searches in MEDLINE based on references in the retrieved papers were done by individual experts. Also, additional papers and reviews previously obtained due to earlier evaluations of some of the substances (e.g. UV filters and parabens) were used.

For substances which are registrered as plant protection products, biocides or pharmaceuticals within the EU, there may be additional documentation for the toxicity of the substances. The authors are aware of this documentation and do not find it likely to influence the proposed categorisation for the substances in this report.

### 2.2 Evaluation, DK criteria for identification of ED

First, evaluations of epidemiological data, *in vitro* data plus animal experimental data, and ecotoxicity data were done by experts at GR (Department of Growth and Reproduction), DTU (National Food Institute, Technical University of Denmark) and SDU, respectively. This was followed by an overall evaluation in the project group and a datasheet comprising study descriptions, evaluation and references was made for each compound (included in appendix 1).

### 2.3 Evaluation, DE-UK potency criteria for identification of ED of very high regulatory concern

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which severe adverse effects are observed (DE-UK, 2011). For subchronic and other medium-term studies (e.g. 2-generation studies) with oral dosing, adverse effects at 10 mg/kg bw/day and below (STOT-RE Cat 1) lead to a classification as an endocrine disrupter of very high regulatory concern. Thus, the overall LOAEL for each substance was assessed, if possible, and evaluated in relation to this cut-off level of 10 mg/kg/day. In some cases, there was effect below 10 mg/kg/day, where e.g. adversity was somewhat uncertain and other clearly adverse effects above 10 mg/kg/day. In such cases, it was difficult to evaluate whether the DE-UK potency criteria could be considered fulfilled or not. Consequently, for these compounds the term ‘unclear’ was used.

# 3. Results

## 3.1 Evaluation based on DK criteria for identification of ED

An overview of the separate evaluations based on epidemiological data, *in vitro* data plus animal experimental data, and ecotoxicity data as well as the overall evaluations is shown in table 4. Among the 22 substances, 15 are evaluated as ED in category 1 and 6 as suspected ED in category 2a. Perchloroethylene is not categorized due to lack of relevant data on endocrine disrupting effects.

**Table 4 Overview of the preliminary evaluations based on epidemiological data, *in vitro* data plus animal data, and ecotoxicity data and the overall evaluation**

| Chemical | Tox | Hum | Eco | Overall |
|---|---|---|---|---|
| 3-benzylidene camphor | 1 | No data | 1 | 1 |
| 4-methylbenzylidene camphor | 1 | Inconclusive | 2a | 1 |
| 4-nitrophenol | 1 or 2a | No data | No data | 2a |
| 4,4´-dihydroxybenzophenone | 1 or 2a | No data | No evidence | 2a |
| Benzophenone-1 | 2a | No data | 2a | 2a |
| Benzophenone-2 | 1 | No data | 1 | 1 |
| Benzophenone-3 | 2a | 2b | 2a | 2a |
| Butylparaben | 1 | Weak associations | 2a | 1 |
| Dicyclohexyl phthalate (DCHP) | 1 | No data | No evidence | 1 |
| Diethyl phthalate (DEP) | 2a | 2a | 2a | 2a |
| Dihexyl phthalate (DHP) | 1 | No data | No evidence | 1 |
| Ethylhexyl methoxycinnamate (OMC) | 1 | 2b | 2a | 1 |
| Metam natrium | 1 | No relevant data | No evidence | 1 |
| Methyl tertiary butyl ether (MTBE) | 1 | No relevant data | 2a | 1 |
| Pentachlorophenol(PCP) | 1 or 2a | Some evidence | 2a | 1 |
| Perchloroethylene | No evidence | No evidence | No evidence | No evidence |
| Propylparaben | 2a | Weak associations | 2a | 2a |
| Quadrosilan | 1 | 1 | No data | 1 |
| Resorcinol | 2a | 1 | 2b | 1 |
| Tert-butylhydroxyanisole | 1 | No data | No data | 1 |
| Thiram | 1 | No data | 2b | 1 |
| Zineb | 1 (incl. read-across) | No relevant data | No data | 1 |

Tox = *in vitro* and *in vivo* toxicity studies related to human health; Hum = human data; Eco= ecotoxicological and environmental data

Detailed datasheets with the evaluations, study summaries and references for each compound are shown in the Appendix together with a table with the overall evaluations. Below substance summaries are given based on the datasheets.

**3-benzylidene camphor (3-BC), CAS 15087-24-8**

There are no relevant human data.

Only few *in vitro* studies have been performed with 3-BC, however these show some evidence of endocrine disrupting modes of action, especially estrogenic mode of action.

The available *in vivo* studies show strong evidence of estrogenic effects. In a screening study for estrogenic effect, 3-BC has been shown to increase uterine weight in immature rats, and in reproductive studies, perinatal 3-BC exposure has been shown to cause delayed sexual maturation, decreased relative epididymis and seminal vesicle weights in adult male offspring, while female offspring showed irregular oestrous cyclicity and strongly impaired sexual behaviour.

In fish, 3-BC has been shown to induce vitellogenin and cause significant effects on reproduction.

**Evaluation: ED in Category 1.**

**4-methylbenzylidene camphor (4-MBC), CAS 36861-47-9**

The limited epidemiological studies do not indicate biologically significant effect on levels of reproductive hormones or on the hypothalamic-pituitary-thyroid axis in humans following dermal treatment with a mixture of three UV-filters, including 4-MBC.

*In vitro*, there is strong evidence of estrogenic activity, as 4-MBC has been shown to bind to the ER, alter gene transcription and cause proliferation of MCF-7 cells. No androgenic or anti-androgenic effects *in vitro* were seen in one study, while anti-androgenic activity and strong progesterone activity was seen in another. 4-MBC can also affect the thyroid system *in vitro*, by binding to the thyroid receptor.

The evidence of estrogenic activity from short term *in vivo* studies is conflicting, however increases in uterine weights and histopathological effects in uterus and vagina have been observed after longer exposure scenarios. Furthermore a large number of endocrine sensitive endpoints such as reproductive organ weights, timing of sexual maturation, impaired sexual behaviour have been shown to be affected in the developmental studies. Also, changes in LH, FSH and GnRH levels have been observed.

In fish, 4-methylbenzylidene camphor at high concentrations induces estrogen-responsive gene products including vitellogenin.

**Evaluation: ED in Category 1.**

**4-nitrophenol, CAS 100-02-7**

No relevant human data or ecotoxicity studies were found.

4-nitrophenol has exhibited estrogenic activity and anti-androgenicity *in vitro*.

*In vivo* studies with 4-nitrophenol in rats have shown evidence for endocrine disrupting effects, e.g. increase in uterus weight, decrease in weights of male reproductive organs (Uterotrophic and Hershberger, respectively) and changed hormone levels in immature male rats.

In a short term study, 4-nitrophenol impaired reproductive function in immature male rats by disturbing the hypothalamic–pituitary–testicular axis.

**Evaluation: Suspected ED in Category 2a.**

**4,4´-dihydroxybenzophenone, CAS 611-99-4**

No relevant human data was found.

The *in vitro* data show strong evidence that 4,4'-dihydroxybenzophenone has estrogenic and possibly also anti-androgenic mode of action. Only one *in vivo* study investigating endocrine disruption has been performed. It showed a significant increase in uterine weight in an Uterotrophic assay using immature rats, showing that an estrogenic effect is also be present *in vivo.*

4,4'-dihydroxybenzophenone did not result in significant vitellogenin induction in the fish.

**Evaluation: Suspected ED in Category 2a.**

**Benzophenone-1 (BP-1), CAS 131-56-6**

No relevant human data was found.

*In vitro* results show strong evidence that BP-1 has an estrogenic mode of action, while *in vitro* data for anti-androgenicity are conflicting.

BP-1 has been shown to increase uterine weight in several Uterothrophic assays in rats, showing an estrogenic effect *in vivo*. No developmental toxicity studies with Benzophenone-1 have been found in the open literature.

Two ecotoxicology studies have shown that benzophenone-1 induces vitellogenin in fish.

**Evaluation: Suspected ED in Category 2a.**

**Benzophenone-2 (BP-2), CAS 131-55-5**

No relevant human data was found.

There is strong evidence that BP-2 has estrogenic and possibly also an anti-androgenic mode of action *in vitro*. BP2 can also affect the thyroid system *in vitro*, by inhibiting the enzyme thyroid peroxidase (TPO), by binding to the thyroid receptor and by affecting thyroid hormone signalling in the testes.

There is furthermore strong evidence that BP-2 increases uterus weight in the Uterotrophic assay, showing estrogenic activity *in vivo*.

Only one developmental study of BP-2 has been described in the open literature. It showed significantly increased incidence of hypospadias in male mouse foetuses. No effect on anogenital distance in the male offspring was seen, indicating that the adverse effect was not mediated by an anti-androgenic mechanism. BP2 can also affect the thyroid hormone system *in vivo*, as shown by reduced thyroid hormone levels in adult rats from several studies

BP-2 induces vitellogenin in fish. One study showed significant estrogenic effects of BP-2 on vitellogenin induction, secondary sex characteristics, gonadal development, and reproduction in fish. The induction of vitellogenin demonstrates an estrogenic mode of action. Cessation of spawning (and thereby reproduction) is an adverse apical effect. It has been shown that benzophenone-2 decreases intrafollicular T4-content in fish which classifies BP-2 as a thyroid gland function disruptor in fish.

**Evaluation: ED in Category 1.**

**Benzophenone-3 (BP-3), CAS 131-57-7**

The studies examining effect of dermal treatment with benzophenone-3 on levels of reproductive hormones and on the hypothalamic-pituitary-thyroid axis in humans are inconclusive due to their limited duration, but indicate some effect. Epidemiological studies indicate wide human exposure to benzophenone-3, intersex difference of effect, possible correlation with BMI on pubertal development and alterations in birth parameters following perinatal exposure to BP-3. Reduced birth weight, was the only significant effect found in humans, which is an apical endpoint, but not endocrine specific.

A large number of *in vitro* studies with BP-3 has been performed. Many of them show estrogenic modes of action, while this mode of action is not seen in others. Antagonism of the androgen receptor and the progesterone receptor has also been shown and BP-3 has also been shown to affect the thyroid system *in vitro*, by binding to the thyroid receptor.

*In vivo* there is only limited evidence of estrogenic activity. Only one study has shown increased uterine weight in the Uterotrophic assay, whereas other studies have not found this, however, all these later studies tested doses below the LOAEL for the uterotrophic effect.

Benzophenone-3 induces vitellogenin in fish in one study but not in two other studies. The study showing a response on vitellogenin also shows reduced percentage of hatching of fish eggs.

**Evaluation: Suspected ED in Category 2a.**

**Butylparaben, CAS 94-26-8**

A few human studies have indicated weak associations between increased paraben exposure and markers for human reproductive health. However, the knowledge in this area is very limited.

The available data for butylparaben show strong evidence that this compound has estrogenic effects *in vitro*.

The available data for butylparaben show strong evidence that this compound has estrogenic effects *in vivo* in Uterotrophic assays performed in immature females. One *in vivo* study has shown adverse effects on sperm counts following perinatal exposure, while there are conflicting results on the influence of butylparaben on sperm count/quality following exposure of young male rats.

Butylparaben causes vitellogenin induction in fish.

**Evaluation: ED in Category 1.**

**Dicyclohexyl phthalate (DCHP), CAS No: 84-61-7**

No relevant human studies were found.

*In vitro* studies have shown estrogenic effects of DCHP.

Rat studies show that DCHP is an anti-androgen comparable to e.g. DEHP, DBP, DiBP and BBP. Impaired masculinization of male pups is observed following perinatal exposure. Fetal/neonatal anogenital distance is reduced in males, the prevalence of nipple retention is increased, male puberty is delayed, and in adults weights of male reproductive organs are decreased, and altered testis histology and sperm quality is observed. In adult animals (F0 of a two-generation study), estrous cycle duration was increased. No studies have examined whether DCHP impairs

testosterone production in fetal testes, but this is suggested to be the mode of action as judged by read-across to compounds such as DEHP, DBP, DiBP and BBP.
In adult rats (F0 of a two-generation study), DCHP was found to increase thyroid weights. This indicates interference with thyroid hormone system and based on read-across to other structurally comparable phthalates that have also been found to disrupt thyroid function (DEHP, DnOP, DnHexylP), it is likely that DCHP is also a disrupter of the thyroid hormone system.
*In vivo* ecotoxicity data provide no relevant evidence on endocrine disrupting properties or effects.
**Evaluation: ED Category 1.**

**Di-ethyl phthalate (DEP), CAS 84-66-2**
Associations between DEP exposure and clinical outcomes related to endocrine disruption (AGD in boys, infertility, and insulin resistance) have been reported in human studies. For some outcomes the same associations were seen as well for other phthalate metabolites present at the same time.
Some *in vitro* studies show weak estrogenic effects, whereas others do not, i.e. results are conflicting.
In experimental animals findings of reduced testosterone levels, delayed vaginal opening and increased incidence of abnormal sperm in a two-generation study point to endocrine disruption. Several studies show that DEP does not share the same mode of action as DEHP, DBP, BBP, DPP and DiBP and does not affect e.g. anogenital distance, fetal testosterone production, fetal testicular gene expression, nipple retention, and reproductive organ weights. Two other studies describe effects of DEP on semen quality, but it is not the same parameters that are altered in the three studies. Other studies including an enhanced 28-day study did not detect any sperm quality changes. Thus, the possibility of effects of DEP on sperm quality is controversial and although evidence of endocrine disruption has been shown, any evidence of adverse effects is less clear.
Vitellogenin induction in fish has been found.
**Evaluation: Suspected ED in Category 2a.**

**Dihexyl phthalate (DnHP), CA S 84-75-3**
No relevant human studies were found.
No relevant *in vitro* studies were found.
In experimental animals, DnHP is an anti-androgen comparable to e.g. DEHP, DBP, DiBP and BBP. Impaired masculinization of male pups is observed following fetal exposure. Fetal/neonatal anogenital distance is reduced in males, the prevalence of nipple retention is increased, and reproductive tract malformations and altered testis histology is observed in adulthood. An older continuous breeding study showed male infertility, impaired semen quality and reduced weight of reproductive organs at high doses and these effects are compatible with an anti-androgenic mode of action. Two studies showed histological changes in thyroids of rats following short-term exposure. In a NTP monograph on DnHP this finding is considered as ”sufficient data” to show that DnHP can cause thyroid toxicity.
In early life-stage studies with rainbow trout, no effects were observed on hatchability, survival, or growth.
**Evaluation: Endocrine Category 1.**

**Ethylhexyl methoxycinnamate (OMC), CAS 5466-77-3**

An epidemiological study indicates some, but no biologically significant effect on levels of reproductive hormones and on the hypothalamic-pituitary-thyroid axis in humans following dermal treatment with a mixture of benzophenone-3, 4-methylbenzylidene camphor and 2-ethylhexyl 4-methoxy cinnamate in the recommended amount. The study is limited in its duration and does not provide information about the effect of dermal treatment with ethylhexyl methoxycinnamate alone.

Some *in vitro* studies of OMC have shown binding to the estrogen receptor, while others have not, resulting in conflicting evidence on estrogenic mode of action. Other modes of action such as binding to the thyroid and progesterone receptor *in vitro* have also been seen.

There is strong evidence that OMC can affect the endocrine system *in vivo*. Slight but significant increases in uterine weights have been seen in both intact immature and adult ovarietectomized rats. In a 2-generation study, a significant decrease in sperm cell number was seen, while another reproductive study has shown developmental OMC exposure to cause several adverse reproductive effects in the offspring, including reduced reproductive organ weights, reduced reproductive hormone levels, reduced sperm counts and neurobehavioural effects. OMC can also interfere with the hypothalamo-pituitary-thyroid (HPT) axis *in vivo*, as a number of studies have shown reduced levels of thyroxine in the blood.

OMC affects the transcription of genes involved in hormonal pathways including vitellogenin in most fish studies.

**Evaluation: Endocrine disrupter in Category 1.**

**Metam natrium, metam sodium (SMD), CAS 137-42-8.**

The only study on humans does not deal with an endocrine specific endpoint.

No relevant data *in vitro* data was found.

SMD have been shown to block ovulation in rats, however there is probably a temporal window in the day of prooestrous during which SMD is most effective in blocking ovulation. A study in rats have demonstrated a dose dependent suppression of the rise in LH, a dose related decrease in serum prolactin and a decrease in percentage of ovulating rats. MITC (the metabolite of sodium natrium) produce a decrease in foetal body weight and size. Doses below those resulting in maternal toxicity produce pre- and post-implantation loss of embryos.

Metam sodium shows teratogenic effects in embryonic zebra fish. *In vivo* ecotoxicity studies on endocrine related endpoints were not found.

**Evaluation: Endocrine disrupter Category 1**

**Methyl tertiary butyl ether (MTBE), CAS 1634-04-4**

Three human studies have been found on MTBE, they are, however, unsuitable for inclusion in an evaluation of the endocrine disrupting potential of MTBE.

A study on mouse spermatogenic cells *in vitro* suggest that a high dose could exert a direct toxic effect on Sertoli cells that would impair their function and subsequently impair spermatogenesis or even cause cell death. MTBE decreased testosterone in rat Leydig cells, but only with extremely high doses.

The effects observed in animal studies of adult rats include: decreased relative ovary and pituitary weights and increased oestrous cycle length, decreased prolactin and T3 levels, increased abnormal sperm percent and irregular histopathology of testes and altered levels of testosterone, LH and FSH. In zebra fish, Vtg-induction and significant effect on sperm parameters were observed.
**Evaluation: Endocrine disrupter Category 1.**

**Pentachlorophenol (PCP), CAS 87-86-5**
Two studies indicate associations to adverse effects on thyroid system in newborns of women with higher levels of PCP. In addition there are hypothesis generation studies indicating effects on female reproduction and miscarriages.
The *in vitro* results indicate that PCP shows anti-androgen, anti-estrogen and thyroid disrupting modes of action and can interfere with steroidogenesis.
*In vivo* studies with PCP in rats have shown decreased T4 and decreased spermatid counts, where several ED modes of action are highly plausible.
A number of ecotoxicological studies studies examine the effect of pentachlorophenol in mammals, i.e. mink and sheep. Pentachlorophenol affects reproduction in some of the experimental setups but not in others. The studies show thyroid function disrupting effects of pentachlorophenol. Likewise, pentachlorophenol interferes with the thyroid system of *Xenopus laevis* and multiple hormone systems in fish.
**Evaluation: Endocrine disrupter in Category 1.**

**Perchloroethylene (PCE), CAS: 127-18-4**
The available literature on human data does not substantiate endocrine disruptive effects of PCE in humans.
No relevant *in vitro* data on endocrine disruption were found.
Three animal studies have been performed in which rats have been exposed to PCE for varying periods of time. Reproductive effects as well as effects on the rat brain have been seen, however no effects pointing to endocrine disruption have been found.
No endocrine specific endpoints or mechanistic data have been tested for PCE in wildlife. Deformities in amphibians could be via some kind of thyroid disruption but this needs to be further investigated.
**Evaluation: PCE is not placed in either ED Category 1, 2a or 2b as no available data pointing in the direction of endocrine disruption was found.**

**Propylparaben, CA S 94-13-3**
A few human studies have indicated weak associations between increased paraben exposure and markers for human reproductive health. However, our knowledge in this area is very limited.
There is strong evidence for estrogenic effects *in vitro*. Anti-androgenic effects have also been shown in a few *in vitro* studies.
Propylparaben has estrogenic effects *in vivo* using Uterotrophic assays or exposure of immature females. One study shows effect of propylparaben on sperm count/quality following exposure of

young males, but some doubt has been raised on the quality of this study. No studies with perinatal exposure to propylparaben have been published.
In ecotoxicological studies propylparaben has caused Vtg induction in multiple studies and caused adverse effect on daphnia reproduction,
**Evaluation: Suspected endocrine disrupter in Category 2a**

**Quadrosilan, CAS 33204-76-1**
There is no doubt that Quadrosilan shows estrogenic, antigonadotropic and thereby anti-androgenic effects in humans following oral exposure to pharmacological doses.
Based on *in vivo* studies, quadrosilan is a potent reproductive toxicant with effects at 0.1 mg/kg bw/day in the rabbit.
No relevant *in vitro* or ecotoxicity were data found.
**Evaluation: Endocrine disrupter in Category 1.**

**Resorcinol, CAS 108-46-3**
According to human case reports, resorcinol exerts anti-thyroid functions. Data are old, but quite clear: long-term administration of resorcinol to permeable (damaged) skin can cause myxoedema (reduced thyroid function). Cessation of exposure causes the myxoedema to disappear. In the human study investigating dermal uptake in healthy individuals the dermal barrier avoids uptake of resorcinol.
*In vitro*, resorcinol has been shown to be a very potent inhibitor of the enzyme thyroid peroxide and to inhibit uptake of radioactive iodide, which are both effects that *in vivo* could lead to decreased thyroid hormone levels.
The results from the *in vivo* studies are somewhat inconsistent. A number of older studies using relatively few animals per dose group found adverse effects on the thyroid hormone system if dosing was performed in a way that allowed for a slow and continuous release of resorcinol to the systemic circulation (i.e. sc injections in oily solution). Some more recent extensive rat studies investigating the effects on the thyroid hormone system using more animals and several dose levels have been performed. Here rats have been dosed by gavage or in the drinking water but no or only very few effects on the thyroid system have been found, indicating that route of exposure is very important. This is most probably because rapid metabolism in most cases prevents resorcinol from reaching concentrations which are toxic for the thyroid gland.
In the ecotoxicology study, anti-thyroid effect in zebra fish embryos was seen. It should be noted that this study could be viewed as an *in vitro* study instead of *in vivo* due to the EU legislation about fish embryos.
**Evaluation: Endocrine disrupter in Category 1.**

**Tert-butylhydroxyanisole (BHA), CAS 25013-16-5**
No relevant human data or ecotoxicity endocrine data was found.
Several studies have shown (weak) estrogenic effect of BHA *in vitro*.
In rats fed with BHA reduced levels of testosterone and thyroid hormones and malformed sperm was found. Their offspring were smaller, had delayed sexual maturation and smaller reproductive

organs compared to controls. These reproductive effects could also be caused by general reproductive toxicity, but since decreased levels of testosterone and T4 have also been observed, much evidence points in the direction of endocrine disruption. In pigs a decrease in weight gain, liver and thyroid weight have been observed.
**Evaluation: Endocrine disrupters in Category 1.**

**Thiram, CAS 137-26-8**
No relevant human data was found.
*In vivo*, thiram has been shown to block ovulation and LH surge and thereby reduce pregnancy rate and increase number of resorptions. Thiram is a dialkyldithiocarbamate and the observed adverse effects on reproduction are similar to the effects of metam natrium, so read across to metam natrium has been included in the evaluation.
Thiram have been shown to cause a down regulation of sox9a during zebra fish development and disrupt corticosterone action on the glucocorticoid receptor in zebra finches.
**Evaluation: Endocrine disrupter in Category 1.**

**Zineb, CAS 12122-67-7**
Zineb is a dithiocarbamate and seem to have the same thyroid hormone disrupting effects as some other dithiocarbamates (e.g. mancozeb, maneb and probineb). Dithiocarbamates are degraded to the known substances thyroid hormone disrupting ethylenethiourea (ETU) and propylthiouracil (PTU) which inhibits the formation of T4 in the thyroid.
The available human studies are unsuitable for inclusion in an evaluation of the endocrine disrupting potential of zineb.
*In vitro*, zineb show similar mechanism of action as mancozeb, where inhibition of thyroid peroxidase (TPO) leads to decreased thyroidal synthesis of T3 and T4.
*In vivo* studies in rats have shown some evidence for endocrine disrupting effects, e.g. increase in thyroid weight and decrease in T3. Overall from the *in vivo* studies it was found that Zineb is disrupting the thyroid hormone system in rats and could also be involved pathological changes in the testis in rats.
No endocrine related endpoints have been investigated in ecotoxicity studies.
**Evaluation: Endocrine disrupter in Category 1.**

**3.2 DE-UK potency criteria for identification of ED of very high regulatory concern**
Inclusion of the potency cut off in the UK/DE criteria leads to much fewer substances considered as EDs or suspected EDs than the evaluation based on the DK criteria (table 5).

Among the 15 substances evaluated as EDs in Category 1 based on the DK criteria only 4 appear likely to fulfil the UK/DE potency criteria. Among the remaining 11 substances, 6 are Unclear and 5 are evaluated as not fulfilling the potency criteria.

Among the 6 substances evaluated as suspected EDs in Category 2a based on the DK criteria none appear likely to fulfil the potency criteria in the UK/DE criteria, 2 are Unclear and 4 are evaluated as not fulfilling the potency criteria.

**Table 5. ED Category based on DK criteria and adding the DE-UK potency criteria (LOAEL below 10 mg/kg)**

| **Substance** | **DK criteria** | **LOAEL below 10 mg/kg** |
|---|---|---|
| 3-benzylidene camphor | 1 | **Yes** |
| 4-methylbenzylidene camphor | 1 | **Yes** |
| Quadrosilan | 1 | **Yes** |
| Tert-butylhydroxyanisole | 1 | **Yes** |
| Benzophenone-2 | 1 | Unclear |
| Butylparaben | 1 | Unclear |
| Dicyclohexyl phthalate (DCHP) | 1 | Unclear |
| Pentachlorophenol(PCP) | 1 | Unclear |
| Thiram | 1 | Unclear |
| Resorcinol | 1 | Unclear |
| Dihexyl phthalate (DHP) | 1 | No |
| Ethylhexyl methoxycinnamate (OMC) | 1 | No |
| Metam natrium | 1 | No |
| Methyl tertiary butyl ether (MTBE) | 1 | No |
| Zineb | 1 | No |
| 4-nitrophenol | 2a | Unclear |
| Propylparaben | 2a | Unclear |
| 4,4´-dihydroxybenzophenone | 2a | No |
| Benzophenone-1 | 2a | No |
| Benzophenone-3 | 2a | No |
| Diethyl phthalate (DEP) | 2a | No |
| Perchloroethylene | no evidence | No |

# 4. Discussion

### 4.1 Evaluations using DK criteria

The separate evaluations based on epidemiological data, *in vitro* data plus animal data, and ecotoxicity data in several cases showed consistency between animal data and ecotoxicity data (table 4). The separate evaluations of 3-benzylidene camphor and benzophenone-2 lead to ED Category 1 and for benzophenone-2, benzophenone-3 and propylparaben to suspected ED Category 2a based on both types of data. However, in several other cases, the separate evaluations differed e.g. the substance was preliminary evaluated as ED Category 1 based on *in vitro* and *in vivo* toxicity data and as suspected ED Category 2a based on ecotoxicity data. In these cases, it can be seen that it reflects absence of studies on relevant adverse ecotoxicity effects.

The overall evaluations illustrate that a substance can be considered an ED (Category 1) based on different kind of data. *In vivo* assays providing data on effects clearly linked to endocrine mechanisms was the background for the evaluation of the two phthalates dicyclohexylphthalate (DHCP) and dihexyl phthalate (DHP), for which the evidence for ED is similar to other endocrine disrupting phthalates (DEHP, DBP etc.). The evaluation of resorcinol illustrates the use of evidence from human cases as main basis for placing a substance in Category 1. The importance of an overall weight of evidence is illustrated by pentachlorophenol. The preliminary evaluations each lead to suspected ED in Category 2a or some evidence from humans, but the overall evaluation was ED in Category 1, because all evidence pointed in the same direction, i.e. adverse effects on thyroid system.

### 4.2 SIN List evaluations vs. evaluation using DK criteria

The evaluation using the DK criteria in most cases leads to the same overall conclusion as the SINList evaluation, as 15 of the 22 substances are categorized as EDs in Category 1. Among the remaining 7 compounds, 6 are categorized as suspected EDs in Category 2a.

The suspected EDs are in most cases substances for which there is sufficient evidence for endocrine mode of action *in vivo*, but absence of relevant studies on adverse effects. In such cases, we evaluate that adverse effects are very likely to be found if the optimal study design is used. Thus, the difference between the evaluations in this report and the SINList evaluations is likely due to different criteria and the way lack of data is handled.

The major difference between the two evaluations is seen on perchloroethylene, which based on the DK criteria is not categorized in any of the ED Categories due to lack of data on both relevant mode of action and effect. We found that the available human data did not substantiate endocrine disruptive effects in humans and no relevant *in vitro* data on endocrine disruption were found. The animal studies showed reproductive effects and some effects on the rat brains, but no effects pointing to endocrine disruption were seen. Also, no endocrine specific endpoints or mechanistic data have been tested for PCE in wildlife. Deformities observed in amphibians could be via some

kind of thyroid disruption but this needs to be further investigated. Thus, although perchloroethylene may be a reproductive toxicant, there is no available data pointing in the direction of endocrine disruption.

### 4.3 UK/DE criteria

Inclusion of the potency cut-off in the DE-UK criteria leads to much fewer substances considered as endocrine disrupters of very high regulatory concern than the evaluation based on the DK criteria for identification of ED (table 4). Only 4 of the 15 substances (27%) evaluated as EDs in Category 1 based on the DK criteria appear likely to fulfil the potency criteria, i.e. 3-benzylidene camphor, 4-methylbenzylidene camphor, quadrosilan and tert-butylhydroxyanisole. Actually, it can be questioned whether the two UV-filters will be considered as endocrine disrupters of very high regulatory concern, as there are some limitations in the toxicity studies on which the LOAELs are based.

The remaining 11 EDs in Category 1 include substances such as dicyclohexylphthalate (DHCP) and dihexyl phthalate (DHP), for which the evidence for ED is similar to other endocrine disrupting phthalates (DEHP, DBP etc.) that are already regulated due to adverse reproductive toxicity effects. Also, the UV-filters such as benzophenone-2 and ethylhexyl methoxycinnamate (OMC), for which human exposure potentially may be high, would most likely not be considered as endocrine disrupters of very high regulatory concern if the DE-UK potency criteria are used. Overall, this indicates that use of the potency criteria may lead to inadequate protection for some endocrine disrupting substances. In contrast, the DK criteria do not specify whether the identification of a substance as an ED should lead to high regulatory concern or not, and further evaluation of exposures can be used to prioritize which of the identified EDs or suspected EDs that are of high regulatory concern. Furthermore, some compounds identified as being of very high regulatory concern according to DE-UK criteria may not be used or lead to negligible exposures and therefore be of less regulatory concern.

None of the 6 substances evaluated as suspected EDs in Category 2a based on the DK criteria appear likely to fulfil the potency criteria in the DE-UK proposal. This is not very surprising as most of the suspected EDs were allocated to this Category based on mode of action data *in vivo* and lack of robust adverse effect data, and the DE-UK potency criteria is a cut-off in relation to adverse effects.

### 4.4 Next steps, e.g. further testing

For the suspected EDs in Category 2a further testing may be considered to obtain data for evaluating whether these substances are actually EDs in Category 1. For the majority of the suspected substances *in vivo* data showed endocrine mode of action, but there was a lack of *in vivo* data on adverse endocrine effects. In such cases we evaluated that adverse effects are very likely to be found if the optimal study design is used and especially for ecotoxicity adverse effects are clearly expected. We find that this is important to include when considering the need for further studies of

these substances. However, below we give some proposals for further testing if this is considered needed for regulatory purposes.

***Studies in humans***

For the majority of the substances evaluated in this project, there are only few, if any, relevant human data. Therefore, the categorization of the substances lies primarily on animal experimental studies and ecotoxicological studies. This is of no surprise, as it lays inherently in the phenomenon of risk assessment that the aim is to identify risk before a situation occur where human data can unequivocally prove endocrine disrupting effects.

Still, for the substances in question, comprehensive, well-designed epidemiological investigations can indeed be used to support or contradict conclusions drawn from the available experimental studies. Epidemiological studies have the capability of associating human health outcome to certain exposure scenarios. In the analysis of epidemiological data, various known confounders can be taken into account, minimizing the risk of false associations. Still, evidence of linkage between exposures and outcomes from epidemiological data sets will always be indirect. For most of the substances investigated here, such epidemiological data are lacking.

The real life scenario, where people are exposed to a combination of substances, sometimes from the very same exposure sources, makes it extremely difficult to distinguish the contribution from a certain chemical from that of other related substances. This is for example extremely relevant for the groups of phthalates and parabens. Also, certain exposures are closely linked to certain lifestyles, which make it difficult, in spite of inclusion of confounding factors, to discern effects due to lifestyle from exposure-induced effects. Therefore, even though large and well-designed epidemiological studies are performed, these will not always be able to point at a certain chemical. This is of course also due to the fact that real life is more complex than what we are able to account for in epidemiological studies. In this respect, new types of data management, such as systems biology methodologies may prove capable of pointing out factors or combination of factors which contribute significantly to different disease trends observed in the population.

Also, to be able to compare different exposure scenarios in human studies, the included population has to be exposed to various extents. In addition, the variation of the concentration of a given chemical within a given individual has to be smaller than the concentration variation between the included subjects. This is not the case for all substances and especially not for those where exposure is more or less ubiquitous. In such cases, occupational studies, where a subgroup of people are exposed to very high levels of a given compound or group of substances, are very useful.

***Toxicity studies, human health***

All the substances categorized as suspected EDs in Category 2a have an expected estrogenic mode of action shown as effect in the Uterotrophic assay and/or vitellogenin (VTG) assays. However, also other modes of endocrine actions are indicated based on *in vitro* studies for a number of these substances. If further data are needed, the substances are all proposed to be tested in OECD TG 443 Extended one-generation reproductive toxicity study and not the in TG 416 Two-generation study, because both the design and the endpoints in the TG 443 in contrast to TG 416 has been enhanced with focus on detection of effects of endocrine disrupters. It is highly recommended to include the

optional assessment of mammary gland development in TG 443, because there are indications for high sensitivity of this endpoint for especially substances with estrogenic mode of action. Also, effects on mammary gland development may indicate increased risk for breast cancer later in life, i.e. a common disease in humans for which the incidence appears to be increasing.

***Toxicity studies, environment***

In table 5, a simple ecotoxicity testing strategy has been proposed for the substances evaluated as suspected in Category 2a and for resorcinol, which is placed in Category 1 based mainly on human data. All these substances have an expected estrogenic mode of action except resorcinol which is an anti-thyroid. The substances which have shown vitellogenin (VTG) induction in one or more species but lack the connection to an adverse effect are all proposed to be tested in OECD TG 234 (Fish Sexual Development Test) where the VTG concentration can be linked to an adverse effect on the sex ratio. Substances with estrogenic mode of action, where VTG effects are divergent between studies or lacking, are proposed to be tested in a short term 7-days test with adult male zebrafish (not yet proposed as an OECD TG) to confirm VTG induction (if a OECD TG is required, TG 229 (Fish Short Term Reproduction Assay) or TG 230 (21-Day Fish Assay) are proposed). If the VTG induction appears it is proposed to continue with TG 234 to test the link to adverse effects. The anti-thyroidal acting resorcinol is proposed to be tested in TG 231 (Amphibian Metamorphosis Assay) or the LAGDA (Larval Amphibian Growth and Development Assay) still under development as a draft test guideline in OECD to confirm the adverse effects on the thyroid system observed in humans.

**Table 5 Simple ecotoxicity testing strategy for substances evaluated as suspected ED in category 2a**

| Chemical | Overall evaluation | Mode of action | Proposed testing strategy |
|---|---|---|---|
| 4-nitrophenol | 2a | estrogenic | TG 234 (Fish Sexual Development |
| 4,4´-dihydroxybenzophenone | 2a | estrogenic | *Short term VTG fish test. If VTG induction then TG 234 |
| Benzophenone-1 | 2a | estrogenic | TG 234 |
| Benzophenone-3 | 2a | estrogenic | *Short term VTG fish test. If VTG induction then TG 234 |
| Diethyl phthalate (DEP) | 2a | estrogenic | TG 234 |
| Propylparaben | 2a | estrogenic | TG 234 |
| Resorcinol | 1 | anti-thyroid | TG 231 (Amphibian Metamorphosis Assay) |

* If existing data are divergent then conduct a short term VTG fish test: e.g. 7-days in male zebrafish (not yet proposed as OECD TG) or TG 229 (Fish Short Term Reproduction Assay) or TG 230 (21-Day Fish Assay to confirm VTG induction).

## 5. Summary and conclusions

DK-EPA has asked the Danish Centre for Endocrine Disrupters to assess the 22 substances on the SIN List 2.0. These substances have been identified by the NGO ChemSec as Substances of Very High Concern according to the criteria in REACH, solely due to their endocrine disrupting properties. The overall aim is to categorize the 22 substances on the basis of the Danish proposal for criteria for endocrine disrupters. This means that the evaluation is based on results from studies in humans, *in vitro* studies and *in vivo* studies related to both human toxicity and environmental effects. Furthermore, all 22 substances are evaluated according to the potency cut-off criteria of 10 mg/kg/day proposed by DE-UK for identification of endocrine disrupters (EDs) of very high regulatory concern.

The evaluation using the Danish criteria in most cases leads to the same overall conclusion as the SINList evaluation, as 15 of the 22 substances are categorized as EDs in Category 1. Among the remaining 7 compounds, 6 are categorized as suspected EDs in Category 2a. The major difference between the two evaluations is seen on perchloroethylene, which although it may be a reproductive toxicant is not categorized in any of the ED Categories based on the Danish criteria as there is no available data pointing in the direction of endocrine disruption.
Inclusion of the potency cut-off in the DE-UK criteria leads to much fewer substances considered as endocrine disrupters of very high regulatory concern than the evaluation based on the DK criteria for identification of ED, as only 4 of the 15 substances appear likely to fulfil the potency criteria. This indicates that use of the potency criteria may lead to inadequate protection for some endocrine disrupting substances. The DK criteria do not specify whether the identification of a substance as an ED should lead to high regulatory concern or not, and further evaluation of exposures can be used to prioritize which of the identified EDs or suspected EDs that are of high regulatory concern.

## References

Danish EPA, 2011: Establishment of Criteria for Endocrine Disruptors and Options for Regulation, 17 May 2011. (Criteria proposal and Annex A, B and C).

DE-UK, 2011: Joint DE-UK Position Paper. Regulatory Definition of an Endocrine Disrupter in relation to Potential Threat to Human Health. Proposal applicable in the regulatory context of Plant Protection Products, Biocidial Products, and Chemicals targeted within REACH, 16 Mai 2011. www.bfr.bund.de

Hass U, Christiansen S, Boberg J, Vinggaard AM, Andersson A-M, Skakkebæk NE, Katrine Bay, Holbech H, Bjerregaard P (2011). Criteria for Endocrine Disrupters. Report from Danish Centre on Endocrine Disrupters for Danish EPA.

OECD TG 443(2001). OECD Test Guideline 443: Extended One-Generation Reproductive Toxicity Study. http://www.oecd-ilibrary.org/environment/test-no-443-extended-one-generation-reproductive-toxicity-study_9789264122550-en

WHO/IPCS 2004. IPCS Risk Assessment Terminology.

Note that references for the substance evaluations are included in each of the datasheets in the Appendix.

## Abbreviations

| | |
|---|---|
| ADME: | Absorption, Distribution, Metabolism, and Excretion |
| AGD: | Anogenital Distance |
| CF: | Conceptual Framework |
| CLP: | Classification, Labelling and Packaging |
| ED: | Endocrine disrupter/disrupting |
| EDs: | Endocrine disrupters |
| EDC: | Endocrine disrupting chemicals |
| EU: | European Union |
| GHS: | Globally Harmonised System of Classification and Labelling of Chemicals |
| IPCS: | International Programme on Chemical Safety |
| NOAEL: | No Observed Adverse Effect Levels |
| NR: | Nipple retention |
| SIN: | Substitute It Now |
| STOT: | Specific Target Organ Toxicity |
| SVHC: | Substance of Very High Concern |
| OECD: | Organisation for Economic Co-operation and Development |
| QSAR: | Quantitative Structure-Activity Relationship |
| REACH: | Registration, Evaluation, Authorisation and Restriction of Chemicals |
| TG: | Test Guideline |
| WHO: | World Health Organisation |

# Annex
# Overview of the evaluations and datasheets with substance evaluations

**Overview of the evaluation of 22 SIN List 2.0 substances according to the Danish proposal on criteria for endocrine disrupters**

| Substance | Category |
|---|---|
| 3-benzylidene camphor | 1 |
| 4-methylbenzylidene camphor | 1 |
| 4-nitrophenol | 2a |
| 4,4´-dihydroxybenzophenone | 2a |
| Benzophenone-1 | 2a |
| Benzophenone-2 | 1 |
| Benzophenone-3 | 2a |
| Butylparaben | 1 |
| Dicyclohexyl phthalate (DCHP) | 1 |
| Diethyl phthalate (DEP) | 2a |
| Dihexyl phthalate (DHP) | 1 |
| Ethylhexyl methoxycinnamate | 1 |
| Metam natrium | 1 |
| Methyl tertiary butyl ether (MTBE) | 1 |
| Pentachlorophenol(PCP) | 1 |
| Perchloroethylene | No |
| Propylparaben | 2a |
| Quadrosilan | 1 |
| Resorcinol | 1 |
| Tert-butylhydroxyanisole | 1 |
| Thiram | 1 |
| Zineb | 1 |

***Explanation:***
***Category 1 - Endocrine disrupter***
***Category 2a - Suspected ED***
***Category 2b – Substance with indications of ED properties (indicated ED)***
***No - The substance is placed neither in ED Category 1, 2a nor 2b as there is no available data pointing in the direction of endocrine disruption***

**3-benzylidene camphor, CAS 15087-24-8**
**Synonyms:** 3-BC

**Human data**
An exposure study based on mothers' cohorts recruited in 2004-2006 in Basel, Switzerland has recently been published (Schlumpf et al. 2010). 54 samples of human breast milk were primary collected before 30 days post partum and were analyzed for eight different UV-filters (benzophenone-2, benzophenone-3, ethyl-hexyl cinnamate, homosalate, 3-(4-methyl-benzylidene) camphor, 3-benzylidene camphor, octocrylene and octyl-dimethyl PABA), sixteen different synthetic musks, seven PCBs and six PBDEs. None of mothers reported use of cosmetic products containing 3-benzylidene camphor, and the compound was not detected in any milk samples. On the base of declaration list for cosmetic products, mothers in Switzerland were not exposed to 3-benzylidene camphor.

***In vitro* data**
*In vitro* studies of 3-BC have shown that the compound can act as an estrogen, by causing proliferation of MCF-7 cells (Schlumpf et al 2004a), and by binding to the estrogen receptor (beta) (Schlumpf et al 2004a, Schreurs et al 2005). Furthermore a study has shown antagonism of the androgen and the progesterone receptor *in vitro* (Schreurs et al 2005), while no androgenic or anti-androgenic effect *in vitro* was seen in another study (Ma et al 2003). Nashev et al (2010) have further shown 3-BC to inhibit 17b-HSD, an enzyme that metabolizes estrogens and androgens. Overall the *in vitro* results show that 3-BC has endocrine disrupting modes of action and especially estrogenic mode of action.

***In vivo* studies, human health**
In an *in vivo* screening study for estrogenic effect, 3-BC has been shown to increase uterine weight in immature LE rats (Schlumpf et al 2004a). The study was performed in 21-23 days old female rats, which were dosed for 3 days by oral gavage (n=4-9 in the dose groups and 24 in the control). 9 doses of the compound were tested (0.8, 2, 4, 9.4, 18.75, 37.5, 75, 150 and 300 mg/kg/day, and significant increases in uterine weight were seen from between 2-4 mg/kg and above, depending on the statistical analysis performed. The ED50 in this study was 45 mg/kg, indicating that this compound is more potent in the uterotrophic assay than many other tested UV-filters.

Some reproductive studies, testing the developmental toxicity of 3-BC have also been performed. Different parts of the studies have been reported in different publications (Schlumpf et al 2004b, Schlumpf et al 2008a,b, Hofman et al 2008, Faass et al 2009), making it difficult to evaluate exactly how many studies have been performed and when which dose levels have been tested. The endocrine disrupting effects of perinatal 3-BC exposure on male offspring was delayed sexual maturation and decreased relative epididymis and seminal vesicle weights in adulthood, while females showed irregular estrous cyclicity and strongly impaired sexual behaviour. Depending on which endpoints were chosen, the LOAEL value for 3-BC was between 0.24 and 2.4 mg/kg/day. At the dose of 2.4 mg/kg many results pointing in the direction of endocrine disruption were seen, whereas the only effect seen 0.24 mg/kg, was irregular oestrous cycles in females. As the study was performed with only about 3 litters per dose group, and it is recommended that a group size of 20 is used for examination of oestrous cyclicity (Cooper and Goldman 1999) the significant change seen at this very low dose may not reflect a real biological effect. Therefore the LOAEL in this evaluation of 3-BC is set at 2.4 mg/kg and the NOAEL at 0.7 mg/kg/day.

Detailed summary of the methods and findings described in the following publications (**Schlumpf et al 2004b, Schlumpf et al 2008a and b, Hofman et al 2008, Faass et al 2009**), are presented below:
The following experimental setup was used in each study. Male and female LE rats were dosed with the compound, by adding it to the feed. The parental generation was exposed for 10 weeks before mating, exposure of dams has continued throughout gestation and lactation, and the offspring were further dosed until adulthood. The following doses have been investigated: 0.07, 0.24, 0.7, 2.4 and 7 mg/kg/day. The number of dams in each dose group was unfortunately not been stated in the Schlumpf et al publications, where most of the results on endocrine sensitive endpoint are reported. However, in papers by Hofman et al (2008) and Faass et al (2009) it is stated that between 3-7 litters per dose group were used, and this was likely also the case for the studies described in Schlumpf et al (2004, 2008).
Doses of 0.24 and above all caused irregular oestrous cycles in the adult female offspring (Schlumpf et al 2008b, Faass et al 2009), however only between 5-11 female rat offspring were used for this study, and they only represented 3 litters each. Adult prostate weights were reduced in the 0.24 mg/kg dose group but not in any of the higher groups, indicating that this might be a chance finding. At the dose of 2.4 mg/kg/day decreased postnatal survival rate and delayed sexual maturation in male offspring was observed. Body weights at puberty were normal in dosed males, indicating that the delay of puberty did not result from nutritional effects (Schlumpf et al 2008b). In Schlumpf et al (2004) it was reported that a dose of 0.24 mg/kg/day also caused delayed puberty in males. However in a later publication from this group, delayed preputial separation was only reported to occur in the 2.4 and 7 mg/kg/day groups (Schlumpf et al 2008). Timing of sexual maturation of the female offspring was not affected by any dose of 3-BC (Schlumpf et al 2004b). The dose of 2.4 mg/kg/day also caused decreased relative epididymis and seminal vesicle weights in adult males. These effects were however not seen at the higher dose of 7 mg/kg, and might therefore be a chance finding. Adult testes weights were not affected at any dose levels and no effects on volume of accessory sex glands or prostate were seen on PND 1 (Hofkamp et al 2007). Thyroid gland weights were not reported, and it is unclear whether they were not measured or whether no significant effects were seen. The immune system of the animals was probably not affected by 3-BC exposure, as thymus weights were not different from controls. Decreased adult body weight was seen in females at the dose of 2.4 mg/kg and in adult males at 7 mg/kg. The highest dose further caused decreased litter size. Female sexual behaviour, measured both as proceptive and receptive behaviour was strongly impaired in offspring exposed to 2.4 and 7 mg/kg (Schlumpf et al 2008b, Faass et al 2009), while this endpoint was not investigated in any other dose groups. Furthermore, 3-BC caused alterations in gene expression in the uterus as well as in sexually dimorphic areas of the brain on PND 6 in all dose groups (Schlumpf et al 2008b, Schlumpf et al 2008a, Faass et al 2009).

***In vivo*, ecotoxicity**
3-benzylidene camphor has been shown to induce vitellogenin in fish, which shows an estrogenic mode of action. In addition to vitellogenin induction, one study also shows significant effects of 3-benzylidene camphor on the reproduction of fish. This is an adverse apical effect.

Summaries of the ecotoxicology studies are provided below:
**Holbech et al. 2002**: *In vivo* exposure of rainbow trout (*Oncorhynchus mykiss*). Two experiments were performed: Experiment 1 with intraperitoneal injections of 3-benzylidene camphor on day 0, 3, and 6 and Experiment 2 with injections on day 10 also. Experiment 1 comprised 5 groups. One group of 5 fish was injected with 683 mg/kg/injection 17β-oestradiol (positive control) and 4 groups of 10 fish each were injected with peanut oil (vehicle control), 27, 205 or 410 mg/kg/injection of 3-benzylidene camphor respectively. Experiment 2 comprised 8 groups of 10 fish each. The trout were injected with peanut oil, 683

mg/kg/injection 17β-oestradiol and 2.7, 8.2, 14, 27, 68 or 137 mg/kg/injection of 3-benzylidene camphor, respectively.
A clear relationship was demonstrated between the dose of injected 3-benzylidene camphor and the concentration of plasma vitellogenin with a 105-times induction from 68 mg 3-benzylidene camphor/kg/injection and above compared to the control vitellogenin level. The study shows that 3-benzylidene camphor induces vitellogenin in fish. Induction of vitellogenin is not an adverse apical effect but a biomarker of estrogenic exposure and thus provides information about the endocrine mode of action.
**Kunz et al. 2004**: The study investigates whether 3-benzylidene camphor interferes with the thyroid and sex hormone system during frog metamorphosis. *Xenopus laevis* tadpoles were exposed to 1.5 and 50 µg/L 3-benzylidene camphor for 35 days (NF stage 52–66).
The rate of metamorphosis was not affected, and no obvious differences in body and tail length compared to controls were observed. 3-benzylidene camphor did not lead to effects on the sex ratio or gross gonad morphology of *X. laevis* at stage 66.
The results indicate that 3-benzylidene camphor does not negatively affect the thyroid system and sex ratio of frogs at the tested concentrations.
**Kunz et al. 2006**: Fathead minnows were exposed to 3-benzylidene camphor for 14 days at four different aqueous exposure concentrations: 9 µg/L (actual), 100 µg/L (nominal), 435 µg/L (actual) or 953 µg/L (actual). Vitellogenin was induced with a LOEC of 435 µg/L.
The study shows that 3-benzylidene camphor induces vitellogenin in fish. Induction of vitellogenin is not an adverse apical effect but a biomarker of estrogenic exposure and thus provides information about the endocrine mode of action.
**Kunz et al. 2006a**: This study evaluates whether 3-benzylidene camphor affects reproduction of fish *Pimephales promelas*. Reproductively mature fathead minnows were exposed to increasing concentrations of 3-benzylidene camphor for 21 days in a static-renewal procedure. Actual 3-benzylidene camphor concentrations were 0.5, 3, 33, 74, and 285 µg/l.
3-benzylidene camphor affected reproduction in a dose-dependent manner with weak effects on fecundity at 3 µg/l, a significant decrease at 74µg/l, and a cessation of reproduction at 285 µg/l. Dose-dependent demasculinization in secondary sex characteristics of male fish and dose-dependent induction of plasma vitellogenin occurred, which was significant at 74 µg/l and higher. 3-benzylidene camphor had a profound and dose-dependent effect on the histology of gonads of male and female fish at 3 µg/l and higher. At 74 and 285 µg/l, oocyte and spermatocyte development was inhibited in male and female gonads. No toxic side effects (i.e., lethargy, uncoordinated swimming, loss of equilibrium, hyperventilation) were observed.
This study shows significant effects of 3-benzylidene camphor on reproduction of fish. This is an adverse apical effect but not endocrine specific. The study also shows that 3-benzylidene camphor induces vitellogenin in fish. Induction of vitellogenin is not an adverse apical effect but a biomarker of estrogenic exposure and thus provides information about the endocrine mode of action. Thus this study both shows adverse apical effects of 3-benzylidene camphor and demonstrates the endocrine mode of action.
**Scheil et al. 2008**: This study investigates the effects of 3-benzylidene camphor in the freshwater amphipod *Gammarus fossarum* at the cellular and molecular level.
Stress protein (Hsp70) responses and reactions of hepatopancreatic cells and cells of gut appendices were investigated after short-term exposure (4 days) to five different concentrations of 3-benzylidene camphor (33 ng/L, 330 ng/L, 3.3 µg/L, 33 µg/L, 330 µg/L) and two control conditions (water and solvent ethanol). Male as well as female gammarids showed increased Hsp70 levels after exposure to low concentrations of 3-benzylidene camphor, with a maximum response at 3.3 µg/L, while the higher concentrations resulted in lower Hsp70 levels. Strong cellular responses and cellular damage were obtained in epithelia of the hepatopancreas and the gut appendices after treatment with 330 µg/L 3-benzylidene camphor.

The effects observed in this study may reflect a general toxic stress situation rather than a specific endocrine disrupting mode of action.

**Schmitt et al. 2008:** *In vivo* sediment tests with the mudsnail *Potamopyrgus antipodarum* and the blackworm *Lumbriculus variegatus* showed that 3-benzylidene camphor increased embryo production in the snail (NOEC: 0.06 mg/kg dry weight; LOEC: 0.28 mg/kg dry weight) but decreased the reproduction of *L. variegatus* (NOEC: 1.49 mg/kg dry weight; LOEC: 6.47 mg/kg dry weight).

The study shows that 3-benzylidene camphor affects reproduction in the mudsnail and the blackworm. This is an apical effect but not endocrine specific. At the highest concentrations tested, 3-benzylidene camphor caused increasing mortality.

**Kunz and Fent 2009:** Juvenile fathead minnows were exposed to actual concentrations of 9, 45, 83.7, 215.4, 420.5 or 412.6 µg/L 3-benzylidene camphor for 14 days to determine vitellogenin induction. Significant vitellogenin-induction occurred at 420 µg/L.

The study shows that 3-benzylidene camphor induces vitellogenin in fish. Induction of vitellogenin is not an adverse apical effect but a biomarker of estrogenic exposure and thus provides information about the endocrine mode of action.

**Sieratowicz et al. 2011**: This study determines the effects of 3-benzylidene camphor on the green alga *Desmodesmus subspicatus* and the crustacean *Daphnia magna*. Exposure to 3-benzylidene camphor resulted in growth inhibition of *D. subspicatus* with a 72 h IC10 value of 0.27 mg/L. The EC50 concentration in the acute immobilisation test with *D. magna* was 3.61 mg/L. Chronic exposure of *D. magna* (OECD guideline 211) caused a concentration dependent and significant decrease in the length of adults and the number of offspring with a NOEC of 0.1 mg/L and a LOEC of 0.2 mg/L. The study shows, that 3-benzylidene camphor affects reproduction in daphnia. This is an adverse apical effect but not endocrine specific.

**Weight of evidence for ED and Category**

Data with endocrine specific endpoint is missing, and 3-benzylidene camphor can therefore not be evaluated for endocrine disruption based on the present human data.

When evaluating the results from the *in vitro* assays and the *in vivo* toxicological studies related to human health, 3-benzylidene camphor fulfills the criteria of being an endocrine disrupter, as *in vivo* studies have found several adverse effects (irregular estrous cyclicity and impaired female sexual behaviour) that are compatible with the estrogenic mode of action found both in the *in vitro* studies and *in vivo* in the Uterotrophic assay. These findings lead to a categorization in Category 1.

Based on the ecotoxicological studies, 3-benzylidene camphor shows an estrogenic mode of action, as it induces vitellogenin in fish (OECD CF level 3). In addition to vitellogenin induction, one study also shows significant effects of 3-benzylidene camphor on the reproduction of fish. This is an adverse apical effect. Based on *in vivo* ecotoxicity data, 3-benzylidene camphor is categorized as an ED (Category 1) because of the combination of adverse apical effects and endocrine mode of action specific test results.

Based on the combined evidence from the ecotoxicological, *in vitro*, *in vivo* and epidemiological studies, 3-benzylidene camphor is evaluated as an endocrine disrupter in **Category 1**.

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which effects are observed, i.e. effects need to be observed at an oral dose of 10 mg/kg/day. For 3-BC, the LOAEL is 2.4 mg/kg or lower based on the reproductive toxicity studies

performed by Schlumpf and co-workers. Therefore 3-BC is likely to be classified as an endocrine disrupter of very high regulatory concern according to DE-UK potency criteria.

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**4-methylbenzylidene camphor, CAS 36861-47-9**
**Synonyms:** 3-(4-methylbenzylidene) camphor, 4-MBC

**Human data**
The effects of 4-MBC exposure in humans has been investigated in one study, and reported in two papers. It was shown that the compound permeated rapidly in human systemic circulation and reached maximum concentration 3-4 hours after dermal application. The study did not find any biologically significant effect on reproductive hormones after dermal application of a mixture of benzophenone-3, 4-methylbenzylidene camphor and 2-ethylhexyl 4-methoxy cinnamate mixture, but indicated a slight reducing effect on testosterone in males (Janjua et al 2004). The hypothalamic-pituitary-thyroid axis in humans did not show any biologically significant effects either, but results indicated a slight effect on thyroid hormones (Janjua et al 2007). However, results of this study are inconclusive due to too short duration of the experiment.

Detailed study summaries are provided below:
**Janjua et al 2004**: An experimental single blinded Danish study on Caucasian volunteers: 15 males and 17 postmenopausal females, examined effect of sunscreen mixture on reproductive hormones levels in humans. A control cream was applied daily for 4 days during the 1. week. A sunscreen formulation with 10% of benzophenone-3, 10% of 3-(4-methyl-benzylidene) camphor and with 10% of 2-ethylhexyl 4-methoxy cinnamate was applied daily for 4 days during the 2. week. Both creams were applied in recommended amount: 2 mg/cm$^2$ by staff. Maximum plasma concentration of 3-(4-methyl-benzylidene) camphor was reached 3-4 hours after application and was 20 ng/ml in both sexes. No significant alterations were seen in FSH, SHBG or LH in any sexes. Estradiol in females was unaltered between first and second week. Significant increase in Inhibin B in males and in testosterone in females was seen already at time 0 in the second week. Estradiol in males decreased significantly 2 and 4 hours, but not 3 hours after application of sunscreen formulation. Testosterone in males decreased significantly 4 hours after application of sunscreen formulation which is in a line with time for maximal plasma concentrations. No hormone levels during the first day later than 4 hours are available.
**Janjua et al. 2007**: An experimental single blinded Danish study on Caucasian volunteers: 15 males and 17 postmenopausal females, examined effect of sunscreen mixture on the hypothalamic-pituitary-thyroid axis in humans. A control cream was applied daily for 4 days during the 1. week. A sunscreen formulation with 10% of benzophenone-3, 10% of 3-(4-methyl-benzylidene) camphor and with 10% of 2-ethylhexyl 4-methoxy cinnamate was applied daily for 4 days during the 2. week. Both creams were applied in recommended amount: 40±3 g for male and 35±3 g for female.No alterations in TSH were seen in any sexes. TBG and T4 in males were unaltered between first and second week. Significant decrease in FT3 in males was seen already at time 0 in the second week. FT3, FT4 and T3 in females decreased sporadically. T3 and FT4 in males and TBG and T4 in females decreased significantly 3-4 hours after dermal application, which is in a line with time for maximal plasma concentrations (Janjua et. al. 2004). No hormone levels during the first day later than 4 hours are available.

***In vitro* data**
Several *in vitro* studies with 4-MBC have been performed. The compound has been shown to act as an estrogen by altering gene transcription in (Heneweer et al 2005) and causing proliferation of MCF-7 cells (Tinwell et al 2002, Schlumpf et al 2001, Schlumpf et al 2004, Matsuomo et al 2005). It has been shown to bind to the ER (Minh et al 2008, Matsuomo et al 2005 Seidlowa-Vuttke et al 2006a, Gomez et al 2005, Schreurs et al 2005) and it has been reported to be a preferential ER-beţa ligand with limited ER-alfa

binding capacity *in vitro* (Mueller et al., 2003; Schlumpf et al. 2004a). In other studies no clear-cut difference in the effect of 4-MBC on ERalfa and ERbeta was evident (Schreurs et al., 2002). Morohoshi et al 2005 also found no ER binding in the two yeast receptor assay. No androgenic or antiandrogenic effect *in vitro* was seen in a study by Ma et al 2003, while presence of weak anti-androgenic activity and strong progesterone activity was seen by Schreurs et al. 2005. Furthermore, 4-MBC can also affect the thyroid system *in vitro*, by binding to the thyroid receptor (Hoffmann et al 2009). These results show that 4-MBC probably has multiples endocrine disrupting modes of action, including estrogenic action.

***In vivo*, human health**

4-MBC has been shown to exert estrogenic effects *in vivo* in the uterotrophic assay (Schlumpf et al. 2001, 2004a; Tinwell et al. 2002). However, when Ashby and coworkers tried to repeat their finding of estrogenic activity in the uterotrophic assay reported in Tinwell et al 2002, they were not able to do so, and concluded that the effects they had observed previously were due to low uterine weight in their previous control group. Increases in uterine weighs have also been observed after longer exposure scenarios, as three months of dosing of adult female rats caused significant increases in uterine weights (Seidlova-Wuttke et al 2006 a,b). The effects on uterus weight were not very marked but histopathological effects in uterus and vagina were observed, and the 4MBC dosing also decreased $T_4$ levels and increased TSH and LH in serum. Schmutzler et al 2004 showed that 4-MBC could also affect the thyroid system as T4 levels were decreased and TSH levels increased after 3 months of dosing. Carou et al 2008 treated adult male rats with sc injections of 4MBC at low doses (2-20 mg/kg/day) and found that significant decreases in the LH and FSH serum concentration, and decreases in hypothalamic GnRH release. They also performed developmental studies where doses of 100 mg/kg/day caused changes in LH, FSH and GnRH levels and earlier vaginal opening in females.
A number of studies examining the developmental toxicity of 4-MBC have also been performed by Schlumpf and coworkers. Different parts of the studies have been reported in different publications (Schlumpf *et al.* 2004, Durer et al 2005, Maerkel et al 2005, Maerkel et al 2007, Durrer et al 2007, Hofkamp et al 2008, Schlumpf *et al.* 2008, Schlumpf *et al.* 2008, Faass et al 2009), making it difficult to evaluate exactly how many studies have been performed and when which doses and endpoints have been investigated. The effects of perinatal 4-MBC seen in these studies have in male offspring included alterations in reproductive organ weight at birth, on day 14 and in adulthood, delayed sexual maturation, and altered gene expression in prostate and brain, while effects observed in females include increased uterus weights, changes in gene expression of estrogen regulated genes in brain and uterus, as well as strongly impaired sexual behaviour. The LOAEL for most of these effects was 7 mg/kg/day, and the NOAEL 0.7 mg/kg/day.

Detailed study summaries of the *in vivo* studies are provided below:

**Schlumpf *et al.* 2001**: Two uterotrophic assays were performed. In one study, immature 21-day old LE rats (n=4-19) were dosed with 66, 119, 211, 337 or 402 mg/kg/day in the feed for four days. In the other study 4MBC was dissolved in olive oil, and applied dermally to the skin of hairless rats for 6 days (n=4-11). In the feeding study a significant increase in uterine weight was seen from 119 mg/kg/day and above and the ED50 was 309 mg/kg/day, while a dermally applied dose of which was calculated to correspond to 37 mg/kg/day , significantly increase in uterine weight in the other study.
**Tinwell et al 2002:** Two uterotrophic assays in immature Wistar rats were performed. 20 day old rats (n=12) were either dosed orally with 500 or 800 mg/kg/day for three days or subcutaneously using doses of 500 or 1000 mg/kg/day for three days. Increased uterine weight was apparent in both studies at both dose levels, making 500 mg/kg/day a LOAEL while no NOAEL was found.

**Ashby et al 2004:** An attempt to repeat their previously reported uterotrophic effects of 4MBC, (reported in the paper by Tinwell et al 2002), failed. Further evaluation led the authors to conclude that 4MBC is uterotrophic only when the control uterine weights are at the low end of their normally encountered range.
**Schmutzler *et al.* 2004**: Adult ovariectomized SD rats (n=8-11) were treated with 4MBC in the feed for 12 weeks at doses of approximately 260 and 1240 mg/kg/day (only listed as 66 and 310 mg/animal/day, and no BW), and endpoints relevant for regulation via the thyroid axis were measured. At both doses thyrotropin (TSH) levels were elevated and thyroxine (T4) serum levels were decreased. In the liver, type I 5-deiodinase was decreased by all treatments, whereas the enzyme thyroid peroxidase (TPO) was not inhibited in an *in vitro* test.
**Siedlova-Wuttke *et al.* 2006a**: Adult ovariectomized SD rats (n=11) were treated with 4MBC in the feed for 12 weeks at doses of 223 and 1023 mg/kg/day. Both doses caused decreased $T_4$ levels and increased TSH. Opposite the effect of E2, the highest dose further caused significant increased serum LH levels, 4MBC also caused reduced animal weight gain, and caused reductions in the size of fat depots and reductions in serum leptin.
**Siedlova-Wuttke *et al.* 2006b:** Adult ovariectomized SD rats (n=11) were treated with 4-MBC in the feed for 12 weeks at doses of approximately 223 and 1023 mg/kg/day. The high dose increased uterine weight and in the uterus and vagina both doses of 4MBC affected histopathology. In the bone, 4MBC shared the antiosteoporotic effects of E2 but the mechanism of action of 4MBC, appeared to be different than by E2
**Carou et al 2008**: Wistar male adult rats (n=10-12) were either injected sc with 4-MBC for 5 days (at doses of 2 or 10 mg/kg) or for 2 days (at doses of 2 and 20 mg/kg). In all rats serum prolactin, LH and FSH concentration were assayed. The hypothalamus's of rats injected during 2 days were also dissected to study GnRH release. Rats that received 2 and 10 mg/kg of 4-MBC for 5 days showed a decrease in the LH and FSH serum concentration. In rats injected for 2 days, serum LH decreased with 2 and 20 mg/kg and FSH decreased with 2 mg/kg. *In vitro* hypothalamic GnRH release also decreased in these animals. These results show that low doses of 4-MBC may inhibit the reproductive axis in adult male rats.
**Carou et al 2009a:** 4-MBC was administered (sc) to female rats from pregnancy onset in doses of 20, 100 and 500 mg/kg/ day. The litters were sacrificed at 15 or 30 days old to determine testicular weight, gonadotropin and prolactin serum levels and also GnRH and amino acids release from the hypothalamus. The exposure to 20 mg/kg/day only increased the LH serum levels in 30-day-old males. Doses of 100 and 500 mg/kg/ day caused a decrease in testicular weight and in LH, GnRH and glutamate levels, in prepubertal rats (15-day-old specimens), and an increase in, gonadotropin (LH and FSH) concentration and aspartate levels in peripubertal rats (30-day-old specimens), without changes in testicular weight. The results show that administration of4-MBC during development may affect the endocrine system in male rats during the prepubertal and pubertal stages. The study however has some shortcomings, as the number of animals used is stated as n= 8-13, but it is impossible to evaluate how many litters these offspring come from, and furthermore is seems that litter effects have not been included in the statistical analysis of the data. However, the paper by Carou et al 2009b focuses on a group that received 4MBC at a dose of 100 mg/kg/day and if these are the same animals as used in the present study, then only one pup per litter has been used.
**Carou et al 2009b**. The 4MBC was administered (sc) to female rats (n=10) during pregnancy, in doses of 100mg/kg every other day. Timing of vaginal opening was investigated in the female offspring and the litters were sacrificed at 70 days to determine gonadotrophin serum levels and also GnRH release from the hypothalamus. The male rats showed a decrease in serum LH and FSH concentration in adulthood, and also in GnRH secretion. The female rats showed an increase in serum LH and FSH concentrations, whereas hypothalamic GnRH release was not modified. All these changes were accompanied by an advance (3 days) on the vaginal opening in 4MBC rats group, but no change in adult uterine weight. In conclusion, prenatal administration of 4MBC disrupted the gonadal axis in a sexual dimorphic mode that could be connected with

the physiological sexual differences in the development of gonadotrophin secretion hypothalamic control mechanisms.

A number of developmental studies of 4MBC from the same research group have been reported in the following publications **(Schlumpf *et al.* 2004, Durer et al 2005, Maerkel et al 2005, Maerkel et al 2007, Durrer et al 2007, Hofkamp et al 2008, Schlumpf *et al.* 2008, Schlumpf *et al.* 2008, Faass et al 2009)**. It is difficult to evaluate exactly how many studies have been performed, when which doses and endpoints have been investigated and when data from different studies has been investigated separately or together. However, the following experimental setup was used in each case. Male and female LE rats were dosed with the compound by adding it to the feed. The parental generation was exposed for 10 weeks before mating, exposure of dams continued throughout gestation and lactation, and the offspring were further dosed until adulthood. The following doses have been investigated: 0.7, 7, 24 and 47 mg/kg/day. A dose of 70 mg/kg was also used initially, but was discontinued because it was too high for the offspring and resulted in reduced postnatal survival. Unfortunately, the number of litters used in the studies, has not been stated in some of the publications which report many of the important results used for evaluating endocrine disrupting effects (Schlumpf et al 2004, 2008a), but in the papers that do include this information the number of litters in each dose group was between 5-8 (Maerkel et al 2007, Faass et al 2009) and in some publications data from more studies has been analyzed together yielding litter numbers between 12-17 (Durrer et al 2007). The dose of 7 mg/kg/day caused proliferative effect on prostate growth. On PND 1 an increase of 70% in accessory sex glands and prostate volume was seen, caused by increasing number and volume of ducts in the prostate (Hofkamp et al 2008). On PND 14 decreased relative testes weight was seen from the dose of 7 mg/kg and higher (Schlumpf et al 2008b), and the same was true for delayed start of puberty in males (Durrer et al 2007). Body weight at puberty was normal in males, indicating that the delay of puberty did not result from nutritional effects (Durrer et al 2007, Schlumpf et al 2008b). In adulthood, males from this group had decreased prostate weights and altered gene expression in the prostate (Durrer et al. 2007, Schlumpf et al. 2008) and in the hypothalamus (Fass et al 2009), while adult females showed increased uterus weights (Schlumpf et al 2008b) and changes in gene expression of estrogen regulated genes in brain (Fass et al 2009) and uterus (Durrer et al 2005). Sexual behaviour of female offspring exposed to 7 and 24 mg/kg was also strongly impaired as a decrease in both proceptive and receptive behavior was observed (Faass et al 2009) At the dose of 24 mg/kg/day effects were also seen on litter size and survival rate, decreased thymus and increased thyroid weights, altered levels of TSH and T3 (Maerkel et al 2007), whereas the highest dose of 47 mg/kg/day further caused increased testes weights in adulthood (Schlumpf et al 2004, 2008b). Furthermore, 4-MBC caused alterations in gene expression in sexually dimorphic areas of the brain in all dose levels (Maerkel et al 2005, Maerkel et al 2007, Schlumpf et al 2008, Fass et al 2009). Timing of sexual maturation of the female offspring was not affected by any dose of 4-MBC, nor was oestrous cyclicity (Faass et al 2009, Schlumpf et al 2008)).

***In vivo*, ecotoxicity**

4-methylbenzylidene camphor at high concentrations induces estrogen-responsive gene products including vitellogenin in fish. This demonstrates an estrogenic mode of action (although studies with lower concentrations show no estrogenic actions). 4-methylbenzylidene camphor affects reproduction in invertebrates, which is an adverse apical effect. However, general toxic effects cannot be excluded since growth inhibition and mortality occurred.

Detailed study summaries are provided below:

**Schreurs et al. 2002**: Zebrafish, in which an estrogen responsive luciferase reporter gene has been stably introduced, were used for *in vivo* testing of UV-filters. 4-methylbenzylidene camphor showed no estrogenic activity in this transgenic zebrafish assay at the tested concentration: 1 µM (254 µg/L).
**Inui et al. 2003**: In this study, the estrogenicity of 4-methylbenzylidene camphor was examined using male medaka (*Oryzias latipes*) in regard to production of vitellogenin (VTG) and choriogenin (CHG) which are known to be estrogen-responsive gene products. Exposure concentrations were: 0.039, 0.39 and 3.9 mM. Exposure duration was 7 days. An increase in vitellogenin plasma concentrations was observed. Increase in mRNA expression levels of vitellogenin subtypes VTG-1 and VTG-2 and choriogenin subtypes CHG-L and CHG-H in liver due to exposure to 4-methylbenzylidene camphor was also seen. In addition, increased mRNA expression levels of estrogen receptor (ER) α in the liver due to exposure to 4-methylbenzylidene camphor were also found. LOEC in this study was 0.039 mM (9.9 mg/L).
This study shows that 4-methylbenzylidene camphor has estrogenic activity in fish - and thus gives information about the endocrine mode of action. The observed effects are not adverse apical effects.
**Kunz et al. 2004:** The study investigates whether 4-methylbenzylidene camphor interferes with the thyroid and sex hormone system during frog metamorphosis. *Xenopus laevis* tadpoles were exposed to 1, 5 and 50 µg/L of 4-methylbenzylidene camphor for 35 days (NF stage 52–66).
The rate of metamorphosis was not affected, and no obvious differences in body and tail length compared to controls were observed. 4-methylbenzylidene camphor exposure did not lead to effects on the sex ratio or gross gonad morphology of *X. laevis* at stage 66.
The results indicate that 4-methylbenzylidene camphor do not negatively affect the thyroid system and sex ratio of frogs at the tested concentrations. The tested concentrations are, however, lower than the LOEC for vitellogenin induction in fish.
**Kunz et al. 2006**: In this study it was analyzed whether 4-methylbenzylidene camphor shows estrogenic activity in fathead minnows by the induction potential of vitellogenin after 14 days of aqueous exposure. Fish were exposed to four different concentrations of 4-methylbenzylidene camphor: 9 (median measured), 100 (nominal), 415 (median measured) or 753µg/L (median measured). No significant differences were found between exposed and control groups.
**Schmitt et al. 2008**: *In vivo* sediment tests with the mudsnail *Potamopyrgus antipodarum* and the blackworm *Lumbriculus variegatus* showed that 4-methylbenzylidene camphor increased embryo production in the snail (NOEC: 0.26 mg/kg dry weight; LOEC: 1.71 mg/kg dry weight) but decreased the reproduction of *L. variegatus* (NOEC: 1.47 mg/kg dry weight; LOEC: 6.18 mg/kg dry weight). The study shows that 4-methylbenzylidene camphor affects reproduction in the mudsnail and the blackworm. This is an apical effect. At the highest concentration tested, however, 4-methylbenzylidene camphor caused mortality.
**Fent et al. 2010**: This study reports on acute and chronic effects of 4-methylbenzylidene camphor on *Daphnia magna*. In the acute toxicity test (OECD guideline 202), the LC50 value (48 h) was 0.56 mg/L. The chronic toxicity of 4-methylbenzylidene camphor was determined in a 21 d reproduction study performed according to OECD guideline 211. At the highest concentration of 4-methylbenzylidene camphor, 50 µg/L, reproduction and body length were reduced. Apparently no adverse effect on the sex of the offspring was observed.
The study shows effects of 4-methylbenzylidene camphor on the reproduction of daphnia at the highest concentration tested. However, it cannot be ruled out that this is a toxic effect not related to endocrine disruption since the growth was also reduced and apparently no effects were seen on the sex ratio of the offspring.
**Sieratowicz et al. 2011**: This study determines the effects of 4-methylbenzylidene camphor on the green alga *Desmodesmus subspicatus* and the crustacean *Daphnia magna*. Exposure to 4-methylbenzylidene camphor resulted in growth inhibition of *D. subspicatus* with a 72 h IC10 value of 0.21 mg/L. The EC50

concentration in the acute immobilisation test with *D. magna* was 0.80 mg/L. Chronic exposure of *D. magna* (OECD guideline 211) caused a concentration dependent and significant decrease in the length of adults with a NOEC of 0.1 mg/L and a LOEC of 0.2 mg/L. No significant effects were seen on the number of offspring. The study provides data on the growth inhibiting and toxic concentrations of 4-methylbenzylidene camphor towards green alga and daphnia.

**Weight of evidence for ED and Category**

The present epidemiological studies do not indicate biologically significant effect on levels of reproductive hormones or on the hypothalamic-pituitary-thyroid axis in humans following dermal treatment with a mixture of three UV-filters, including 4-MBC. The present studies are however limited in their duration and do not provide any information about the effect of dermal treatment with 4-methylbenzylidene camphor alone. The results are therefore inconclusive.

When evaluating the combined results from the *in vitro* and *in vivo* data, 4-MBC can be grouped as an endocrine disrupter in Category 1. The evidence of estrogenic activity from the short term *in vivo* studies with adult animals is conflicting, however the large number of endocrine sensitive endpoints that have been shown to be affected in the developmental studies, together with *in vitro* data showing a plausible endocrine disrupting mode of actions, makes 4-MBC fulfill the criteria of being an endocrine disrupter in Category 1.

Based on ecotoxicological studies, 4-MBC induces estrogen-responsive gene products - including vitellogenin - in fish, at high concentrations. This demonstrates an estrogenic mode of action (although studies with lower concentrations show no estrogenic actions). Furthermore, 4-MBC affects reproduction in invertebrates, which is an adverse apical effect. However, general toxic effects cannot be excluded since growth inhibition and mortality did occur. Based on *in vivo* ecotoxicity data, 4-methylbenzylidene camphor is categorized as a suspected ED (Category 2a).

Based on the combined evidence from *in vivo*, *in vitro*, ecotoxicology and epidemiological studies, 4-MBC is evaluated as an ED as **Category 1**.

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern, is based on the dose level at which effects are observed, i.e effects need to be observed at an oral dose of 10 mg/kg/day. For 4-MBC, developmental effects which were probably caused by endocrine disruption, were seen at a dose of 7 mg/kg/day, and 4-MBC can therefore also be considered an endocrine disrupter of very high regulatory concern according to DE-UK potency criteria.

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**4-nitrophenol, CAS 100-02-7**
**Synonyms:** p-nitrophenol, PNP

**Human data**
No relevant data found.

***In vitro* data**
The endocrine disrupting properties of 4-nitrophenol *in vitro* have been investigated in some test systems. 4-nitrophenol has in a human estrogen receptor (hER)-yeast screen assay exhibited estrogenic activity and anti-androgenicity in a human androgen receptor (hAR)-yeast screen assay (Taneda et al 2004). Overall this study indicates that 4-nitrophenol has modes of action that are compatible with both an estrogenic and an anti-androgenic mode of action.

***In vivo*, human health**
Overall, *in vivo* studies with 4-nitrophenol in rats have shown evidence for endocrine disrupting effects, e.g. increase in uterus weight, decrease in weights of male reproductive organs (Uterotrophic and Hershberger respectively) and changed hormone levels in immature male rats.
4-nitrophenol has been shown to affect uterus weight (weight increased) in one published study performing the uterotrophic assay and weights of reproductive organs were decreased in the Hershberger assay (Li et al 2006), indicating that the compound possesses estrogenic and anti-androgenic activity *in vivo*. The LOAEL for the study was between 0.01 and 10 mg/kg/day; while NOAELs were found at 1 mg/kg/day (no NOAEL in the Hershberger study was found). Study descriptions of this study are summarized below.
In a short term study PNP has impaired reproductive function in immature male rats by disturbing the hypothalamic–pituitary–testicular axis. The present findings demonstrate that PNP has an endocrine-disruptive effect on male reproductive function in this model (Li et al 2009).

Study descriptions of the *in vivo* studies:
***Li et al (2006)*** performed an uterotrophic assay in ovariectomised 25 day old immature female Wistar-Imamichi rats, which were subcutaneously injected with 1, 10, 100 mg/kg PNP for seven days. They found increased uterine weight at 10 and 100 mg/kg, giving a LOAEL of 10mg/kg/day and a NOAEL of1mg/kg/day.
In Hershberger castrated male assay the rats were exposed to 0.01, 0.1, 1 mg/kg for 5 days (with testosterone). At all doses the ventral prostate weight were decreased. At 0.1 mg/kg, seminal vesicle plus coagulating gland weights decreased. At 0.01 and 0.1 mg/kg levator ani-bulbocavernosus muscle weight decreased and at 0.1 and 1.0 mg/kg glans penis weight was decreased. Males showed increased FSH and LH with inverted-U dose responses.
***Li et al (2009)*** performed a study where 28-day-old male rats were injected s.c. to the doses; 0.01, 0.1, 1.0, 10. At 0.01, 0.1, 1.0, 10 mg/kg LH decreased and corticosterone increased while at 0.1, 1.0, 10 mg/kg FSH decreased and inhibin increased. Moreover, at 10 mg/kg both testosterone and prolactin increased. 4-Nitrophenol treatment had no effect on the normal growth of rats, nor did it cause differences in the weights of the testes or the accessory reproductive organs, even though testosterone levels increased.

***In vivo*, ecotoxicity**
No relevant data found.

**Weight of evidence for ED and Category**

When evaluating the combined results from the *in vitro* and *in vivo* data on human health, there are arguments both for ED Category 1 and ED Category 2a. The *in vitro* study have evaluated endocrine disrupting properties showing clear sign of both estrogenic and an anti-androgenic mode of action. *In vivo* studies in rats have shown evidence for endocrine disrupting effects, e.g. increase in uterus weight, decreases in weights of reproductive organs (Uterotrophic and Hershberger assay, respectively) and changed hormone levels in immature male rats. No studies have investigated adverse *in vivo* effects and thus 4-nitrophenol is evaluated as a suspected ED in **Category 2a**.

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which adverse effects are observed, i.e. effects need to be observed at an oral dose of 10 mg/kg/day. For 4-nitrophenol, effects on reproductive organ weights in the Uterotrophic assay and the Hershberger assay were seen at lower or exactly at 10 mg/kg. In the study with immature male rats changes in hormone levels were seen from the lowest dose observed: 0.01mg/kg. However, these effects may not be considered as adverse and thus it is unclear whether 4-nitrophenol will be considered as an endocrine disrupter of very high regulatory concern according to DE-UK criteria.

**References, human data**

No references

**References, *in vitro* and *in vivo***

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**4,4´-dihydroxybenzophenone, CA S 611-99-4**
Synonyms: 4DHB

**Human**
No relevant data found.

***In vitro***
The endocrine disrupting properties of 4,4'-dihyroxybenzophenone have been investigated in several *in vitro* tests systems. The compound has been shown to cause proliferation of MCF-7 cells (Suzuki et al 2005), it has been shown to display estrogenic activity in both the yeast two-hybrid system, a fluorescence polarization system and in the E-screen (Hashimoto et al 2001) and to bind to the estrogen receptor in several other *in vitro* studies (Yamasaki et al 2003, Blair et al 2000, Kawamura et al 2005, Kunz & Fent 2006), indicating estrogenic activity. Furthermore some studies have also shown antagonism of the androgen receptor *in vitro* (Kawamura et al 2005, Kunz& Fent et al 2006, Suzuki et al 2005). Overall these studies show strong evidence that 4,4'-dihyroxybenbophenone has estrogenic and possibly also anti-androgenic mode of action *in vitro*.
Dihydrobenzophenone has furthermore been shown to interact with the mitotic spindle during metaphase of cell division, and may thereby possibly be able to affect reproductive success (Pfeiffer et al 1997). This effect has however not been included in the evaluation of the endocrine disrupting effects of 4,4-dihydroxybenzophenone.

***In vivo*, human health**
An Uterotrophic assay was performed by Yamasaki et al 2003. Immature 19-day-old SD rats (n=6) were subcutaneously injected on 3 consecutive days with 8, 40 or 200 mg/kg/day. At 200 mg/kg/day a significant increase in the uterine blotted weight was seen, indicating estrogenic effect. In a Hershberger assay doses of 50, 200 and 600 mg/kg /day were administered to male rats for 10 days, but no dose-dependent effects on male reproductive organs were seen (Yamasaki et al 2003).

***In vivo*, ecotoxicity**
Kunz et al. 2006 exposed fathead minnows to 4,4'-dihydroxybenzophenone for 14 days at the following concentrations: 10 µg/L (median measured), 100 µg/L (nominal), 500 µg/L (nominal), 900 µg/L (median measured) or 5011 µg/L (median measured).4,4'-dihydroxybenzophenone did not result in significant vitellogenin induction in the fish.

**Weight of evidence for ED and Category**
Only one *in vivo* study has been performed, however here a significant increase in uterine weight was seen at 200 mg/kg in an uterotrophic assay using immature rats. Furthermore, there is strong evidence of estrogenic mode of action *in vitro* for dihydroxybenzophenone. Structurally the compound is similar to the other benzophenones, some of which have been evaluated in this report as endocrine disrupters in Category 1. The compound could therefore in principle fulfill the criteria of being an endocrine disrupter. However, the single *in vivo* assay does not give clear evidence for adverse effects and therefore the substance is evaluated as Category 2a (suspected ED).

Based on *in vivo* ecotoxicity data from one study, 4,4'-dihydroxybenzophenone there is no evidence for endocrine disrupting properties.

Based on the combined evidence from all studies 4,4'-dihydroxy-benzophenone is evaluated as a suspected ED in **Category 2a.**

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which effects are observed, i.e. effects need to be observed at an oral dose of 10 mg/kg/day. For 4,4-dihydroxybenzophenone, the effect on uterine weight seen in the uterotrophic assay was at 200 mg/kg, and BP-1 cannot be considered an endocrine disrupter of very high regulatory concern according to DE-UK potency criteria.

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**Benzophenone-1, CAS 131-56-6**
**Synonyms:** BP-1, 2,4-dihydroxybenzophenone, resbenzophenone

**Human data**
No relevant data found.

***In vitro* data**
The endocrine disrupting properties of BP-1 *in vitro* have been investigated in a number of test systems. BP-1 has been shown to act as an estrogen by altering gene transcription (Heneweer et al 2005) and causing proliferation of MCF-7 cells (Nakagawa et al 2002, Suzuki et al 2005, Schlumpf et al 2004, Matsuomo et al 2005), by showing estrogenic activity in the yeast two-hybrid assay (Kawamura et al 2003, Takatori et al 2003) and by binding to the estrogen receptor (Molina-Molina et al 2008, Nakagawa et al 2002,Kunz et al 2006, Yamasaki et al 2004, Blair et al 2000, Matsuomo et al 2005). Furthermore some studies have also shown antagonism of the androgen receptor *in vitro* (Molina-Molina et al 2008, Suzuki et al 2005, Nashev et al 2010), while others have not seen this anti-androgenic effect (Kawamura et al 2005). Overall the *in vitro* results show strong evidence that BP-1 has an estrogenic mode of action, while *in vitro* data for anti-androgenicity are conflicting.

***In vivo*, human health**
Benzophenone-1 has been shown to increase uterine weight in several uterothrophic assays in rats, indicating an estrogenic effect *in vivo*. In a study by Schlumpf et al (2004) the chemical was administered for three days by oral gavage to immature female LE rats. The authors found increased uterine weight after three days of exposure. However, the investigated dose levels were not reported. In a study by Yamasaki et al 2004, immature female SD rats were treated for three days with subcutaneous injections of BP1, and here all three investigated doses (100, 300 and 1000 mg/kg/day) caused an increase in uterine weight, giving a LOAEL of 100 and no NOAEL. Suzuki et al (2005) treated ovariectomized 6 week old female F344 rats with intraperitoneal doses of BP1 for three days and found that doses of 500 mg/kg resulted in significantly increased uterine weights, while the increase seen at a dose of 100 mg/kg/day was not statistically significant, and was therefore the NOAEL. Finally Koda et al (2005) investigated the effects of BP1 in ovariectomized 13 week old female SD rats injected subcutaneously for three days, and reported increased relative uterine weight at 625 mg/kg/day with a NOAEL of 250 mg/kg/day. In summary, LOAELs between 100-625 mg/kg and NOAELs between 100-250 mg/kg have been found for increased uterus weight in the uterus assay, with the differences probably illustrating the differences in animals strain and dosing scheme being used. No developmental toxicity studies with benzophenone-1 have been found in the open literature.

***In vivo*, ecotoxicity**
Two ecotoxicology studies have shown that benzophenone-1 induces vitellogenin in fish. Kunz et al. (2006) have shown that when fathead minnows were exposed to benzophenone-1 for 14 days at five different aqueous exposure concentrations: 9 µg/L (actual), 100 µg/L (nominal), 500 µg/L (nominal), 981 µg/L (actual) or 4919 µg/L (actual), vitellogenin was induced with a LOEC of 4919 µg/L. Kunz and Fent (2009) exposed juvenile fathead minnows to actual concentrations of 95, 593, 1202, 2668, 3666 or 5384 µg/L benzophenone-1 for 14 days to determine vitellogenin induction. Significant vitellogenin-induction occurred at 2668 µg/L.

**Weight of evidence for ED and Category**

*In vivo* studies in both immature and adult ovariectomized animals have shown effects, e.g. increased uterus weight, demonstrating ED mode of action, and *in vitro* data show estrogenic activity. However, the fact that all of the studies are screening studies without endpoints for clearly adverse effects, indicates that the evidence from the experimental animals is not sufficiently convincing to place the substance in Category 1, but rather in a Category 2a (suspected ED).

The two ecotoxicology studies showed that benzophenone-1 induces vitellogenin in fish. Induction of vitellogenin is not an adverse apical effect but a biomarker of estrogenic exposure and thus provides information about the endocrine mode of action. Based on *in vivo* ecotoxicity data, benzophenone-1 is categorized as a suspected ED (Category 2a).

Based on the combined evidence from the ecotoxicological studies, the *in vitro* and the *in vivo* studies, benzophenone-1, is evaluated as a suspected endocrine disrupter in **Category 2a.**

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which adverse effects are observed, i.e. adverse effects need to be observed at an oral dose of 10 mg/kg/day. For BP-1, effects on uterine weight in the Uterotrophic assay may not be considered as adverse and the effects were seen at 100-625 mg/kg. Consequently, BP-1 cannot be considered an endocrine disrupter of very high regulatory concern according to DE-UK potency criteria.

**References *in vitro* and *in vivo***

Blair,R.M., Fang,H., Branham,W.S., Hass,B.S., Dial,S.L., Moland,C.L., Tong,W.D., Shi,L.M., Perkins,R., and Sheehan,D.M., 2000. The estrogen receptor relative binding affinities of 188 natural and xenochemicals: Structural diversity of ligands. Toxicological Sciences 54, 138-153.

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**References ecotoxicology**

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**Benzophenone-2, CAS 131-55-5**
**Synonyms:** BP-2, 2,2',4,4'-tetrahydroxybenzophenone

**Human data**
No relevant data found.

***In vitro* data**
The endocrine disrupting properties of BP-2 *in vitro* have been investigated in a number of test systems. BP-2 has been shown to cause proliferation of MCF-7 cells (Suzuki et al 2005, Schlumpf et al 2004, Matsuomo et al 2005), and to bind to the estrogen receptor (Kawamura et al 2005, Schlecht et al 2004, Morohoshi et al 2005, Molina-Molina et al 2008, Kunz & Fent 2006, Yamasaki et al 2003a, Matsuomo et al 2005 Seidlowa-Vuttke et al 2004, 2005), showing strong evidence of estrogenic activity. Furthermore some studies have also shown antagonism of the androgen receptor *in vitro* (Kawamura et al 2005, Molina-Molina et al 2008). Overall these studies show that BP-2 has estrogenic and possibly also an anti-androgenic mode of action *in vitro*. Furthermore, BP2 can also affect the thyroid system *in vitro*, by inhibiting the enzyme thyroid peroxidase (TPO) (Schmutzler et al 2007), by binding to the thyroid receptor (Hoffmann et al 2009) and by affecting thyroid hormone signalling in the testes (Kim et al 2011).

***In vivo*, human health**
BP-2 has been shown to affect uterus weight in all published studies performing the uterotrophic assay (Schlumpf et al 2004, Yamasaki et al 2003a, Koda et al 2005, Seidlova-Wuttke et al 2004, Jarry et al 2005, Schlecht et al 2004, Schlecht et al 2006), giving strong evidence for estrogenic activity *in vivo*. The LOAEL for all studies was between 100 and 250 mg/kg/day irrespective of rat strain and study design, while NOAELs were found at values between 33 and 40 mg/kg/day. Study descriptions of each of the studies showing increased uterine weight are summarized on the next page. No significant effects on male reproductive organs have been seen in the Hershberger assay (Yamasaki et al 2003b).
Only one developmental study of BP-2 has been described in the open literature. It was performed in mice (n=16-20), and showed that in utero exposure to 6.25 mg BP2 /kg/day by oral gavage to pregnant dams from GD12-17, significantly increased incidence of hypospadias in male foetuses, when investigated on GD18 (Hsieh et al 2007). 8 male foetuses (from 6 of the 20 litters present in this dose group) showed hypospadias, while no offspring in the control group showed any genital malformations. Furthermore, the study included a group of mice that were treated with BP-2 together with the estrogen receptor antagonist (EM-800) (n=7) and a group that only received EM-800 (n=5) and in both groups, no hypospadias were observed. As male genital tubercles had significantly higher levels of ER-beta than controls, the authors suggested that the increased incidence of genital malformations was mediated through an estrogen receptor dependent mechanism. No effect on anogenital distance in the male offspring was seen, indicating that the effect was not mediated by an anti-androgenic mechanism. Only one dose levels was investigated in the present study, why no dose-response relationship was established. The LOAEL for this study was 6.25 mg/kg/day.
BP2 can also affect the thyroid hormone system *in vivo*, which was shown in the study by Jarry et al (2004). Here five days of BP-2 dosing resulted in significant decreases in T4 at both tested dose levels (250 and 1000 mg/kg/day) and a decrease in T3 was seen at 1000 mg/kg. Similarly Seidlova-Wuttke et al (2005) showed that a dose of 677 mg/kg for three months mixed in the feed of adult ovariectomised rat decreased T3 and T4 levels, with a NOAEL of 153 mg/kg. In 2007 Schmutzler et al further showed a decrease in T4 and elevated TSH levels in 2 months old ovariectomised SD rat (n=12) exposed to BP2 by oral gavage for 5 days. The NOAEL for the thyroid effects *in vivo* was 100 mg/kg/day, while significant effects on T4 and TSH levels

were seen at 333 mg/kg and above. In summary, NOAELs for effects on the thyroid hormone system were between 100-153 mg/kg/day, while LOAELs were between 250-677 mg/kg/day.

Study descriptions of each of the studies showing increased uterine weight:
**Schlumpf et al (2004)** administered BP2 by oral gavage to immature female LE rats for three days. The authors found increased uterine weight after three days of exposure, however, the investigated dose levels were not reported.
**Yamasaki et al (2003a)** dosed immature female SD rats for three days with subcutaneous injections of BP2, and here the two highest doses (200 and 800 mg/kg/day) caused an increase in uterine weight, while a NOAEL of 40 mg/kg/day was seen.
**Koda et al (2005)** performed an uterotrophic assay in ovariectomised 13 week old female SD rats, which were subcutaneously injected with BP-2 for three days. They found increased relative uterine weight at all three tested doses (100, 250 and 625 mg/kg/day), giving a LOAEL of 100mg/kg/day and no NOAEL.
**Seidlova-Wuttke et al (2004)** showed that dosing of ovariectomised female SD rats (n=12) with BP-2 at doses of 185 or 925 mg/kg/day for three months in the feed, caused increased uterus weight, histological changes in the uterus, vaginal cornification and altered expression of estrogen receptor in the vagina at both dose levels. All effects were comparable to the effects of their positive control, estradiol benzoate, indicating clear estrogenic effects of BP2. The LOAEL was 185 mg/kg/day, while no NOAEL was found.
**Jarry et al (2005)** dosed two months old ovariectomised female SD rats (n=11) with BP-2 at dose levels of 250 or 1000 mg/kg/day by gavage. After five days the rats had increased uterine weights at both dose levels, again yielding no NOAEL whereas the LOAEL was 250 mg/kg/day. In the highest dose group a significant decreased in serum LH levels was also seen. This finding was seen again in a later publication from the same group, which showed that dosing of adult ovariectomised female SD rats with BP-2 at doses of 153 or 677 mg /kg/ day in the feed for three months, lead to significantly decreased LH levels, compared to controls (Seidlova-Wuttke et al 2005).
**Schlecht et al (2004**) used ovariectomised adult female SD rats, and showed increased uterine weight and altered gene-expression of estrogen receptors in the uterus after 5 days of gavage dosing, with doses of 250 and 1000 mg/kg /day. Both doses gave results comparative to what was seen after a dose 0.6 mg/kg/ day of 17-beta-estrdiol. Again no NOAEL was found, while the LOEAL in this study was 250 mg/kg.
**Schlecht et al (2006)** investigated five doses of BP2 using the same study design (n=12), and again found increased uterine weights and altered gene expression in the uterus, at doses of 100 mg/kg/day and above, while the NOAEL for both endpoint was 33 mg/kg/day.

***In vivo*, ecotoxicity**
Four relevant ecotoxicological studies were found in the open literature. Studies by Kunz et al. (2006) and Kunz and Fent (2009) showed that benzophenone-2 induces vitellogenin in fish. Induction of vitellogenin is not an adverse apical effect but a biomarker of estrogenic exposure and thus provides information about the endocrine mode of action. Weisbrod et al. (2007) showed significant estrogenic effects of benzophenone-2 on vitellogenin induction, secondary sex characteristics, gonadal development, and reproduction in fish. The induction of vitellogenin again demonstrates an estrogenic mode of action. Cessation of spawning (and thereby reproduction) is an adverse apical effect. Thus this study showed both adverse apical effects of benzophenone-2 and demonstrated an endocrine mode of action. Thienpont et al. (2011) have furthermore shown that benzophenone-2 decreases intrafollicular T4-content in fish which provides information on the endocrine mode of action of benzophenone-2, and classifies BP-2 as a thyroid gland function disruptor in fish.

Study descriptions of each of the ecotoxicolocy studies are presented below:
**Kunz et al. 2006**: Fathead minnows (Pimephales promelas) were exposed to benzophenone-2 for 14 days at five different aqueous exposure concentrations: 10µg/L (actual), 100 µg/L (nominal), 500 µg/L (nominal), 1067 µg/L (actual) or 8783 µg/L (actual). Vitellogenin was induced with a LOEC of 8783 µg/L.
**Weisbrod et al. 2007**: In this study, it was evaluated whether benzophenone-2 affects important reproductive parameters such as fecundity, gametogenesis and secondary sex characteristics. Reproductively mature fathead minnows (Pimephales promelas) were exposed to 0.002, 0.1, 1.2, 5.0 and 9.7 mg/L benzophenone-2 for 15 days.
In males, a dose-dependent vitellogenin induction and decrease in the number of nuptial tubercles occurred. LOECs for both parameters were 1.2 mg/L benzophenone-2. Moreover, significant dose-related effects on gonads of male and female fish were observed. At concentrations of 1.2 mg/L and higher, spermatocyte and oocyte development was significantly inhibited in male and female fish, respectively. Testes of exposed males had much fewer spermatocytes; and ovaries of exposed females had much fewer mature and more atretic follicles. Reproduction was negatively affected in a dose-dependent manner with a decrease in egg production at 1.2 mg/L and a complete cessation of spawning activity at 5.0 and 9.7 mg/L benzophenone-2. Although the mortality of fish exposed to 5.0 and 9.7 mg/L benzophenone-2 was slightly, but not statistically significant, increased the authors assume that toxicity can be disregarded as the main reason, as no indication on the condition factor for a toxic effect at 9.7 mg/L was found in the females.
**Kunz and Fent 2009**: Juvenile fathead minnows were exposed to actual concentrations of 91, 483, 980, 2228, 4715 or 9356 µg/L benzophenone-2 for 14 days to determine vitellogenin induction.
Significant vitellogenin-induction occurred at 4715µg/L.
**Thienpont et al. 2011**: This study utilizes a T4 immunofluorescence quantitative disruption test (TIQDT) to measure impairment of the thyroid function as a decrease in the intrafollicular T4-content in zebrafish eleutheroembryos. Zebrafish eleutheroembryos were exposed to benzophenone-2 from 48 to 120 hpf and intrafollicular T4-content was measured using TIQDT.
Benzophenone-2 caused a significant decrease in intrafollicular T4-content and was thus classified as a thyroid gland function disruptor.

**Weight of evidence for ED and Category**
When evaluating the combined results from the *in vitro* and *in vivo* data on human health, benzophenone-2 fulfils the criteria of being an endocrine disrupter, as increased uterine weight has been seen in several *in vivo* studies and there is strong evidence of estrogenic activity *in vitro.* Also, adverse effects (genital malformations) likely to be mediated through an estrogen receptor dependent mechanism have been found in one study. Furthermore both *in vitro* and *in vivo* studies point to interference with thyroid function. These findings lead to ED in Category 1.

Based on *in vivo* ecotoxicity studies, benzophenone-2 induces vitellogenin in fish which shows an estrogenic mode of action. In addition to vitellogenin induction, one study also shows significant effects of benzophenone-2 on the reproduction of fish. This is an adverse apical effect. Moreover, one study demonstrates that benzophenone-2 decreases thyroid hormone content in fish, thereby suggesting that benzophenone-2 also shows a thyroid-disrupting mode of action. Based on *in vivo* ecotoxicity data, benzophenone-2 is categorized as an ED (Category 1) because of the combination of adverse apical effects and endocrine mode of action specific test results.

Based on the combined evidence from all studies, benzophenone-2 is evaluated as an ED in **Category 1**.

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which effects are observed, i.e. effects need to be observed at an oral dose of 10 mg/kg/day. For BP-2, effects on uterine weight in the uterotrophic assay were seen at much higher dose levels. In the reproductive study in mice, increased incidence of hypospadias were seen at a dose of 6,25 mg/kg/day, however only one dose was tested, and no other studies confirm this finding. Whether this finding is enough to evaluate BP-2 an endocrine disrupter of very high regulatory concern according to DE-UK potency criteria is unclear.

**References, *in vitro* and *in vivo***

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**Benzophenone-3, CAS 131-57-7**
**Synonyms:** BP-3, oxybenzone, 2-hydroxy-4-methoxybenzophenone

**Human data**
Benzophenone-3 is permeated rapidly in human systemic circulation and reached maximum concentration 3-4 hours after dermal application. No biologically significant effect of dermal application of a mixture of UV-filters incl. BP-3 was seen on reproductive hormones but indicated a slight reducing effect on testosterone in males (Janjua et al 2004). The mixture also did not cause any biologically significant effect on the hypothalamic-pituitary-thyroid axis in humans, but indicated a slight effect on thyroid hormones. (Janjua et al 2007). However, results of this study are inconclusive due to too short duration of the experiment. Wolff et al (2008) showed that the majority of pregnant women in USA are exposed to benzophenone-3. Maternal exposure to benzophenone-3 during pregnancy, was significantly associated with decrease in birth weight among girls and with increase in birth weight among boys. Alteration in birth weight of infants is a marker for general toxicity/reproductive toxicity, but is not endocrine specific. Intersex variation indicates hormone mediated effect. A study by Wolf et al (2010) indicates that benzophenone-3 might interfere with pubertal breast development in girls in cooperation with BMI, a known endogenous hormonal factor for pubertal development. Finally, a study by Philippat et al (2011) showed that pregnant women in France were widely exposed to benzophenone-3. Maternal exposure to benzophenone-3 during pregnancy, was associated with increase in birth weight and increase in head circumference at birth among boys, but was not significant. Alteration in birth weight of infants is a marker for general toxicity/reproduction toxicity, but is not endocrine specific.

Detailed study summaries are provided below:
**Janjua et al. 2004**: An experimental single blinded Danish study on Caucasian volunteers: 15 males and 17 postmenopausal females, examined effect of sunscreen mixture on reproductive hormones levels in humans. A control cream was applied daily for 4 days during the 1. week. A sunscreen formulation with 10% of benzophenone-3, 10% of 3-(4-methyl-benzylidene) camphor and with 10% of 2-ethylhexyl 4-methoxy cinnamate was applied daily for 4 days during the 2. week. Both creams were applied in recommended amount: 2 mg/cm$^2$ by staff. Maximum plasma concentration of benzophenone-3 was reached 3-4 hours after application and was 200 ng/ml in females and 300 ng/ml in males. No significant alterations were seen in FSH, SHBG or LH in any sexes. Estradiol in females was unaltered between first and second week. Significant increase in Inhibin B in males and in testosterone in females was seen already at time 0 in the second week. Estradiol in males decreased significantly 2 and 4 hours, but not 3 hours after application of sunscreen formulation. Testosterone in males decreased significantly 4 hours after application of sunscreen formulation which is in a line with time for maximal plasma concentrations. No hormone levels during the first day later than 4 hours are available.
**Janjua et al. 2007**: An experimental single blinded Danish study on Caucasian volunteers: 15 males and 17 postmenopausal females, examined effect of sunscreen mixture on the hypothalamic-pituitary-thyroid axis in humans. A control cream was applied daily for 4 days during the 1. week. A sunscreen formulation with 10% of benzophenone-3, 10% of 3-(4-methyl-benzylidene) camphor and with 10% of 2-ethylhexyl 4-methoxy cinnamate was applied daily for 4 days during the 2. week. Both creams were applied in recommended amount: 40±3 g for male and 35±3 g for female. No alterations in TSH were seen in any sexes. TBG and T4 in males were unaltered between first and second week. Significant decrease in FT3 in males was seen already at time 0 in the second week. FT3, FT4 and T3 in females decreased sporadically. T3 and FT4 in males and TBG and T4 in females decreased significantly 3-4 hours after dermal application which is in a

line with time for maximal plasma concentrations (Janjua et.al., 2004). No hormone levels during the first day later than 4 hours are available.

**Wolff et al. 2008:** Epidemiological study of multiethnic prospective cohort of 404 pregnant women-infant pairs in New York, recruited from Martch 1998 to Martch 2002. Maternal urine samples, collected mostly during the third trimester were analyzed for five phenols (benzophenone-3, triclosan, benzophenol A, 2,5-dichlorophenol, 2,4-dichlorophenol), ten different phthalates and sums of di(2-ethylhexyl) metabolites, monoester metabolites of high-molecular weight and monoester metabolites of low molecular-weight. Benzophenone-3 was detected in 97.8% of urine samples. Median concentration was 7.5 µg/l. The third tertile of benzophenone-3 concentrations in maternal urine (26-104,000µg/g Creatinine or 7-92,700 µg/l urine) were significantly positive correlated with adjusted mean birth weight for boys (p=0.026) but inversely correlated with adjusted mean birth weight for girls (p=0.021) compared to first tertile. Birth weight predicting model showed significant sex-specific difference in association between birth weight and maternal benzophenone-3 concentrations (44 g; 95% CI: 5.4-84g; p<0.05). Results were adjusted for race, gestational age, In-creatinine, prenatal smoking, maternal education, maternal marital status and pre-pregnancy BMI. Only samples with > 20 mg/dl creatinine were used in analyzes.

**Wolff et. al. 2010**: Multiethnic longitudinal study of 1,151 girls without underlying endocrinological conditions, recruited at 6-8 years of age from New York, Ohio and northern California during 2004-2007. Urine samples, collected at enrollment, were analyzed for seven phenols (benzophenone-3, bisphenol A, 2,5-dichlorophenol, triclosan, methyl-, butyl- and propyl parabens), nine phthalates and three phytoestrogens. Concentrations were creatinine corrected to adjust for urine dilution. Girls were examined for breast developmental (n=985) and presents of pubic hair (n=967) at the time of urine collection and one year later. Age and sex specific BMI. No association between urinary benzophenone-3 concentration and breast development or pubic hair stage were seen when adjusted for age, race, guardian education, seaso of urine collection, site and sex- and age specific BMI%. However, benzophenone-3 concentrations in the fifth quintile were positively associated with breast stage in girls with low BMI (n=469) (adjusted prevalence ratio (PR): 1.08; 95% CI 0.97-1.20; p-trend 0.15) and inverse associated with breast stage in the girls with high BMI (p-trend 0.38) compared to benzophenone-3 concentrations in the first quintile.

**Philippat et al. 2011**: Case-control study of Eden mother-child cohort, recruited before gestational week 28 during 2003-2006 in France. Only male infants were included in the study: 48 Cases: boys with hypospadias and cryptorchidism. 143 Controls: boys without genital malformations. 191 maternal urine samples, collected between gestational week 24 and 30, were analyzed for five phenols (2,4-dichlorophenol, 2,5-dichlorophenol, Bisphenol A, benzophenone 3, triclosan), four parabens and eleven phthalates. Benzophenone-3 was detected in 80.5% of urine samples. Median concentration was 1.3 µg/l. The third tertile of benzophenone concentrations in maternal urine was positively associated with birth weight (105g; 95% CI -40 – 250g) and with head circumference at birth (0.5cm 95% CI: -0.0-1.0) in boys compared to the first tertile. There was no difference in results after exclusion of cases. Results were adjusted for maternal pre-pregnancy weight and height, gestational age, In-creatinine, prenatal smoking, maternal education, parity, recruitment center and sampling conditions. Head circumference was adjusted for mode of delivery.

***In vitro* data**

A large number of *in vitro* studies with BP-3 have been performed, most of which show estrogenic but also other modes of action for this compound. BP-3 has been shown to act as an estrogen by altering gene transcription (Heneweer et al 2005) and causing proliferation of MCF-7 cells (Schlumpf et al 2001, Suzuki et al 2005, Matsuomo et al 2005), by showing estrogenic activity in the yeast two-hybrid assay after incubation with S9 extract (Kawamura et al 2003, Ogawa et al 2006, Takatori et al 2003) and by binding to the estrogen receptor in other test systems ( Morohoshi et al 2005, Schreurs et al 2005, Matsuomo et al 2005, Kawamura

et al 2005, Kunz & Fent 2006).There are however also studies which have not found *in vitro* estrogenic action of BP-3 (Gomez et al 2005, Blair et al 2000, Molina-Molina et al 2008, Nakagawa et al 2002). Despite the studies showing weak or no estrogenic activity of BP3 itself, it is likely that the chemical is metabolized *in vivo* to the more potent estrogenic benzophenone-1 as it occurs *in vitro* after incubation with the liver S9 extract (Takatori et al., 2003).
Furthermore some studies have shown antagonism of the androgen receptor *in vitro* (Araki et al 2005, Schreurs et al 2005, Ma et al 2003, Kawamura et al 2005, Kunz & Fent 2006,Molina-Molina et al 2008, Suzuki et al 2005) and the progesterone receptor (Schreurs et al 2005). Furthermore, BP-3 has been shown to affect the thyroid system *in vitro*, by binding to the thyroid receptor (Hofmann et al 2009), to increase baseline corticosterone production in adrenocortical cell suspensions from female rats (Ziolkowska et al 2006) and to dose-dependently inhibit prostaglandin production in human embryo palatal mesenchyme cell (Jannesson et al 2004). Overall these studies indicate that BP-3 has modes of action that are compatible with an estrogenic mode of action and possibly also other endocrine modes of action *in vitro*.

***In vivo*, human health**
Relatively few *in vivo* studies with BP-3 have been performed. The compound was shown to yield a weak uterotrophic response, by causing a slight increase in uterine weight in immature LE rats (n=5-9) treated for three days by oral gavage (Schlumpf et al 2001). The LOAEL was 1525 mg/kg/day, while a dose of 937 mg/kg did not cause any significant changes in uterine weight, and was therefore the NOAEL in this study. In response to these data, Bolt et al (2001) refer to an unpublished industry study performing an uterotrophic assay. Here immature Wistar rats were dosed orally with daily doses of 500 or 1000 mg/kg of BP-3, and no significant effects on uterine weight were seen. Number of animals used was not stated. In another uterotrophic assay performed with BP-3, no significant changes in uterine weights were seen at doses of 250 and 1000 mg/kg /day (Schlecht et al 2004). In this study ovariectomized adult female SD rats (n=11) were dosed by gavage for 5 days. The authors also investigated gene-expression of estrogen receptors indifferent tissues. They found significant decrease in ERβ mRNA expression in the uterus and ERα mRNA expression in pituitary, at both investigated dose levels, and concluded that it was still unknown whether these changes had any functional significance. An uterotrophic study was also performed by Suzuki et al (2005). They dosed adult ovariectomized F344 rats (n=5) for 3 days with 20, 100 or 500 mg/kg/day and found no effect on uterine weight. Giving that the NOAEL was set to 937 mg/kg/day in the first study, the fact that no effects on uterine weight were seen in the following studies is not surprising.
A study has also examined the effects of BP-3 on male reproduction parameters. Daston et al (1993) dosed male mice (n=10) topically with 10, 20, 100 or 400 mg/kg/day for 90 days, and reproductive organ weights, testicular histology and different sperm quality parameters were assessed. None of the had any significant effects on the investigated endpoints.

***In vivo*, ecotoxicity**
Benzophenone-3 induces vitellogenin in fish in one study (rainbow trout and Japanese medaka) but not in another study (fathead minnow).This may be related to species-specific differences in vitellogenin induction and/or metabolism of benzophenone-3. The study showing a response on vitellogenin also shows reduced percentage of hatching of fish eggs.

Study summaries are provided below:
**Schreurs et al. 2002**: Zebrafish, in which an estrogen responsive luciferase reporter gene has been stably introduced, were used for *in vivo* testing of UV-filters.

Benzophenone-3 showed no estrogenic activity in this transgenic zebrafish assay at the tested concentration: 10 µM (2282 µg/L).
**Kunz et al. 2006**: In this study it was analyzed whether benzophenone-3 shows estrogenic activity in fathead minnows by the induction potential of vitellogenin after 14 days of aqueous exposure. Fish were exposed to five different concentrations of benzophenone-3: 12 (median measured), 100 (nominal), 500 (nominal), 766 (median measured) or 3900 µg/L (median measured).
No significant differences were found between exposed and control groups.
**Coronado et al. 2008**: The effects of benzophenone-3 on estrogenic activity and reproduction were evaluated using a 14-day juvenile rainbow trout assay for plasma vitellogenin and a subsequent 21-day Japanese medaka reproduction assay.
Significant induction of vitellogenin was observed in the rainbow trout at the 1000 µg/L nominal concentration (749 µg/L median measured concentration) of benzophenone-3. Vitellogenin induction was also observed in the 1000 µg/L nominal concentration (620 µg/L median measured) of benzophenone-3 in male Japanese medaka (*Oryzias latipes*) after 21 days of exposure. The number of eggs produced per female per day exposed to the same concentration (620 µg/L) was significantly lower after 7 days, but returned to control values after 21 days. The overall percentage of fertilized eggs collected during the 21-day exposure that hatched was significantly lower in the 620 µg/L benzophenone-3 concentration.
These data show that benzophenone-3 induces vitellogenin in two fish species and reduces the percentage of hatching of fish eggs.
**Sieratowicz et al. 2011**: This study determines the effects of benzophenone-3 on the green alga *Desmodesmus subspicatus* and the crustacean *Daphnia magna*. Exposure to benzophenone-3 resulted in growth inhibition of *D. subspicatus* with a 72 h IC10 value of 0.56 mg/L. The EC50 concentration in the acute immobilisation test with *D. magna* was 1.67 mg/L. In a chronic exposure test with *D. magna* (OECD guideline 211), benzophenone-3 showed no effects on neonate production or the length of adults. The study provides data on the growth inhibiting and toxic concentrations of benzophenone-3 towards green alga and daphnia.

**Weight of evidence for ED and Category**
The studies examining effect of dermal treatment with benzophenone-3 on levels of reproductive hormones and on the hypothalamic-pituitary-thyroid axis in humans are inconclusive due to their limited duration, but indicate some effect. Epidemiological studies indicate wide human exposure to benzophenone-3, intersex difference of effect, possible correlation with BMI on pubertal development and alterations in birth parameters following perinatal exposure to benzophenone-3. Reduced birth weight, was the only significant effect found in humans, which is an apical endpoint, but not endocrine specific. Benzophenone-3 is evaluated as ED Category 2b based on human data.

Based on the relatively few number of studies investigating *in vivo* effects, and the fact that only one study has shown estrogenic effects in the uterotrophic assay at a dose of 1525 mg/kg/day, evidence from the experimental animals is not sufficiently convincing evidence to place the substance in Category 1. There is however a large number of *in vitro* studies showing estrogenic activity as well as other modes of action which could affect the endocrine system. Changes in gene expression of the estrogen receptors have also been observed *in vivo*, after a few days of dosing with 250 mg/kg/day. Furthermore, the compound is metabolized to benzopheone-1, a compound which has been shown to affect the endocrine system *in vivo*. Based on this information, according to the Danish criteria, benzophenone-3 is evaluatewd as a suspected endocrine disrupter in Category 2a.

Based on *in vivo* ecotoxicity studies, benzophenone-3 induces vitellogenin in fish in one study but not in another study. The study showing a response on vitellogenin also shows reduced percentage of hatching of fish eggs. Based on *in vivo* ecotoxicity data, benzophenone-3 is evaluated as a suspected ED in Category 2a because vitellogenin induction reveals an endocrine mode of action. Whether the reduced hatching of fish eggs is an adverse effect needs to be established.

Based on the combined evidence from the ecotoxicological studies, *in vitro* and *in vivo* data from human health, benzophenone-3 is evaluated as a suspected ED in **Category 2a**.

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which effects are observed, i.e. effects need to be observed at an oral dose of 10 mg/kg/day. For BP-3, effects on uterine weight in the uterotrophic assay were seen at much higher dose levels and BP-3 is therefore not evaluated as an endocrine disrupter of very high regulatory concern according to DE-UK potency criteria.

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**Butylparaben, CAS 94-26-8**
**Synonyms**:. n-Butyl-4-hydroxybenzoat

**Human data**
Humans are exposed to several different parabens. In urine samples from 60 young Danish men four common parabens were measured in nearly all samples: methyl paraben (MP) in 98% of the samples (median level = 18 ng/ml ); propyl paraben (PB) in 98% of the samples (median level = 3.6 ng/ml ); ethyl paraben (EP) in 80% of the samples (median level = 2.0 ng/ml ); butyl paraben (BP) in 83% of the samples (median level = 0.2 ng/ml ) (Frederiksen et al., 2911). Similar trend was observed in an U.S. population (NHANES 2005-2006), although MP and PB were observed in about a threefold higher level in the U.S. samples compared to the Danish samples and furthermore EP and BP were detected less frequently than in Danish samples, 42% and 47%, respectively (Calafat et al., 2010).

Only few human studies have investigated possible endocrine disrupting effects of parabens. One study analysed the association between urinary concentration of MP, PP and BP and markers for male reproduction health (Meeker et al., 2011). No associations were observed between the three parabens and serum hormone levels or semen quality parameters. However even though BP only was detected in 32% of samples, a positive association between urinary BP concentration and sperm DNA damage was observed. Similar association was not found for MP or PP, but for bisphenol A (BPA) in same study group. Thus it was suggested that combined exposure to BP and BPA may have an additive effect on DNA damage. Another study found no associations between the urinary concentration of parabens (MP, PP and BP) and pubertal stage; breast development and pubarche in U.S. girls (Wolff et al., 2010). Finally two unpublished studies "Urinary paraben concentrations and *in vitro* ferterlization (IVF) outcomes" (Sabatini et al,. 2011) and "The association of urinary paraben concentrations with measures of ovarian reserve among patients from a fertility canter" (Smith et al., 2011), both presented as poster abstracts indicated that 1) increased urinary MP and PP were associated with increased incidence of poor embryo quality and 2) that there was a suggestive evidence for an association between PP and higher serum follicle stimulating hormone (FSH) and lower antral follicle count (AFC) on day three of the menstrual cycle.
In conclusion, a few human studies have indicated weak associations between increased paraben exposure and markers for human reproductive health. However, our knowledge in this area is very limited.

***In vitro* data**
The available data for butylparaben show strong evidence that this compound has estrogenic effects *in vitro*. In general, references to these statements can be found in the review by Boberg et al., 2010. There are also reports of weak antiandrogenic effects *in vitro* (Chen et al., 2007, Kjaerstad et al., 2010).
The literature review for butylparaben is based on comprehensive paraben reviews published from 2008 to 2010 (CIR review 2008, Darbre and Harvey, 2008, Boberg et al., 2010, Cowan-Ellsbury et al., 2009) and detailed study descriptions are therefore limited to reviews of the most important studies on endocrine disrupting effects. The following table from Boberg et al., 2010, collects information from studies on estrogenic and androgenic activity *in vitro*.

**Table 1** *In vitro* studies published on the estrogenic and antiandrogenic activity of parabens. Boberg et al., 2010. +ve refers to positive effect in an assay

| **Estrogenic effect *in vitro*** | **Antiandrogenic effect *in vitro*** |
|---|---|
| +ve (yeast + receptor binding) (Routledge 1998, Miller 2001, Schultis and Metzger 2004, Morohoshi 2005) | +ve (recombinant hAR) (Sato 2005) |
| +ve (human MCF7) (Okubo 2001, Byford 2002, Schultis and Metzger 2004, Vanapyris 2006, Pugazhendhi 2005) | +ve (transfected CHO-K1 cells) (Sato 2005) |
| +ve (rat uterus receptor binding) (Blair 2000, Lemini 2003) | +ve (transfected HEK 293 cells) (Chen 2007) |
| +ve (human HeLa overexpressing ER) (Gomez 2005) | |

The evidence of estrogenic effects is based on several reports of weak estrogenic effects *in vitro* using MCF-7 assay, reporter gene assay, or recombinant yeast screen assay (Byford et al., 2002, Gomez et al., 2005, Routledge et al., 1998, Kang et al., 2002b, Vo et al., 2011). Antiandrogenic effects have also been shown in a few *in vitro* studies (for references see Table 1).

***In vivo*, human health**

The available data for butylparaben show strong evidence that this compound has estrogenic effects *in vivo*. In general, references to these statements can be found in the review by Boberg et al., 2010. The literature review for butylparaben is based on comprehensive paraben reviews published from 2008 to 2010 (CIR review 2008, Darbre and Harvey, 2008, Boberg et al., 2010, Cowan-Ellsbury et al., 2009) and detailed study descriptions are therefore limited to reviews of the most important studies on endocrine disrupting effects. The following table from Boberg et al., 2010, collects information from *in vivo* studies on uterotrophic effects.

**Table 2**. Summary of results of uterotrophic assays. Modified from Boberg et al., 2010. Route: subcutaneous (SC) unless otherwise stated.

| Study | Response in immature rats (effective doses in mg/kg) | Response in immature mice (effective doses in mg/kg) | Response in ovariectomized mice (effective doses in mg/kg) |
|---|---|---|---|
| Routledge 1998 | LOEL 400<br>NOEL 200 | | |
| Hossaini 2000 | LOEL 100, sc | No effect at 100 | |
| Lemini 2003 | LOEL 70<br>NOEL 20 | LOEL 7<br>NOEL 0.7 | LOEL 21<br>NOEL 7 |
| Lemini 2004 | | | LOEL 70 |
| Shaw 2009 | | | No effect up to 1000 |
| Vo 2009 | LOEL 1000<br>NOEL 250 | | |
| Vo 2010 | LOEL 62.5 | | |

The evidence of estrogenic effects is based on several reports of weak estrogenic effects *in vivo*, using uterotrophic assays or exposure of immature females (Lemini et al., 2004, Routledge et al., 1998, Vo et al., 2009, Vo et al., 2010) (Table 2). One study by Vo et al., 2010, showing estrogenic effects of butylparaben is presented in detail below, as this was performed in immature female rats and thus a relevant model for

exposure of developing humans, i.e. more relevant than uterotrophic assays in ovariectomized animals. No NOAEL was determined in that study but a LOAEL of 62.5 mg/kg bw/day was determined for effects on uterine and ovarian histology. One *in vivo* study has shown adverse effects on sperm counts following subcutaneous exposure in the perinatal period (Kang et al., 2002a), while there are conflicting results on the influence of butylparaben on sperm count/quality following dietary exposure of young males (Hoberman et al., 2008, Oishi et al., 2001). No effects on antiandrogenic endpoints such as fetal anogenital distance were seen in rat studies using fetal exposure (Kang et al., 2002a, Boberg et al., 2008, Taxvig et al., 2008). There are no consistent signs of thyroid toxicity (Vo et al., 2010).
The evidence of effects on male reproduction is based primarily on the findings by Kang et al., 2002a, showing effects on semen quality following perinatal exposure. In contrast, the studies by Oishi et al., 2001 and Hoberman et al., 2008, are not considered sufficient to reach conclusions on whether butylparaben can affect male rat reproduction following peripubertal exposure. Subcutaneous exposure was applied in the study by Kang et al., 2002, and this is considered a relevant exposure route as humans are exposed to parabens by dermal application. However, when evaluating studies using subcutaneous as well as oral exposure it should be noted that internal doses in the experimental animal will be different from internal doses in humans following dermal application. In general, however, effects seen at either exposure route can be considered relevant in the hazard identification.

Detalied study summaries are provided below:
**Kang et al., 2002**. Pregnant SD rats were exposed subcutaneously to 100 or 200 mg/kg bw/day of butylparaben from GD 6 to PND 20. Reduced sperm count and reduced motility of sperm was observed in both dose groups, and no NOAEL could be determined. Testis and prostate weights relative to brain weights were reduced, but not in a dose-related manner and the authors did not list organ weights relative to body weights. No effects on male or female anogenital distance were observed. The age of vaginal opening was significantly earlier in the 100 mg/kg group than in controls, but only slightly earlier in the 200 mg/kg group compared to controls. This study shows clear adverse reproductive effects of butylparaben. The lack of effect on anogenital distance and the possible effect on vaginal opening may point to the mode of action being estrogenic rather than anti-androgenic.
**Oishi et al., 2001**. Three-week old male Wistar rats were exposed to dietary doses of 0.01%, 0.1% and 1.0% of butylparaben corresponding to approximately 10, 100 and 1000 mg/kg bw/day for 8 weeks. Daily sperm production (testis sperm counts) as well as epididymal cauda sperm counts were reduced in a dose-related manner at all applied doses. Serum testosterone was reduced at 100 and 1000 mg/kg bw/day showing a dose-response relationship. Body weight was not statistically significantly reduced. Relative epididymis weight was reduced at 100 and 1000 mg/kg bw/day with a dose-response relationship. Absolute, but not relative weight of the seminal vesicle was reduced at 1000 mg/kg. Thus, the LOAEL was 10 mg/kg bw/day, and no NOAEL could be determined. This study has some shortcomings as rat body weights at study start were unusually high; 50–52 g at PND 19–21 compared to the expected 40 g at that age. Standard deviations for testosterone measurements were unusually low and mean sperm counts in rats were in the high end of historical control data. As the raw data were not available for review by the European Union Scientific Committee on Consumer Products (SCCP), they acknowledge some doubt about the result of this study (SCCP 2006).
**Hoberman et al., 2008.** Hoberman et al. performed a repeat study of the study by Oishi from 2001 by exposing 3-week old male Wistar rats to dietary doses of 10, 100 and 1000 mg/kg bw/day of butylparaben for 8 weeks. This study was performed under GLP conditions and included a higher number of animals than the Oishi study. The authors reported “no adverse effects” at all dose levels concluding a NOAEL of 1000 mg/kg bw/day. However, serum testosterone was reduced significantly after 3 weeks of dosing at 100 and

1000 mg/kg bw/day. In the paper is stated that this was due to two high outliers in the control group, but examination of raw data reveals that the effect is still statistically significant in the highest dose group after removing the two outliers from the control group. The effects on testosterone levels at week 3 may be important to the masculinization of the rats taking place at this age (prepuberty, 6 weeks of age) and may be regarded as adverse. The SCCP have evaluated the study report on this butylparaben study and conclude that due to several shortcomings the study "cannot be considered as scientifically valid" (SCCP 2006).
**Taxvig et al., 2008**.Pregnant Wistar rats were orally exposed to 200 or 400 mg/kg bw/day of butylparaben from gestation day 7 to 21. No changes in anogenital distance or serum levels of testosterone or progesterone were observed in fetuses, and no changes in plasma T3, T4, 17alpha-hyroxyprogesterone or progesterone levels were detected. Butylparaben increased growth in the T-screen assay indicating interference with thyroid receptors. Progesterone levels were elevated at the highest tested dose (30 uM) in an *in vitro* assay investigating influences on hormone production in adrenal cells (H295R assay), whereas no effects on estradiol or testosterone production were seen.
**Vo et al., 2010**. Effects of 6 parabens were compared in a female pubertal assay in rats. Female SD rats were orally exposed to 62.5, 250 or 1000 mg/kg bw/day of butylparaben from postnatal day 21 to 40 (4 weeks), and reproductive endpoints were examined at postnatal day 40. No changes in age at vaginal opening or changes in estrous cycles were observed for butylparaben, but the uterine epithelium was thickened at all three doses. This was also seen for isobutylparaben, propylparaben and isopropylparaben but not for the short-chain parabens methyl- and ethylparaben. Liver weights were increased at all doses and relative thyroid weight was increased at the lowest dose only, but serum T4 was not significantly altered. Histological evaluation of ovaries showed increased numbers of cystic follicles, and decreased numbers of corpora lutea.

***In vivo*, ecotoxicity**
Three relevant ecotoxicology studies with butylparaben have been performed. Increases in average plasma vitellogenin levels were seen at oral exposure to 9 mg butylparaben/kg/2 day in rainbow trout. Vtg induction after injection of butylparaben had a LOEC of 100 mg/kg and Vtg induction after water exposure to BP had a LOEC of 201 µg/l. Daphnia magna reproduction declined with a LOEC of 2.6 mg/l.

Study summaries are provided below:
**Pedersen et al 2000**: The widely used phenolic preservative butylparaben was tested for the ability to evoke an oestrogenic response *in vivo*. Vitellogenin induction in sexually immature rainbow trout (*Oncorhynchus mykiss*) was used as an oestrogen-specific endpoint after two injections of the compound (day 0 and 6). Vitellogenin was measured in blood at day 0, 6 and 12. Butylparaben had oestrogenic potency comparable to bisphenol A with a LOEC of 100 mg/kg.
**Alslev et al 2005**: The estrogenic effect of butylparaben was investigated in a rainbow trout *Oncorhynchus mykiss* test system. Butylparaben was administered orally to sexually immature rainbow trout every second day for up to 10 days in doses between 4 and 74 mg/kg/2 day and in the water at 35 and 201 mg/l for 12 days. Plasma vitellogenin was measured before and during the exposures and the concentrations of butylparaben in liver and muscle were determined at the end of experiments. Increases in average plasma vitellogenin levels were seen at oral exposure to 9 mg butylparaben/kg /2 day. The ED50 values for increase in vitellogenin synthesis were 46, 29 and 10.5 mg butylparaben/kg/2 day, respectively, at day 3, 6 and 12. Exposure to 201 µg butylparaben/l increased vitellogenin synthesis, but exposure to 35 µg/l did not. Butylparaben showed little tendency to bioaccumulation in rainbow trout; less than 1‰ of the total amount of butylparaben administered orally at 51 mg/kg/2 day over the 12 days experimental period was retained in liver at the end of the experiment. After 12 days exposure to 35 and 201 µg butylparaben/l, plasma

concentrations were 9 and 183 µg/l, respectively, and for the fish exposed to 201 µg/l there was a positive correlation between concentrations of vitellogenin and butylparaben in the plasma.
**Dobbins et al 2009**: Standardized acute and subchronic endpoints in larval fish (*Pimephales promelas*) and cladoceran (*Daphnia magna*) models were examined for seven different parabens (methyl-, ethyl-, isopropyl-, propyl-, isobutyl-, butyl-, benzylparaben), which encompassed a range of log P values. Butylparaben 48 h median lethal concentration values (LC50) were 5.3 mg/L in D. magna and 4.2 mg/L in fathead minnow. Growth and reproduction in D. magna had lowest-observed-effect concentrations (LOECs) of 0.2 mg/L and 2.6 mg/L, respectively. Fathead minnow growth was adversely affected at 1.0 mg/L.

**Weight of evidence for ED and Category**
A few human studies have indicated weak associations between increased paraben exposure and markers for human reproductive health. However the knowledge in this area is very limited.

Based on *in vivo* and *in vitro* studies, butylparaben can be placed in Category 2a, as a suspected endocrine disrupter, as there is evidence of an estrogenic mode of action *in vivo* that is suspected to be linked to adverse effects *in vivo*. However, the findings of reduced sperm counts following perinatal exposure is considered as an adverse effect where an ED mode of action is highly plausible and therefore Category 1 is considered appropriate.

In ecotoxicological studies, based on the confirmed Vtg induction in multiple studies and the adverse effect on daphnia reproduction, butylparaben could be evaluated as an ED in Category 1 but as reproduction is not endocrine specific, a test with an endocrine specific adverse endpoint is needed (e.g. sex ratio) before this paraben could be evaluated as an ED in category 1. Therefore, butylparaben is evaluated as suspected ED (Category 2A).

Based on the combined evidence from *in vivo*, *in vitro*, ecotoxicological and human studies, butylparaben is evaluated as an ED in **Category 1**.

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which severe adverse effects are observed. For subchronic studies with oral dosing, effects at 10 mg/kg bw/day and below leads to a classification as an endocrine disrupter of very high regulatory concern. No NOAEL can be determined for butylparaben. A LOAEL of 100 mg/kg bw/day in the study by Kang et al., 2002, provides the main evidence of adverse effects, but as no NOAEL is determined in that study, this study cannot be used for a robust conclusion on potency. A study by Oishi et al., 2001, showed effects on semen quality at 10 mg/kg bw/day after 4 weeks dosing, but uncertainties on this study makes it impossible to judge whether this LOAEL can be regarded as an overall LOAEL. If this LOAEL for an adverse reproductive effect related to endocrine disruption is used, then this fulfills the DE/UK pontency criteria for an endocrine disrupter of high regulatory concern. However, this is regarded as unclear.

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**Dicyclohexyl phthalate (DCHP), CAS No: 84-61-7**
**Synonyms**: Phthalic acid, dicyclohexyl ester; 1,2-Benzenedicarboxylic acid, Dicyclohexyl ester; Benzene-1,2-dicarboxylic acid, Dicyclohexyl ester;

**Human data**
No relevant human data were found.

***In vitro* data**
*In vitro* studies have shown estrogenic effects of DCHP in an assay evaluating growth of estrogen-sensitive MCF-7 cells (Obuko et al., 2003, Hong et al., 2005), whereas studies on estrogen receptor alpha binding did not support an estrogenic mode of action (Yamasaki et al., 2002). A study in H295R adrenal cells revealed that DCHP inhibited CYP21B activity and decreased cortisol secretion (Nakajin et al., 2001). This enzyme is responsible for conversion of 17-OH progesterone to cortisol, and as such is not involved in sex hormone synthesis. It is known that other phthalates can inhibit CYP17, which converts 17-OH progesterone to androstenedione before its conversion to testosterone. CYP17 activity was possibly slightly inhibited by DCHP (Nakajin et al., 2001) and this could further support the proposed mechanism of action that DCHP can inhibit testosterone production.

***In vivo*, human health**
An overall evaluation of the available literature on DCHP leads to the conclusion that DCHP is an anti-androgen comparable to e.g. DEHP, DBP, DiBP and BBP. Impaired masculinization of male pups is observed following perinatal exposure. Fetal/neonatal anogenital distance is reduced in males, the prevalence of nipple retention is increased, male puberty is delayed, and in adults weights of male reproductive organs are decreased, and altered testis histology and sperm quality is observed (Hoshino et al., 2005, Saillenfait et al., 2009; Yamasaki et al., 2009). In adult animals (F0 of a two-generation study), estrous cycle duration was increased (Hoshino et al., 2005), and this could be interpreted as either an estrogenic or anti-androgenic effect. Exposure of immature rats to DCHP (or other phthalates) did not alter gene and protein levels of the estrogen-regulated protein CaBP-9k, whereas known estrogenic compounds did (Hong et al., 2005), indicating that effects of DCHP are not likely to be related to an estrogenic mode of action. The lowest LOAEL of the studies examining antiandrogenic effects is 20 mg/kg bw/day as observed in the study by Yamasaki et al., 2009. No NOAEL could be determined. No studies have examined whether DCHP impairs testosterone production in fetal testes, but this is suggested to be the mode of action as judged by read-across to compounds such as DEHP, DBP, DiBP and BBP.
In adult animals (F0 of a two-generation study), DCHP was found to increase thyroid and liver weights (Hoshino et al., 2005). This increase in thyroid weight may indicate interference with thyroid hormone system and based on read-across to other structurally comparable phthalates that have also been found to disrupt thyroid function (DEHP, DnOP, DnHexylP), it is likely that DCHP is also a disrupter of the thyroid hormone system. The overall LOAEL for endocrine disrupting effects is 20 mg/kg bw/day based on the study by Yamasaki et al., 2009.

Detailed study descriptions are provided below:
**Hoshino et al., 2005**. A two-generation study tested the reproductive toxicity of DCHP. Dietary doses of 0, 240, 1200, 6000 ppm were applied corresponding to 0, 14, 70 and 349 mg/kg bw/day during gestation (F0 females) and 0, 21, 104, and 511 for the total study period (F0 females). At 1200 and 6000 ppm, reduced testicular sperm numbers were observed (F1 males), anogenital distance was reduced and nipple retention

increased (F2 males). Anogenital distance and nipple retention was also altered in F1 males at the highest dose level. In the high dose group, increased thyroid weights were seen in d F0 and F1 males, and reduced protstate weight and diffuse or focal atrophy of seminiferous tubules were seen in F1 males. In females, prolonged estrous cycle was noted in the high dose group. This study indicates adverse reproductive effects related to endocrine disruption that can be attributed to an anti-androgenic mode of action. Also the observed effect on thyroid weight indicates that DCHP may be a disrupter of the thyroid.

**Saillenfait et al., 2009.** A developmental study in SD rats was applied to test for developmental toxicity of DnHP and DCHP and included assessment of anogenital distance as an endocrine sensitive endpoint and evaluation of the degree of trans-abdominal testicular migration. Dams were exposed to 0, 250, 500 or 750 mg/kg bw/day of test compounds from gestation day 6 to 20, and fetuses were examined at gestation day 21. Anogenital distance of male fetuses was reduced by DCHP in all dose groups, and this was statistically significant with and without correction for body weight. Decreased body weight of fetuses was only present at the highest dose of DCHP. Testicular migration was not affected by DCHP. This study indicates that DCHP affects masculinization in a similar way as the known endocrine disrupters DEHP, DBP and BBP.

**Yamasaki et al., 2009**. A study using perinatal exposure to DCHP was applied to investigate endocrine-mediated effects in offspring. SD rats were exposed by oral gavage to 0, 20, 100, or 500 mg/kg bw/day of DCHP from gestation day 6 to postnatal day 20 (n=10 dams per dose group). In adult offspring, relative prostate weight was decreased in all dose groups, but the effect was only statistically significant at 20 and 500 mg/kg bw/day. Weight of levator ani/bulbocavernosus muscle (LABC) was decreased at 500 mg/kg bw/day. Histological changes in the testis were described in the high dose group (data not shown). Hypospadia was seen in two males of the high dose group. Delayed preputial separation, reduced andogenital distance and an increase in nipple retention were seen in the high dose group. For females, no abnormalities in age of vaginal opening or estrous cycle were observed. No effects on thyroid weights were observed. This study does not deliver a complete set of data, but the evidence of endocrine disrupting effects at the highest dose appears strong. However, it is less clear if any lower dose group also has endocrine disrupting effects.

**Yamasaki et al., 2002**. A reporter gene assay was performed for evaluating ERalpha mediated transcriptional activation by 23 chemicals. DCHP did not alter transcriptional activity of ERalpha, i.e. did not appear to be an ER alpha agonist. An immature rat uterotrophic assay was applied to examine estrogenic effects of 23 chemicals including DBP and DCHP. Female SD rats were exposed to subcutaneous doses of 2, 20, and 200 mg/kg bw/day of chemicals at postnatal day 20, 21 and 22 (n=6 rats per dose group). Uterus weight was determined on postnatal day 23. No statistically significant change in uterus weight was seen for DCHP or DBP.

**Yamasaki et al., 2005** reviews a set of parallel two-generation studies on potential endocrine disrupters including the study by Hoshino et al., 2005, and concludes that DCHP is tested positive for endocrine disrupting activity in that study. The observed effects are considered indicative of endocrine disrupting activity (thyroid hypertrophy and increased thyroid weights, reduction of prostate weight, atrophy of seminiferous tubules, decreased male anogenital distance and increased male nipple retention).

**Lake et al., 1982**. (only abstract available for review) A study in young SD rats was performed to examine hepatic and testicular toxicity of DCHP. Rats were exposed to 500-2500 mg/kg bw/day of DCHP for 7 days resulting in hepatic enzyme induction and liver enlargement. Histological examination of the testes revealed testicular damage at 2500 mg/kg bw/day. The metabolite MCHP was found to produce marked testicular atrophy. This study supports that DCHP is a reproductive toxicant and the observed effect are known to be in compliance with an endocrine disrupting mode of action.

**Obuko et al., 2003.** *In vitro* studies in human breast cancer estrogen-sensitive MCF-7 cells were applied to test for estrogenic activity of 19 phthalate diesters or monoesters. 17beta-estradiol and 4OH-tamoxifen were included as positive controls. DCHP showed an inverted-U shaped dose response curve similarly to the

known estrogen tamoxifen, but at higher dose level than tamoxifen. Increased proliferation was observed from 10 uM and peaked at 100 uM of DCHP. The monoester for DCHP, MCHP did not induce cell proliferation in that assay. DEHP and BBP were the only other tested phthalates that induced cell proliferation, and this was with lower potency than DCHP. Exposure of cells to the anti-estrogenic compound ICI 182,780 concomitantly with DCHP revealed that DCHP induced cell proliferation could be inhibited by ICI 182,780. This study supports an endocrine disrupting mode of action (estrogen-like effects).
**Hong et al., 2005**. An *in vitro* study in human breast cancer estrogen-sensitive MCF-7 cells was applied to test for estrogenic activity of 5 phthalates. DCHP induced cell proliferation from 10 uM, whereas DEHP and DBP induced cell proliferation at 100 uM, and BBP induced cell proliferation already at 1 uM. An *in vivo* study in immature female SD rats was applied to test for influences of estrogenic phthalates on expression of the estrogen-regulated protein CaBP-9k. Females were exposed to test compounds including DCHP at 600 mg/kg bw/day in corn oil by gavage on days 14, 15 and 16 after birth (n=5 rats per dose group). Rats were euthanized at day 17 and uteri collected for Northern blot and Western blot examination of CaBP-9k mRNA and protein expression levels. Results showed that positive controls Ethinyl estradiol and Diethylstilbestrol as well as the weak estrogens Octylphenol and Bisphenol A induced expression of CaBP-9k mRNA and protein levels, whereas none of the tested phthalates including DCHP altered expression of CaBP-9k. This study supports that DCHP may be estrogenic *in vitro* whereas *in vivo* effects were not detected.
**Nakajin et al., 2001**. An *in vitro* study in human adrenocortical H295R cells was applied to test for influence of several phthalate esters and alkylphenols on adrenocortical steroidogenesis. Levels of cortisol were measured along with enzyme activity measurements for selected enzymes involved in steroidogenesis, but no assessment of sex hormone levels (estrogen, testosterone, progesterone) were made. Among the phthalates, only DCHP reduced cortisol secretion at the applied concentration of 30 uM (to 76% of control). Furthermore, DCHP inhibited the activity of 21-hydroxylase, CYP21B (to 80% of control), but did not affect any other CYP-family enzymes investigated. A slight but not statistically significant decrease in CYP17 activity was observed at the applied concentration levels up to 25 uM (reduction to 85% of control). This study supports and endocrine disrupting effect of DCHP *in vitro*.

***In vivo*, ecotoxicity**
In a study by Sugiyama et al., 2005, interference of dicyclohexyl phthalate with the thyroid system of *Xenopus laevis* was investigated. Dicyclohexyl phthalate showed $T_3$-antagonistic activity in two *in vitro* assays with *Xenopus laevis* cells. However, dicyclohexyl phthalate did not exhibit $T_3$-antagonistic activity in an *in vivo* metamorphosis-based assay.

**Weight of evidence for ED and Category**
An overall evaluation of the available literature on DCHP leads to the conclusion that DCHP is an anti-androgen comparable to e.g. DEHP, DBP, DiBP and BBP. Impaired masculinization of male pups is observed following perinatal exposure. Thus DCHP fulfills the criteria of being an endocrine disrupter, as several *in vivo* studies have found adverse effects related to an anti-androgenic mode of action, and as a few studies further point to estrogenicity and interference with thyroid function. These findings *in vivo* lead to ED in Category 1.

The *in vivo* ecotoxicity data on dicyclohexyl phthalate does not clearly indicate endocrine disrupting properties.

Based on the combined evidence from the ecotoxicological studies and the *in vitro*, *in vivo* and epidemiological studies, DCHP is evaluated as an ED in **Category 1**.

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which severe adverse effects are observed. For studies with oral dosing, effects at 10 mg/kg bw/day and below leads to a categorization as an endocrine disrupter of very high regulatory concern. As DCHP shows endocrine disrupting effects (reduced prostate weight) at 20 mg/kg bw/day, DCHP may not be categorized as an endocrine disrupter of very high regulatory concern according to DE-UK criteria. However, as the lower end of the dose-response curve is poorly examined for DCHP and no NOAEL has been determined, it is possible that relevant effects may be seen at 10 mg/kg bw/day or lower doses. If an uncertainty factor can be included because no NOAEL is determined, then DCHP can be considered an endocrine disrupter of very high regulatory concern according to the DE-UK potency criteria. Overall, it is evaluated as unclear, whether DHCP will be categorized as an endocrine disrupter of very high regulatory concern according to DE-UK potency criteria.

**References, *in vivo* and *in vitro***

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**Di-ethyl phthalate (DEP), CAS 84-66-2**

Synonyms: 1,2-Benzenedicarboxylic acid, diethyl ester; o-Benzenedicarboxylic acid diethyl ester; o-Bis(ethoxycarbonyl)benzene; Diethyl 1,2-benzenedicarboxylate; Diethyl o-phenylenediacetate; Diethyl o-phthalate; Di-n-ethyl phthalate; DPX-F5384; Ethyl phthalate; Phthalic acid, diethyl ester

**Human data**

Associations between DEP exposure and clinical outcomes have been studied in several human studies as summarised in the table below:

| Study | Study group | findings |
|---|---|---|
| Swan et al. 2005 (and 2008) | Prenatal maternal spot urine samples (n=85) | Maternal prenatal urinary MEP concentrations negatively associated (p=0.017) with AGD in the newborn sons. Same association observed for MBP |
| Main et al. 2006 | Breast milk (n=130) from mothers of cryptorchid and control boys | No difference between cases and controls. MEP concentration in breast milk correlated to increased SHBG, decreased free testosterone and increased LH/free testosterone ratio. Same trends were seen for MBP |
| Boas et al. 2010 | Spot urine, children aged 4-9 years (n=845) | MEP urine concentration negatively associated with total $T_3$ serum levels in girls but not boys. Also negatively associated with free $T_3$ when unadjusted but this association is no longer significant after creatinin adjustment |
| Frederiksen et al. 2012 | First morning urine, girls aged 6-19 years | No association between urinary MEP concentrations and timing of breast development or pubic hair were observed although association to delayed pubic hair development were seen for several of the other phthalate metabolites measured. |
| Stahlhut et al. 2007 | NHANES men > 18 y (n=+650) | Urinary MEP concentration significantly positively associated with waist circumference and with insulin resistance (HOMA). Urinary MEP levels explained 0.4% of the variation in waist circumference and 1.5% of the variation of HOMA. Other phthalate metabolites showed similar associations. |
| Duty et al. 2003 | Spot urine, men (n=168) | Urinary MEP concentration (gravidity adjusted) was significantly positively associated with DNA damage of sperm (comet assay). The association was only seen for MEP and not for other phthalates measured. |
| López-Carrillo et al. 2010 | First morning urine from | Urinary MEP concentration significantly higher |

| | | |
|---|---|---|
| | 233 women with breast cancer and 221 aged and area matched controls. Urine sample obtained before start of treatment | in the case group compared to controls. For some of the other phthalate metabolites (e.g. MBP) the reverse is seen. |
| Tranfo et al. 2012 | Spot urine from 56 infertile couples and 56 fertile couples | Higher urinary levels of MEP were found in the infertile couples compared to the fertile couples. Both the male and the female partners contributed to this difference. Causes of infertility in the case group were: endometriosis 32%, unknown female factor 57%, tubal disease 20%, anovulation 32%, abnormalities in the semen 14% |
| Rozati et al. 2008 | Serum from infertile women with and without endometriosis (n=99 cases and 135 age-matched controls) | Authors claim they found significantly higher levels of several diester phthalates including DEP in serum of the women with endometriosis - however, it seems as they used wrong statistics. Also potentially problems with measuring diesters in serum samples. This is not a good study and the results should be excluded . |

In addition, there are also studies with negative findings in relation to sperm quality.
For some of the observed MEP exposure - out-come associations there is no clear explanatory mechanism, e.g. there is no experimental data showing that DEP/MEP cause DNA damage. Also for some out-comes the same associations were seen as well for other phthalate metabolites present at the same time. Often good correlation can be seen between the concentrations of different phthalates in the same sample although generally MEP is less correlated than the metabolites of the more lipophilic phthalates. This could be due to a different exposure pattern of DEP or a different pharmacokinetics of DEP compared to the more lipophilic phthalates. Nevertheless, as MEP is present together with other phthalates showing same associations it may be difficult to differentiate between the contributions of the different metabolites. However, in Stahlhut et al. they modelled the effect of the individual metabolites and MEP in it self seemed to have the strongest association to increased insulin resistance. In Swan et al. and Main et al. very similar associations were seen for MEP and MBP.

***In vitro* data**

*In vitro* studies showing weak estrogenic effects of some phthalate esters such as DEHP, DBP, BBP and DCHP do not show estrogenic effects of DEP (Hong et al., 2005, Obuko et al., 2003). However, other studies show weak estrogenic effect of DEP *in vitro* at high concentrations (Harris et al., 1997, Oh et al., 2006, Parveen et al., 2008). Examination of estrogen responsive genes in an MCF-7 cell proliferation assay show estrogenic activity of DEP and correlations in gene expression patterns were seen for DEP and 17beta-estradiol (Parveen et al., 2008). Reporter gene assays for ERalpha and ERbeta did not show activation by DEP. Thus, DEP may have a weakly estrogenic mode of action, although results are conflicting.

***In vivo* human health**

A large number of studies on endocrine related endpoints have been performed for DEP, and as results are unequivocal, a comprehensive (though not necessarily complete) review of the available literature is presented. Overall, findings of reduced testosterone levels, delayed vaginal opening and increased incidence of abnormal sperm in a two-generation study (Fujii et al., 2005) point to endocrine disruption, but several other studies show that DEP does not share the same anti-androgenic mode of action as DEHP, DBP, BBP, DPP and DiBP and does not affect e.g. anogenital distance, fetal testosterone production, fetal testicular gene expression, nipple retention, and reproductive organ weights. Two other studies describe effects of DEP on semen quality, but it is not the same parameters that are altered in the three studies. Sperm concentration is affected in the one generation study described by Lamb et al., 1987; sperm linearity is altered in the 28-day-study by Kwack et al., 2009, and incidence of abnormal sperm is increased in the two-generation study by Fujii et al., 2005. Other studies including an enhanced 28-day study (Shiraishi et al., 2006) did not detect any sperm quality changes, but as data were not presented in the manuscript, the possibility of effects of DEP on sperm quality remains controversial.

Detailed study summaries are provided below.

**Brown et al., 1978**: A study on dietary exposure of rats showed increased relative testis weights in males exposed for 2, 6 or 16 weeks to 5% DEP in the diet. As these males also had significantly lower absolute weights this was not considered dose related.

**Foster et al., 1980**: This study compared the effects of large doses of 6 phthalate esters including DEP in male rats exposed orally to 2 g/kg bw/day for 4 days. No testicular damage (histology or zink content) was found for DEP, whereas DnPentyl phthalate and DnHP produced testicular atrophy and lowered testicular zink content.

**Foster et al., 1983**. A study comparing the reproductive toxic phthalate DPP with DEP showed that DEP did not alter testicular levels of various CYP450 enzymes (17alpha-hydroxylase, 17-20 lyase, 17beta dehydrogenase), whereas DPP did. In that study, young SD rats were exposed for 2 or 4 days with 7.2 mmol/kg bw/day of DEP or DPP (approximately 1600 and 2200 mg/kg bw/day, respectively). The number of animals per dose group is not noted. This study using high doses further supports the evidence that DEP does not share the same mode of action as other reproductive toxic phthalates. In several subsequent studies, low levels of testicular CYP450 enzymes have been shown in fetal testes of rats exposed to antiandrogenic phthalates by using gene expression analysis or immunohistochemistry.

**Fujii et al., 2005**: A large (n=24) two generation study was performed using dietary exposure to DEP at 600, 3000, and 15000 ppm (dam intake 51, 255, 1297 mg/kg bw/day). An increased incidence of abnormal sperm and tailless sperm was observed in F0 males at 3000 ppm, but not 15000 ppm, and in F1 males at 3000 and 15000 ppm (showing dose-response relationship). No other changes in sperm quality or testicular or epdididymal sperm number were seen.

Body weights of offspring during lactation and age at pinna detachment and incisor eruption was reduced at 15000 ppm. Age at vaginal opening was delayed at 15000 ppm in females, and no change in preputial separation was observed in males. In F0 males, epididymis weight (absolute but not relative) was reduced, and liver weights (relative but not absolute) were increased. In F1 parental males liver weights (abs and rel) were increased at 15000 ppm, thyroid weights (abs and rel) were increased at 3000 ppm, but not at 15000 ppm. Adrenal gland weight was decreased at 15000 ppm in F0 (absolute weight), but decreased in F1 males (relative weight). No changes in reproductive organ weights of adult F1 males were observed. Females had increased liver weights at 15000 ppm (abs and rel, F0 and F1). F1 females had increased kidney weights (abs and rel). Analyses of liver enzymes in F0 males showed significant increases in microsomale CYP3a2 and

CYP4a1 content at 15000 ppm. Hormone analyses showed decreased testosterone in F0 males at 3000 ppm and 15000 ppm, but showing no clear dose-response relationships.
At weaning, body weight was reduced in the highest dose group (F1 and F2), and several organ weights were changed, possibly due to the general reduction in body weight. Among these, it is noted that in F1 males absolute prostate weight was significantly reduced, whereas a slight decrease in relative prostate weight was not statistically significant. In F2 males, only slight reductions in absolute and relative prostate weights were seen, and this was not statistically significant. Weights of thyroids, epididymides, testes and seminal vesicles were not reduced. For females, in should be noted that uterus weight at weaning was reduced in F1 (absolute, not relative), and F2 (abs and rel) in the highest dose group. F2 females also had significantly lower relative uterus weights at weaning in the 3000 ppm group.
This study appears to be robust and shows that if DEP has any effect on reproductive development, then these effects are subtle, but could be related to an endocrine disrupting mode of action. It is noted in the discussion, that a preliminary study showed decreased prostate weight and increased liver weights and attributed this to enhanced activity of steroid-metabolic enzymes in the liver. The authors also note that the increase in CYP3a2 content could be related to changes in steroid metabolism. The decrease in serum testosterone levels, however, is not regarded as an adverse effect by the authors, as no change in reproductive capacity is seen (Fujii et al., 2005). However, it is well known that rats have a better reproductive capacity than humans, and that effects on rat semen quality does not necessarily affect rat reproduction. The observed slight changes in semen quality (increased incidence of abnormal sperm) at the top dose in F0 and F1 males, the reduction of testosterone levels in F0 males, and the subtle changes in prostate weights (F1 males and reported preliminary study) could be dose related and attributed to slight anti-androgenic activity of DEP. This is possibly due to enzyme induction in the liver at the top dose. It should be noted that the possible effect of DEP is seen at much higher doses than effects of other anti-androgenic phthalates, and that effects such as decreased AGD were not found, indicating much lower potency of DEP than e.g. DEHP, DBP, and BBP.
**Gray et al., 2000,** examined the effects of perinatal exposure to 6 phthalates on several endpoints known to be affected by anti-androgens. DEHP, BBP, DINP, DEP, DMP and DOTP were administered at 750 mg/kg bw/day from GD 14 to PND 3 (n=3 for DEP). DEP did not alter male AGD, reproductive organ weights, incidence of reproductive malformations, or induce nipple retention in males, whereas changes in these parameters were seen with DEHP and BBP, and nipple retention and increased incidence of malformations were seen with DINP. It should be noted that only 12 males from 3 litters exposed to DEP were examined in this study, and that this number may be too low to give any useful information on changes in rare findings such as changes in the incidence of malformations. In comparison, the weakly antiandrogenic phthalate DINP induced reproductive malformations in a low number of animals (4 of 52 examined males), but did not have any effects on anogenital distance or reproductive organ weights (similarly to DEP).
This study is not sufficiently large to conclude on effects of DEP on the incidence of reproductive malformations, but clearly does contribute to the evidence that DEP does not have the same reproductive effects as DEHP and BBP at the applied dose.
**Harris CA, et al., 1997**. The estrogenic activity of phthalate esters was tested *in vitro* in a yeast recombinant assay, expressing hER and lac-Z reporter gene. An estrogenic response was seen for five tested phthalate diesters (BBP, DBP, DIBP, DEP and DINP), whereas some monoester metabolites were inactive. The estrogenic potency of DEP was 0.0000005 compared to the potency of 1 for 17beta-estradiol. Maximum response was 30% at 1 mM compared with 17beta-estradiol and thus the maximum response was comparable to that of DBP and DiBP. This study shows that DEP is has weak estrogenic effects *in vitro*.
**Hong et al., 2005.** An *in vitro* study in human breast cancer estrogen-sensitive MCF-7 cells was applied to test for estrogenic activity of 5 phthalates. DEP did not induce cell proliferation, whereas BBP, DCHP,

DEHP, and DBP induced cell proliferation from 1, 10, 10, and 100 uM, respectively. An *in vivo* study in immature female SD rats was applied to test for influences of estrogenic phthalates on expression of the estrogen-regulated protein CaBP-9k. Females were exposed to test compounds including DEP at 600 mg/kg bw/day in corn oil by gavage on days 14, 15 and 16 after birth (n=5 rats per dose group). Rats were euthanized at day 17 and uteri collected for Northern blot and Western blot examination of CaBP-9k mRNA and protein expression levels. Results showed that positive controls Ethinyl estradiol and Diethylstilbestrol as well as the weak estrogens Octylphenol and Bisphenol A induced expression of CaBP-9k mRNA and protein levels, whereas none of the tested phthalates including DEP altered expression of CaBP-9k. These studies indicate no estrogenic activity of DEP.

**Howdeshell et al., 2008**. Pregnant SD rats were exposed to six different phthalates (BBP, DEHP, DBP, DiBP, DPP, or DEP) from GD 8 to 18 (n=3 for DEP in the two highest dose groups). DEP did not affect testosterone production in fetal rats at doses of 100, 300, 600, or 900 mg/kg/day.

This study indicates that DEP does not share the same mode of action as reproductive toxic phthalates, i.e. reducing fetal testicular testosterone production. It may be noted that the study includes a low number of litters, and that only one parameter, testicular testosterone production is investigated, whereas e.g. blood or testis levels of testosterone, anogenital distance or gene expression of enzymes involved in steroidogenesis were not measured.

**Kwack et al., 2009.** This 28-day study in 4-week-old rats compared the toxicity of several phthalate esters and their monoester metabolites at a dose level of 500 mg/kg bw/day for both diesters and monoesters. DEP was found to induce a significant reduction in sperm linearity, but although slight decreases in sperm concentrations and sperm motility were also seen, these apparent decreases were not statistically significant (reduction to 84% and 76% of control values, respectively). However, exposure to the metabolite MEP did reduce sperm concentration and sperm motility significantly (reduction to 59% and 44% of control values, respectively). Sperm linearity was reduced to 82% (*) and 88% (NS) of control values by DEP and MEP, respectively. Weights of testes or epididymides were not changed by DEP or MEP in that study. It may be noted that the molar weight of MEP (194 g/mol) is much lower than the molar weight of DEP (282 g/mol) so that the molar dose is higher for MEP than for DEP, and that this could in part explain the more marked effect of MEP than DEP on sperm count and sperm motility, although differences in bioavailability of the active metabolite (probably MEP) and kinetics may also contribute to the observed differences.

**Lamb et al., 1987**. Toxicities of four phthalates were compared in continuous breeding studies. Increased absolute prostate weight and decreased sperm concentration was seen in adult offspring (F1) exposed to 2.5 % DEP in the diet corresponding to estimated 2500 mg/kg bw/day. No changes in other sperm parameters were affected, whereas other phthalate esters affected several sperm parameters. These measures were only made for the highest dose group. This study shows possible effects of DEP on sperm concentration at high doses, but the pattern of effects is not comparable to the other tested phthalates (DBP, DnHP, DEHP), which also affected sperm motility and abnormality rate, and decreased rather than increased weights of reproductive organs including prostate. This may point to a different mechanism of action of DEP compared to the known potent reproductive toxic phthalates.

**Liu et al., 2005**. A study of gene expression changes in fetal rat testes after exposure to different phthalates revealed that DEP (and DMP and DOTP) did not induce the same changes in gene expression as DEHP, DBP, BBP and DPP. DEP did not change anogenital distance in male fetuses, whereas DEHP, DBP, BBP and DPP did. In that study, pregnant SD rats were exposed to 500 mg/kg bw/day of the respective phthalates from GD 12 to 19, and testicular gene expression was examined by microarray and RT-PCR at GD 19 (n= 5 in phthalate groups and n=10 in control group).

This study supports the evidence that DEP does not influence fetal testicular development in the same manner as the known reproductive toxic phthalates. This study may not be the most sensitive to detect

endocrine disrupting activities, but does show that DEP does not act as an endocrine disrupter when using the applied study design.

**Okubo et al., 2003**. *In vitro* studies in human breast cancer estrogen-sensitive MCF-7 cells were applied to test for estrogenic activity of 19 phthalate diesters or monoesters. 17beta-estradiol and 4OH-tamoxifen were included as positive controls. DEP and the monoester for DEP, MEP did not induce cell proliferation in that assay. DCHP, DEHP and BBP were the only tested phthalates that induced cell proliferation.

**Oh et al., 2006**. An *in vitro* study in the E-screen study in MCF-7 cells showed high proliferation indicating an estrogenic effect. The effect was seen only in the highest tested concentration of 100 uM. This study indicates that DEP has weak estrogenic effects *in vitro*

**Parveeen et al. 2008**. An MCF-7 cell assay was applied to examine the effects of phthalates on estrogen-responsive genes. This assay is normally used as a proliferation assay but here, a DNA microarray was applied to compare the patterns observed and to test whether responses were related to their estrogenic activity. This study is one of several studies showing weak estrogenic effects of DEP *in vitro*.

**Pereira et al, 2007**. A two-generation study of DEP, polychlorinated biphenyls (Clophen A60), or a mixture of Clophen A60 and DEP was perfomed to study effects on adrenal and thyroid gland. 50 mg/kg feed per day was mixed in corn oil into the diet (equal to 2.85 mg/kg bw/day?). All three exposure groups showed shrinkage of thyroid follicles, loss of thyroglobulin and fibrosis of interfollicular epithelium in the parental and F1 generation. No quantitative measures or statistical analyses were presented. It is not clear whether these results do indeed indicate a thyrotoxic effect of DEP.

**Shiraishi et al., 2006**, investigated the effects of DEP in a study based on a draft protocol for the enhanced OECD test guideline 407 (28-day study) including endpoints intended to detect endocrine-mediated effects (n=10 males and 10 females per group). Oral doses of 40, 200 and 1000 mg/kg bw/day of DEP did not lead to changes in sperm quality or estrous cyclicity (data not listed). Adrenal weight was increased in the high dose group in females, but not in males, and estradiol levels were reduced in the high dose group in males, but not in females. No other dose-related changes were seen in organ weights or hormone levels.

**Takeuchi et al., 2005**. Reporter gene assays were applied to test for agonistic or antagonistic activity of serveral phthalate esters on human ERalpha, ERbeta and AR. No effect of DEP was detected, whereas other phthalates did show estrogenic, antiestrogenic and/or antiandrogenic effect.

**Taxvig et al., 2011**. An *in vitro* T-screen assay was applied to test for the ability of selected phthalates and pesticides to bind and activate the thyroid receptor. No effect of DEP was detected indicating that DEP does not bind and activate the thyroid receptor.

**Yamasaki et al 2005** reviews a set of parallel two-generation studies on potential endocrine disrupters including the study by Fujii et al., 2005, and concludes that DEP is tested positive for endocrine disrupting activity in that study. The decreased testosterone levels in males and the delayed vaginal opening in females is considered indicative of endocrine disrupting activity.

***In vivo*, ecotoxicity**

Many ecotoxicological studies have examined the endocrine disrupting effects of Diethylphtalat. Daphnids exposed to the compound took significantly more time to complete four molts than did the controls with NOEC/LOEC of 11.2/22.4 mg/l. Inhibited the activity of chitobiase (also known as N-acetyl-b-glucosaminidase) in the epidermis and hepatopancreas of fiddler crab. Inhibition of larval development to the metamorphosis stage in abalone *Haliotis diversicolor supertexta*. Vitellogenin induction in fish (LOEC = 100 µg/l).

Detailed study summaries are provided below:

**Zou et al 1997**: The effects of diethyl phthalate, on molting of Daphnia magna were investigated. Daphnids exposed to diethyl phthalate took significantly more time to complete four molts than did the controls with NOEC/LOEC of 11.2/22.4 mg/l. The results support the hypothesis that some xenobiotics which disrupt endocrine processes in vertebrates can also interfere with the hormonally regulated molting process in arthropods through acting as antagonists of endogenous ecdysteroids by binding to and thereby blocking the ecdysteroid receptor.

**Zou et al 1999:** Seven-day exposure of fiddler crabs, Uca pugilator, to diethyl phthalate at 50.0 mg/l significantly inhibited the activity of chitobiase (also known as N-acetyl-b-glucosaminidase) in the epidermis and hepatopancreas. The inhibitory effects rendered by diethyl phthalate and PCB29 can at least partly account for the delayed molting they cause because chitobiase is needed to break down the old exoskeleton of crustaceans prior to ecdysis. Since chitinolytic enzymes are apparently the products of ecdysteroid regulated genes in arthropods, the decline in chitobiase activity after exposure to diethyl phthalate, 4-(tert)-octylphenol, and PCB29 along with the delayed molting they cause strongly suggests that these xenobiotics disturb the Y-organ–ecdysteroid receptor axis. Such disturbance may involve an interaction between ecdysteroid receptors and steroid mimics where the steroid mimics act as antagonists of endogenous steroid molting hormones, and/or arise from the interference with synthesis and excretion of ecdysteroids by these compounds.

**Lutz et al 1999**. Binding to liver estrogen receptor in *Xenopus laevis* was investigated *in vitro*. The specificity of estrogen receptors as shown by competitive displacement experiments demonstrated receptors being highly specific just for estrogens, but not for other endogenous steroids having the following ranking of binding affinities: E2 > estrone > dehydroepiandrosterone > aldosterone> testosterone> corticosterone> progesterone. The affinity ranking of environmental chemicals compared to E2 was: E2> tetrachlorbiphenyl> diethylphthalate> 2,2-bis-4-hydroxyphenyl-propan bisphenol A > 4-nonylphenol> 3-t-butyl-4-hydroxyanisole>4-octylphenol> dichlor-diphenyl-trichlor-ethan (4,4'-DDT). Taken together the established radioreceptorassay for $^{3}$H-E2 binding in *Xenopus laevis* liver cytosol is useful to screen estrogen receptor binding of pure compounds or complex mixtures of them, which is the prerequisite for causing either estrogenic or antiestrogenic effects.

**Liu et al 2009**. In the present study, abalone Haliotis diversicolor supertexta larvae were exposed to DEP. The inhibition of larval development to the metamorphosis stage following exposure to PAEs was a more sensitive endpoint than that of embryonic development to the blastula stage. As stated in the introduction, PAEs are regarded as potential EDCs. Therefore the greater sensitivity is possibly due to the disrupting effects of PAEs on the hormonal system of the tested embryos, which plays an important role in the metamorphosis of abalone LOEC for metamorphosis disruption was 0.2 mg/l.

**Dinan et al 2001**.The $B_{II}$ bioassay was developed as a rapid and reliable tool for detecting potential insect growth regulators acting as ecdysteroid receptor (ant)agonists. Based on an ecdysteroid-responsive cell line from *Drosophila melanogaster*, this microplate assay is ideally suited to the evaluation of environmental contaminants as potential endocrine disrupters. Among the industrial chemicals, antagonistic activity was observed for bisphenol A median effective concentration (EC50) of 1.0 x $10^{-4}$ M and diethylphthalate (EC50 of 2.0 x $10^{-4}$ M (44 mg/l)).

**Barse et al 2007**.. This experiment evaluated effects of DEP in adult male (89 g) common carp (*Cyprinus carpio*) by exposing them to fractions of LC50 (1/500–1/2.5) doses with every change of water for 28 days. Vitellogenin induction metabolic enzymes, somatic indices and bioaccumulation were studied on 7th, 14th, 21st and 28th day. The 96th hour LC50 of DEP in fingerlings was found to be 48 mg/L. Compared to control, except increase ($P< 0.01$) in alkaline phosphatase activity (EC 3.1.3.1) and liver size, there was decrease ($P<0.01$) in activity of acid phosphatase (EC 3.1.3.2), aspartate aminotransferase (EC 2.6.1.1), alanine aminotransferase (EC 2.6.1.2) and testiculosomatic index following exposure to 1, 5 and 20 ppm

DEP. Significant (P<0.01) dose dependant vitellogenin induction (in muscle tissue) was observed with exposure of fish to 0.1, 1 and 5 ppm DEP. The bioaccumulation of DEP in testis, liver, brain, gills and more importantly in muscle tissues of fish increased significantly (P <0.01) with increase of dose from 1 to 5 ppm. Significant interaction (P< 0.01) of dose and duration of exposure indicated that exposure period of a week to two was sufficient to bring about changes in quantifiable parameters studied. Fish exposed to 20 ppm DEP became lethargic and discolored during onset of the 4th week. This is the first report describing metabolic changes and vitellogenin induction following exposure of *C. carpio* to DEP dose that is as low as 1/500th fraction of LC50. The observed vitellogenin induction shows endocrine disrupting activity of DEP. Vitellogenin induction is endocrine specific but not defined as adverse.

**Weight of evidence for ED and Category**

Based on human data DEP is evaluated as a suspected ED in Category 2a.

The delayed vaginal opening and increased incidence of abnormal sperm found in a two-generation study (Fujii et al., 2005), and the decreased sperm concentration and prostate weight seen in a one-generation study (Lamb et al., 1987) are adverse effects that may be induced by endocrine disruption. Reports of estrogenic activity are conflicting, and DEP does not appear to act via the same mode of action as other endocrine disrupting phthalates. Therefore, no mode of action has been shown for DEP. DEP can be evaluated as a suspected ED in Category 2a based on adverse effects on sperm quality, but lack of knowledge on mode of action.

The results from the ecotoxicological studies showed that daphnids exposed to diethyl phthalate took significantly more time to complete four molts than did the controls. DEP also caused vitellogenin induction in fish, inhibited the activity of chitobiase in the epidermis and hepatopancreas of fiddler crab, and inhibition of larval development to the metamorphosis stage in abalone *Haliotis diversicolor supertexta* was seen. Some of the invertebrate effects could be characterized as adverse but not as endocrine specific. Vitellogenin induction is endocrine specific but not defined as adverse. DEP is therefore evaluated as belonging to Category 2a (suspected ED).

Based on the combined evidence from the ecotoxicological studies and the *in vitro*, *in vivo* and epidemiological studies showing adverse effects in rodents and estrogenic mode of action in fish, DEP could be considered as an ED in category 1. However, the relationship between these two findings is somewhat unclear and consequently DEP is evaluated as a suspected ED in **Category 2a**.

According to DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which effects are observed, i.e. effects need to be observed at an oral dose of 10 mg/kg bw/day. For DEP, effects on sperm quality were seen at doses of 250 mg/kg or much higher, and DEP cannot be considered an endocrine disrupter of very high regulatory concern according to DE-UK potency criteria.

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**Dihexyl phthalate (DHP), CA S 84-75-3**
**Synonyms:** Di-n-hexylphthalate (DnHP),

**Human data**
No relevant data found.

***In vitro* data**
No relevant data found.

***In vivo* data, human health**
An overall evaluation of the available literature on DnHP leads to the conclusion that DnHP is an anti-androgen comparable to e.g. DEHP, DBP, DiBP and BBP. Impaired masculinization of male pups is observed following fetal exposure (Saillenfait et al., 2009a, Saillenfait et al., 2009b). Fetal/neonatal anogenital distance is reduced in males, the prevalence of nipple retention is increased, and reproductive tract malformations and altered testis histology is observed in adults (Saillenfait et al., 2009a, Saillenfait et al., 2009b). An older continuous breeding study showed male infertility, impaired semen quality and reduced weight of reproductive organs at high doses (Lamb et al., 1987), and these effects are compatible with an antiandrogenic mode of action. A NOAEL of 50 mg/kg bw/day and a LOAEL of 125 mg/kg bw/day could be determined for antiandrogenic effects based on reduced anogenital distance and an increased incidence of malformations (Saillenfait et al., 2009b). Two studies showed histological changes in thyroids of rats following short-term exposure. In a rat study, 3 to 21 days of dietary exposure to DnHP induced histological changes in thyroids and suppressed serum T4 levels (Hinton et al., 1986). In a NTP monograph on DnHP this finding is considered as ”sufficient data” to show that DnHP can cause thyroid toxicity (NTP 2003 and Kavlock et al., 2002).
An overall NOAEL of 50 mg/kg bw/day and a LOAEL of 125 mg/kg bw/day for endocrine disrupting effects is based on the study by Saillenfait et al., 2009b.

Detailed study descriptions are provided below.
**Foster et al., 1980**. In a study comparing several phthalate diesters, testicular atrophy was seen following 4 days of oral exposure to DnHP (2400 mg/kg bw/day). Relative testis weights were reduced compared to controls and histological examination showed atrophy of seminiferous tubules and complete loss of spermatocytes and spermatids. The histological effects of DnHP were slightly less severe than DBP and dipentyl phthalate. Zinc content was reduced compared to controls. This study shows evidence of reproductive toxicity of DnHP at a very high dose level in rats.
**Hinton et al., 1986.** In a rat study on short- and long-term exposure to DEHP clear signs of thyroid hyperactivity were shown. For DnHP a reduction in serum T4 levels was evident after 3, 10 and 21 days of dietary exposure (20,000 ppm) and ultrastructural changes in thyroids were seen and were comparable to effects of DEHP and DnOP. Hepatic peroxisome proliferation seen for DEHP was not seen for DnHP. This study shows evidence of a thyroid-disrupting effect of DnHP comparable to the effect of some other phthalates.
**Howarth et al., 2001.** Male Wistar rats were exposed to DnHP or DEHP or a mixture of DnHP and DEHP for 14 days and parameters for liver and thyroid toxicity were evaluated. Thyroid evaluation showed histological changes as an evidence of hyperactivity, i.e. reduction of follicular size and an increase in the proportion of follicular cells with a columnar appearance. These changes may be associated with an increased rate of thyroglobulin turnover, i.e. changes that may be secondary to liver toxicity. These thyroid changes are sometimes related to the hepatic peroxisome proliferation known for several phthalates, but no

peroxisome proliferation was seen for DnHP. This study points to thyroid hormone disrupting effects of DnHP.

**Kavlock et al., 2002**. See NTP 2003.

**Lamb et al., 1987**. Toxicities of four phthalates were compared in continuous breeding studies in CD-1 mice. For DnHP three dietary doses of 0.3, 0.6, and 1.2 % were applied. A dose-related decrease in infertility was observed and significant adverse reproductive effects were seen at the lowest dose tested. Semen quality and sperm concentrations were significantly decreased at the highest dose group, which also had extensive atrophy of seminiferous tubules. This study shows clear evidence of reproductive toxicity of DnHP in mice.

**Lamb et al., 1997**. This is a short summary of the findings in the study by Lamb et al., 1987, thus reaching the same conclusion that DnHP is a reproductive toxicant in mice.

**NTP 2003**. This comprehensive report by the NTP Centre for the evaluation of risks to human reproduction reviews all available toxicity studies on DnHP and compares human exposure levels to levels at which adverse effects can be seen. The same report is published in a peer reviewed journal with the reference Kavlock et al., 2002. The review concludes that there is "clear evidence of adverse effects" regarding reproductive toxicity and "limited evidence of adverse effects" regarding developmental toxicity. The conclusions on reproductive toxicity are mainly based on the studies by Lamb et al., 1987 and Foster et al., 1980. Further, it is stated that there is not sufficient hazard and exposure information to reach a concluding regarding the potential for DnHP to adversely affect human development or reproduction; and specifically no NOAEL can be determined.

**Saillenfait et al., 2009a**. A developmental study in SD rats was applied to test for developmental toxicity of DnHP and DCHP and included assessment of anogenital distance as an endocrine sensitive endpoint and evaluation of the degree of trans-abdominal testicular migration. Dams were exposed to 0, 250, 500 or 750 mg/kg bw/day of test compounds from gestation day 6 to 20, and fetuses were examined at gestation day 21. Anogenital distance of male fetuses was reduced by DnHP in all dose groups, and this was statistically significant with and without correction for body weight. Decreased body weight of fetuses was only present at the highest dose of DnHP. Testicular migration was affected by DnHP, as malpositioned testes were observed in 2 of 24 litters at the lowest dose, 9 of 21 litters at the middle dose, and in 11 of 17 litters at the highest dose. This study indicates that DnHP affects masculinization in a similar way as the known endocrine disrupters DEHP, DBP and BBP.

**Saillenfait et al., 2009b**. An in utero study was conducted to examine the effects of DnHP on male rat reproductive development. Pregnant SD rats (n=8-11 litters) were exposed to 500 mg/kg bw/day of DEHP or 50, 125, 250 or 500 mg/kg bw/day of DnHP from GD 12 to 21 and male pups were necropsied at PND 70-78 or PND 111-120. At doses from 125 mg/kg bw/day anogenital distance was reduced and malformations of the reproductive tract were observed (hypospadias, underdeveloped testes, undescended testes). At 250 and 500 mg/kg bw/day degeneration of seminiferous tubules and nipple retention were seen. In a preliminary study, reproductive tract malformations (including small penis, cleft prepuce, cleft phallus, vaginal pouch in addition to the above) were observed at 500 and 625 mg/kg bw/day of DnHP. This study shows that DnHP has comparable effects to DEHP and a NOAEL of 50 mg/kg bw/day can be determined.

***In vivo*, ecotoxicity**

In an ecotoxicology by Rhodes et al. (1995) effects of dihexylphtalate was examined in two species. The chronic toxicity studies were performed with *Daphnia magna* (0.008-0.15 mg/L, mean measured concentrations) and rainbow trout (*Oncorhynchus mykiss*) (0.014-0.22 mg/L, mean measured concentrations). In the daphnia test NOEC was 0.084 mg/L and LOEC was 0.15 mg/L for both survival and reproduction. In early life-stage studies with rainbow trout, no effects were observed on hatchability, survival, or growth at the tested concentrations of dihexyl phthalate.

**Weight of evidence for ED and Category**

An overall evaluation of the available literature on DnHP leads to the conclusion that DnHP is an anti-androgen comparable to e.g. DEHP, DBP, DiBP and BBP. Impaired masculinization of male pups is observed following fetal exposure Based on this, DnHP fulfills the criteria of being an endocrine disrupter, as several in vivo studies have found adverse effects on male sexual development and an anti-androgenic mode of action is highly plausible. A few studies also further point to interference with thyroid function. Based on these findings DnHP is evalued as an ED in Category 1.

Dihexyl phthalate affects reproduction in daphnia. However, survival is affected at the same concentration. Thus a general toxic effect cannot be excluded. At the tested concentrations, dihexyl phthalate has no effect on hatchability in early life stage studies with rainbow trout. Based on the *in vivo* ecotoxicity data there are no indications of endocrine disrupting properties.

Based on the combined evidence from *in vivo* and ecotoxicological studies, DnHP is evaluated as an ED in **Category 1**.

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which severe adverse effects are observed. For studies with oral dosing, effects at 10 mg/kg bw/day and below leads to a classification as an endocrine disrupter of very high regulatory concern. As DnHP shows endocrine disrupting effects (reduced anogenital distance and malformations of male reproductive organs) at 125 mg/kg bw/day, DnHP would not be evaluated as an endocrine disrupter of very high regulatory concern according to DE-UK potency criteria.

**References, *in vivo***

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**References ecotoxicology**

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**Ethylhexyl methoxycinnamate, CAS 5466-77-3**
**Synonyms: OMC,** Octyl methoxycinnamate 2-ethyl-hexyl-4methoxycinnamate, Octinoxate, EHMC

**Human data**
Two epidemiological studies with ethylhexyl methoxycinnamate have been performed. OMC permeated rapidly in human systemic circulation and reached maximum concentration 3-4 hours after dermal application (Janjua et al 2004). Dermal application of a mixture of benzophenone-3, 4-methylbenzylidene camphor and 2-ethylhexyl 4-methoxy cinnamate did not cause any biologically significant effect on reproductive hormones, but indicated a slight reducing effect on testosterone in males (Janjua et al 2004), nor did it cause any biologically significant effect on the hypothalamic-pituitary-thyroid axis in humans, but indicated a slight effect on thyroid hormones. However, results of the study are inconclusive due to too short duration of the experiment.

Detailed study summaries are provided below:
**Janjua et al. 2004**: An experimental single blinded Danish study on Caucasian volunteers: 15 males and 17 postmenopausal females, examined effect of sunscreen mixture on reproductive hormones levels in humans. A control cream was applied daily for 4 days during the 1. week. A sunscreen formulation with 10% of benzophenone-3, 10% of 3-(4-methyl-benzylidene) camphor and with 10% of 2-ethylhexyl 4-methoxy cinnamate was applied daily for 4 days during the 2. week. Both creams were applied in recommended amount: 2 mg/cm$^2$. Maximum plasma concentration of 2-ethylhexyl 4-methoxy cinnamate was reached 3-4 hours after application and was 10 ng/ml in females and 20 ng/ml in males. No significant alterations were seen in FSH, SHBG or LH in any sexes. Estradiol in females was unaltered between first and second week. Significant increase in Inhibin B in males and in testosterone in females was seen already at time 0 in the second week. Estradiol in males decreased significantly 2 and 4 hours, but not 3 hours after application of sunscreen formulation. Testosterone in males decreased significantly 4 hours after application of sunscreen formulation which is in a line with time for maximal plasma concentrations. No hormone levels during the first day later than 4 hours are available.
**Janjua et al. 2007**: An experimental single blinded Danish study on Caucasian volunteers: 15 males and 17 postmenopausal females, examined effect of sunscreen mixture on the hypothalamic-pituitary-thyroid axis in humans. A control cream was applied daily for 4 days during the 1. week. A sunscreen formulation with 10% of benzophenone-3, 10% of 3-(4-methyl-benzylidene) camphor and with 10% of 2-ethylhexyl 4-methoxy cinnamate was applied daily for 4 days during the 2. week. Both creams were applied in recommended amount: 40±3 g for male and 35±3 g for female. No alterations in TSH were seen in any sexes. TBG and T4 in males were unaltered between first and second week. Significant decrease in FT3 in males was seen already at time 0 in the second week. FT3, FT4 and T3 in females decreased sporadically. T3 and FT4 in males and TBG and T4 in females decreased significantly 3-4 hours after dermal application, which is in a line with the time for maximal plasma concentrations (Janjua et. al., 2004). No hormone levels during the first day later than 4 hours are available.

***In vitro* data**
*In vitro* studies of OMC have shown evidence of estrogenic but also other modes of action for this compound. OMC has been shown to act as an estrogen by altering gene transcription of (Heneweer et al 2005) and causing proliferation of MCF-7 cells (Schlumpf et al 2001, Schlumpf et al 2004), and by binding to the estrogen receptor (Schreurs et al 2005, Gomez et al 2005), whereas a study by Morohoshi et al (2005)

showed no binding to the estrogen receptor in a yeast two-hybrid assay, and Seidlova-Wuttke et al (2006a) did not find binding of OMC to either the ERα the ERβ or cytosolic E2 binding sites. Androgen receptor antagonism has been tested in two studies, and has not been seen in any of them (Ma et al 2003, Schreurs et al 2005). On the other hand, binding to the thyroid receptor *in vitro* has been reported (Hofmann et al 2009) and so has binding to the progesterone receptor, which was strong compared to ER binding (Schreurs et al 2005). Furthermore, OMC may affect neurotransmitters involved in GnRH release, as it has been shown to decreases GnRH release (Carbone et al 2010), and affect LHRH and amino acid neurotransmitters release from the rat hypothalamus *in vitro* (Szwarcfarb et al 2008). OMC may also affect the immune responses, as it has been shown to affect interferon-gamma and interleukin-10 *in vitro* in a mouse spleen test system (Rachon et al 2006).

***In vivo*, human health**

OMC has been shown to affect the endocrine system *in vivo*. Slight but significant increases in uterine weights have been seen in different experimental setups, using both intact immature and adult ovariectomized rats of different strains (Schlumpf *et al.* 2001, Klammer *et al.* 2005, Siedlová-Wuttke *et al.* 2006b). However, OMC also showed effects, which were opposite those seen after estradiol treatment, as increased serum LH levels (Siedlová-Wuttke *et al.* 2006a) and upregulated expression of the estrogen receptor beta (Klammer *et al.* 2005). In a 2-generation study, developmental exposure to OMC resulted in few adverse effects on reproduction, but a significant decrease in sperm cell number was seen (Schneider et al 2005). Another reproductive study has shown developmental OMC exposure to cause several adverse reproductive effects in the offspring, including reduced reproductive organ weights and reduced reproductive hormone levels, reduced sperm counts and neurobehavioural effects (Axelstad et al 2011). The LOAELs for the effects on reproductive endpoints have been between 333-1035 mg/kg/day and the NOEALs between 100-522 mg/kg/day.

OMC can also interfere with the hypothalamo-pituitary-thyroid (HPT) axis *in vivo*, as a number of studies have shown reduced levels of thyroxine in the blood. This effect has been seen in adult females rats treated with OMC for different periods of time (Axelstad et al 2011, Schmutzler *et al.* 2004, Siedlova-Wuttke *et al.* 2006a, Klammer *et al.* 2007), and in male offspring treated in the perinatal period (Axelstad et al 2011). The LOAELs for reduced T4 levels were between 200-500 mg/kg/day, and the NOAEL was 100 in the study by Klammer et al (2007), and NOAEL has not been determined in the other studies looking at thyroid endpoints *in vivo*.

Summaries of the *in vivo* studies are provided below:

**Schlumpf *et al.* 2001**: An uterotrophic assay was performed with immature 21 day old LE rats. The animals (n=5-13 ) were dosed with 0, 268, 522, 1035 , 1518 or 2667 mg/kg/day in the feed for 4 days. A significant increase in uterine weight was seen in the three highest dose group, and the ED50 was 935 mg/kg/day.

**Klammer et al. 2005**: Adult ovariectomized SD rats (n= 8-12) were treated for 5 days by gavage, with 10, 33, 100, 333 or 1000 mg OMC/kg bw/day. Doses of 333 and above increased uterus weight and altered gene expression in the pituitary. At the highest dose also gene expression of C3 in the uterus was altered in an estrogenic manner. However, the high dose also upregulated expression of the estrogen receptor beta, an effect opposite of what is seen after estradiol treatment.

**Schmutzler et al. 2004**: Adult ovariectomized SD rats (n=8-11) were treated with OMC in the feed for 12 weeks at doses of 200 and 1100 mg/kg/day. Both doses reduced activity of 5'-deiodinase (the enzyme that converts $T_4$ to triiodothyronine ($T_3$ )) in the liver, and the low dose also reduced thyroxine ($T_4$) levels in the blood (in the high dose the reduction was not significant).

**Siedlova-Wuttke et al. 2006a**: Adult ovariectomized SD rats were treated with OMC in the feed for 12 weeks at doses of 200 and 1100 mg/kg/day. Both doses caused decreased $T_4$ levels, although a statistically significant effect was only observed in the low dose group. The highest dose further caused significantly increased serum LH levels and decreased serum leptin and cholesterol levels in a manner similar to E2.
**Siedlova-Wuttke et al. 2006b:** Adult ovariectomized SD rats (n=11) were treated with OMC in the feed for 12 weeks at doses of approximately 200 and 1100 mg/kg/day. The higher dose stimulated uterine growth and increased thickness of the endometrial epithelium, while both doses increased progesterone receptor transcripts in the uterus and vagina similarly to E2.
**Klammer et al. 2007**: Adult ovariectomized SD rats (n=12) were treated for 5 days with 10, 33, 100, 333 or 1000 mg OMC/kg bw/day. The two highest doses reduced $T_4$ levels in serum which probably caused the observed reduction in deiodinase activity in the liver. The highest dose also caused reductions in T3 levels in serum and increased TSH receptor gene expression in the thyroid. No effect on the enzyme thyroid peroxidase was observed.
**Schneider *et al.* 2005**: A two-generation study where pregnant dams (n=20-25) were treated with OMC in doses of 150, 450 or 1000 mg/kg bw/day in the feed. Exposure began before mating and continued throughout gestation, lactation, adolescence, mating of the F1 generation and until weaning of the F2 generation. Exposure caused no adverse effects on reproduction and development, as no significant change in weights of the reproductive organs was seen. Based on this, the authors concluded that OMC displayed no estrogenic potential *in vivo*. However, the total number of spermatids/g cauda epididymis in the F1 generation was actually significantly reduced in animals receiving 1000 mg/kg bw/day. The authors inferred that the effects were caused by anomalously high control values, exceeding historical controls. No behavioral or thyroid endpoints were included in this study.
**Axelstad *et al.* 2011**: A developmental reproductive toxicity study, where Wistar rats (n=14-18) were dosed with OMC (500, 750 or 1000 mg/kg bw/day) during gestation and lactation. Thyroxine ($T_4$) levels showed a very marked decrease during the dosing in dams from all dose groups. T4 reductions were much less severe in the offspring, but significant in the male pups on postnatal day 16 in all three dose groups. ON day 16 males from the high dose group also showed reduced relative prostate and testis weights, and a dose-dependent decrease in testosterone levels. At eight months of age, sperm counts were reduced in all three OMC-dosed groups, and prostate weights were reduced in the highest dose group. The reproductive system of the female offspring was also affected, as both estradiol and progesterone levels were significantly lowered in PND 28 offspring. Behavioral changes were also observed in both male and female adult offspring, and these were probably not mediated solely by early $T_4$ deficiencies, as the observed effects differed from those seen in other studies of developmental hypothyroxinemia.

***In vivo*, ecotoxicity**
Ethylhexyl methoxycinnamate affects the transcription of genes involved in hormonal pathways including vitellogenin in most fish studies. Two studies examine the effects of ethylhexyl methoxycinnamate on daphnia reproduction. In one of the studies, ethylhexyl methoxycinnamate reduces reproduction, which is an adverse apical effect. However, general toxic effects cannot be excluded since growth inhibition also occurred.

Detailed study summaries are provided below.
**Schreurs et al. 2002**: Zebrafish, in which an estrogen responsive luciferase reporter gene has been stably introduced, were used for *in vivo* testing of UV-filters. Ethylhexyl methoxycinnamate (OMC) showed no estrogenic activity in this transgenic zebrafish assay at the tested concentration: 10 µM (2.9 mg/L).

**Inui et al. 2003**: In this study, the estrogenicity of ethylhexyl methoxycinnamate (OMC) was examined using male medaka (*Oryzias latipes*) in regard to production of vitellogenin (VTG) and choriogenin (CHG) which are known to be estrogen-responsive gene products. Exposure concentrations were: 0.034, 0.34, 3.4 and 34 mM. Exposure duration was 7 days.
An increase in vitellogenin plasma concentrations was observed. Increase in mRNA expression levels of vitellogenin subtypes VTG-1 and VTG-2 and choriogenin subtypes CHG-L and CHG-H in liver due to exposure to ethylhexyl methoxycinnamate was also seen. In addition, increased mRNA expression levels of estrogen receptor (ER) α in the liver due to exposure to ethylhexyl methoxycinnamate were also found. LOEC in this study was 0.034 mM (9.87 mg/L). This study shows that ethylhexyl methoxycinnamate has estrogenic activity in fish - and thus provides information about the endocrine mode of action. The observed effects are not adverse apical effects.
**Kunz et al. 2006**: In this study it was analyzed whether ethylhexyl methoxycinnamate (OMC) shows estrogenic activity in fathead minnows by the induction potential of vitellogenin after 14 days of aqueous exposure. Fish were exposed to five different concentrations of ethylhexyl methoxycinnamate: 8 (median measured), 100 (nominal), 500 (nominal), 889 (median measured) or 5025 µg/L (median measured). No significant differences were found between exposed and control groups.
**Fent et al. 2010**: This study reports on acute and chronic effects of ethylhexyl methoxycinnamate (EHMC) on *Daphnia magna*. In the acute toxicity test (OECD guideline 202), the LC50 value (48 h) was 0.29 mg/L. The chronic toxicity of ethylhexyl methoxycinnamate was determined in a 21 d reproduction study performed according to OECD guideline 211 with exposure concentrations of 1.28, 3.2, 8 and 20 µg/L. No adverse effects on either the number or sex of the offspring, or body size were observed after exposure to ethylhexyl methoxycinnamate.
**Christen et al. 2011**: This study evaluates the effects of measured water concentrations of 5.4, 37.5, 244.5 and 394 µg/L ethylhexyl methoxycinnamate (EHMC) on the expression of genes involved in hormonal pathways in the liver, testis and brain of male and female fathead minnows (*Pimephales promelas*). The transcription profile is compared with the plasma vitellogenin content, secondary sex characteristics, and gonad histology. Transcripts of the androgen receptor were significantly down-regulated in the liver of females with a LOEC of 37.5 µg/L ethylhexyl methoxycinnamate. In the liver of females, estrogen receptor α expression was down-regulated in a concentration-dependent manner, being significant at 394 µg/L ethylhexyl methoxycinnamate. Additionally, the 3β-hydroxysteroid dehydrogenase transcript was significantly decreased in the liver with a LOEC of 37.5 µg/L ethylhexyl methoxycinnamate in males and a LOEC of 244.5 µg/L ethylhexyl methoxycinnamate in females. The expressional changes were tissue-specific in most cases, being most significant in the liver. Vitellogenin plasma concentration was significantly increased at 244.5µg/L ethylhexyl methoxycinnamate in males. Ethylhexyl methoxycinnamate induced significant histological changes in testes and ovaries at 394 µg/L. Testes displayed a decrease in spermatocytes, and ovaries a decrease in previtellogenic oocytes. The induction of vitellogenin plasma concentrations and the histological changes in gonads suggest an estrogenic and/or antiandrogenic activity of ethylhexyl methoxycinnamate. On the other hand, the gene expression profile also shows an antiestrogenic (e.g.: downregulation of estrogen receptor α) activity of ethylhexyl methoxycinnamate. In conclusion, the data demonstrate that ethylhexyl methoxycinnamate displays multiple hormonal activities in fish and thus provide information on the endocrine modes of action. The observed effects are not adverse apical effects.
**Sieratowicz et al. 2011**: This study examines the effects of ethylhexyl methoxycinnamate on the green alga *Desmodesmus subspicatus* and the crustacean *Daphnia magna*. Exposure to ethylhexyl methoxycinnamate resulted in growth inhibition of *D. subspicatus* with a 72 h IC10 value of 0.24 mg/L. The EC50 concentration in the acute immobilisation test with *D. magna* was 0.57 mg/L. Chronic exposure of *D. magna* (OECD guideline 211) caused a concentration dependent and significant decrease in the length of adults and the

number of offspring with a NOEC of 0.04 mg/L and a LOEC of 0.08 mg/L. The study shows, that ethylhexyl methoxycinnamate affects reproduction in daphnia. This is an adverse apical effect - but not endocrine specific.
**Zucchi et al. 2011**: To identify molecular effects and modes of action of ethylhexyl methoxycinnamate a gene expression profiling in zebrafish using whole genome microarrays was applied. Transcriptome analysis and validation of targeted genes were performed after 14 days of exposure of male zebrafish. Concentrations of 2.2 µg/L and 890 µg/L ethylhexyl methoxicinnamate lead to alteration of 1096 and 1137 transcripts, respectively, belonging to many pathways. Genes involved in lipid metabolism and estrogenic pathway (vitellogenin 1), lipid biosynthesis (ptgds), vitamin A metabolic process (rbp2a), DNA damage and apoptosis (gadd45b), and regulation of cell growth (igfbp1a) were investigated by qRT-PCR analysis in whole body, liver, brain and testis.
The analysis showed tissue-specific gene profiles and revealed that ethylhexyl methoxycinnamate affects the transcription of genes involved in hormonal pathways including vitellogenin 1, estrogen receptor α, estrogen receptor β, androgen receptor, P450 aromatase B (cyp19b) and hydroxysteroid 17-β dehydrogenase-3. This study provides information on the endocrine modes of action of ethylhexyl methoxycinnamate. The observed effects are not adverse apical effects.

**Weight of evidence for ED and Category**
The present epidemiological study indicates some, but no biologically significant effect on levels of reproductive hormones and on the hypothalamic-pituitary-thyroid axis in humans following dermal treatment with a mixture of benzophenone-3, 4-methylbenzylidene camphor and 2-ethylhexyl 4-methoxy cinnamate in the recommended amount. The study is limited in its duration and does not provide any information about the effect of dermal treatment with ethylhexyl methoxycinnamate alone. The results are therefore inconclusive.

When evaluating the results from the *in vivo* data on human health, ethylhexyl methoxycinnamate fulfills the criteria of being an endocrine disrupter. The effects include increased uterine weight in short term studies and adverse reproductive effects, including decreased reproductive organ weights and decreased sperm count and altered hormone levels in developmental studies. As some of the *in vitro* studies have shown estrogenic mode of action, and furthermore both *in vivo* and *in vitro* studies have shown effects on the thyroid hormone system, OMC is evaluated as an ED in Category 1.

Ethylhexyl methoxycinnamate affects the transcription of genes involved in hormonal pathways including vitellogenin in most fish studies. Two studies examine the effects of ethylhexyl methoxycinnamate on daphnia reproduction. In one of the studies, ethylhexyl methoxycinnamate reduces reproduction, which is an adverse apical effect. However, general toxic effects cannot be excluded since growth inhibition also occurred. Based on *in vivo* ecotoxicity data, ethylhexyl methoxycinnamate is categorized as a suspected ED (Category 2a).

Based on the combined evidence from all the studies, ethylhexyl methoxycinnamate is evaluated as an endocrine disrupter in **Category 1.**

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which effects are observed, i.e. effects need to be observed at an oral dose of 10 mg/kg/day. For OMC the dose levels needed for observation of adverse effects on the endocrine system are

200 mg/kg or higher, and OMC can therefore not be classified as an endocrine disrupter of very high regulatory concern according to DE-UK criteria.

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**Metam natrium, CAS 137-42-8.**
Synonyms: metam sodium (MS), Vapam, sodium methyldithiocarbamate (SMD)
Its metabolite = MITC

**Human data**
The only study on human data does not deal with an endocrine specific endpoint. No direct exposure measurement is provided. Thus the study is unsuitable for inclusion in the evaluation of metam natrium.

**Reynolds et al, 2005:** A population-based case-control study investigated the potential correlation between incidences of childhood cancers and mothers residential proximity to agricultural applications of pesticides at the time of the child's birth. Agricultural application of the pesticide metam natrium was associated with higher leukemia risk when comparing the highest and the lowest category of metam sodium use density (OR = 2.05; 95% confidence interval = 1.01-4.17).

***In vitro* data**
No relevant data found.

***In vivo*, human health**
Overall, metam sodium (SMD) have been shown to block ovulation in rats, however there is probably a temporal window in the day of prooestrous during which SMD is most effective in blocking ovulation. A study in rats have demonstrated a dose dependent suppression of the rise in LH, a dose related decrease in serum prolactin and a decrease in percentage of ovulating rats. MITC (the metabolite of sodium natrium) produce a decrease in foetal body weight and size. Moreover doses below those resulting in maternal toxicity produce pre- and post-implantation loss of embryos.
In a study by Goldman et al. 2007 oral administration of 50-200 mg SMD/kg to 60-day old female Sprague-Dawley rats was found to suppress hypothalamic norepinephrine following single exposure and cause increasing incidence of segments of persistent dioestrus (possibly reflecting pseudo pregnancy) following 3 weeks exposure. No significant changes in LH surge and oocyte release were present in normal cycling animals. Intraperitoneal (i.p.) administration on prooestrous of 25-300 mg SMD/kg to 90-day old female Long Evans rats (Goldman et al. 1994) demonstrated a dose dependent suppression of the rise in LH, a dose related decrease in serum prolactin and a decrease in percentage of ovulating rats (prevented by prior GnRH administration). The authors find it likely that SMD do not block the LH surge but causes a shift in the time of its appearance on prooestrous. Myers et al. (2005) studied the mechanism of thymic atrophy caused by SMD by administering 100-300 mg SMD/kg by oral gavage to 8-12 week old female B6C3F1 mice. Thymic cellularity decreased dose dependently and $CD4^+CD8^+$ thymocytes (immature thymocytes) were most significantly depressed. Serum corticosterone was increased and returned to baseline levels within 6-8 hours. Adrenalectomy and aminoglutethimide treatment (inhibit synthesis of nascent corticosterone) prevented thymic atrophy, indicating that SMD causes thymic atrophy by an adrenal mediated mechanism. The immunotoxic effect of SMD has been confirmed by others (Pruett et al. 1992, 2001, 2005, 2006 and 2009) who demonstrate that oral or dermal administration of 50-300 mg SMD/kg to mice elevate corticosterone levels which cause thymic atrophy with a rapid selective depletion of $CD4^+CD8^+$ cells (immature thymocytes) and a more slowly depletion of mature thymocytes. Pruett et al. also suggest three potential mechanisms of action in inhibiting inflammation and decreasing resistance to infection: 1) acting as a free radical scavenger, 2) acting as a potent copper chelator (affecting signal molecules) and 3) induces a classical neuroendocrine stress response characterized by elevated serum corticosterone concentrations which could affect cytokine production.

Study summaries are reported below.

**Goldman et al. 2007**. This study use 60-day old female Sprague Dawley-rats and administer a single oral exposure of metam sodium (MS) (200 mg MS/kg) at prooestrous or 3 week oral exposure (0, 50, 100 or 200 mg MS/kg). Outcomes measured are: oestrous cyclicity, LH surge, ovulation and hypothalamic and caudate catecholamine (CA) (norepinephrine, NE and dopamine, DA and DOPAC (3,4-dihydroxy-phenylacetic acid) reflecting DA turnover). A single oral administration at prooestrous significantly suppressed hypothalamic (anterior and posterior) NE. With 3 week oral exposure an increasing incidence of segments of persistent dioestrus (possibly reflecting pseudo pregnancy) was observed with 100 and 200 mg MS/kg. Body weights decreased slightly according to dose (for 50, 100 and 200 mg MS/kg BW were 96%, 96% and 92% of controls respectively). No significant changes in LH surge and oocyte release were present in normally cycling animals. A significant decrease in DA turnover of the hypothalamus and a significant fall in ratios of DOPAC to DA in the caudate were found. The authors discuss that MS would not be expected to cause an alteration in the endocrine regulation of reproductive activity in two-generation reproductive tests, 90-day oral toxicity protocol or chronic bioassay because of homeostatic adjustment by the hypothalamus (refers to colleagues showing homeostatic readjustment of reproductive functions in response to extended chemical exposure), however adaptability of CNS regulatory activity depends upon the nature of the insult.

**Goldman et al. 1994**. This study uses 90-day old Long Evans female rats (4-5 months old at time of experimentation) and investigates effect of IP SMD on hormonal control of ovulation. 3 experiments are described. *Experiment 1 (effect of SMD on LH surge)*: bilateral ovariectomies were performed on regularly cycling rats. IP injections of 0, 25, 50 or 100 mg SMD/kg were administered at 1100 h (prior to anticipated rise in serum LH) and LH determined at 1300, 1500, 1700 and 1900 h. Rats were decapitated at 2100 h and pituitary weight and hypothalamic catecholamine (norepinephrine (NE) and dopamine (DA)) concentrations measured. In addition several rats receiving 100 mg SMD/kg were injected with adrenergic receptor agonist clonidine at 1200 h. *Experiment 2 (effect of SMD on ovulation)*: IP injections of 0, 50, 100, 200 or 300 mg SMD/kg were administered at 1300 h on prooestrous. Animals were euthanized the following day between 1200 and 1300 h. In addition a group was also injected with gonadotropin-releasing hormone 1 h prior to SMD (200 mg/kg) administration and a group was given 300 mg SMD/kg at different times over the day of vaginal prooestrous. Oocytes were collected and quantified. *Experiment 3 (SMD effects on changes in brain catecholamines during presurge periode)*: IP injections of 0, 50, 100, 200 or 300 mg SMD/kg at 1300 h on prooestrous, decapitation 1 or 3 hours later. Determination of serum LH, serum estradiol and hypothalamic catecholamines. In addition a group was dosed PO at 1300 h prooestrous with MITC (and vehicle control and SMD) representing 300 mg SMD/kg. Either determination of changes in hypothalamic catecholamines at 3 h post-treatment or ovulation evidence at 1200 h the following day. IP injections of SMD suppress the rise in LH dose-dependently (100 mg SMD/kg completely suppresses LH). Additional clonidine injection causes a pulsatile reelevation of LH. Hypothalamic NE concentrations decline with increasing dose. A dose-related decrease in percentage of ovulating rats for 50-300 mg SMD/kg was prevented by prior GnRH administration. No differences in serum estradiol but a dose-related suppression in serum prolactin. Hypothalamic epinephrine declines comparably with NE accompanied by a prompt elevation in DA (consistent with suppression in the conversion of DA to NE). PO administration of SMD and MITC showed similar alterations in hypothalamic catecholamine but did not block ovulation. There is probably a temporal window in the day of prooestrous during which SMD is most effective in blocking ovulation. It seems likely that SMD do not block the LH surge but causes a shift in the time of its appearance on prooestrous. Compares SMD with disulfiram and its metabolite diethyldithiocarbamate with regard to interfering with catecholamine neurotransmitter metabolism.

**Myers et al. 2005**. This study investigates the mechanism by which SMD causes thymic atrophy in mice. Eight-twelve weeks old female B6C3F1 mice received 300, 200 or 100 mg SMD/kg by oral gavage, restraint for 2 hours and adrenalectomy or administration of aminoglutethimide SC (30mg/kg) (to inhibit synthesis of nascent corticosterone). Cell populations in thymic and splenic tissues were quantified by flow cytometry and serum corticosterone was measured. Thymic cellularity decreased in a dose dependent manner with maximum atrophy at 300 mg SMD/kg. DP thymocytes (CD4+CD8+) were most sensitive and most significantly depressed. 300 mg SMD/kg caused an increase in serum corticosterone which returned to baseline levels by 6-8 hours. Aminoglutethimide treatment and adrenalectomy prevented thymic atrophy by corticosterone in mice receiving 200 mg SMD/kg. Results indicate that SMD causes thymic atrophy by an adrenal mediated mechanism, both peak of serum corticosterone and duration of exposure seem to be important in the induction of thymic atrophy by a stressor.

**Pruett 1992.** In this study female B6C3F1 mice, 6-10 weeks of age, received 300 mg SMD/kg by daily gavage for 3, 5, 10 or 14 days (n=6) or 50, 100, 150, 200, 225 or 300 mg SMD/kg for 7 days and were euthanized 24h after the last dose. In one experiment SMD was administered dermally for 4 days (n=5) with euthanasia at day 5. Oral administration of 300 mg SMD/kg resulted in transient behavioral changes shortly after administration and decreases in body weights by all durations of 300 mg SMD/kg administration (10% or greater at 10 days of treatment indicating generalized toxicity). Oral administration of SMD resulted in a rapid selective depletion of the major subpopulation of thymocytes (CD4+CD8+) and a more slowly depletion of mature lymphocytes in thymus and spleen. Thymus weight were decreased for all durations and doses of SMD, haemoglobin and hematocrit decreased following 10 and 14 days of 300 mg SMD/kg (neutrophils increased and lymphocytes decreased), spleen weight and number of macrophage forming colonies in the bone marrow increased dose dependently which is interpreted as a homeostatic mechanism to compensate for the loss of lymphocytes. Liver and kidney weights were unaffected. *In vivo* antibody response was not affected. Splenic NK activity was significantly and dose-dependently decreased following 7 daily doses. Dermal administration significantly suppressed NK activity at 200 and 300 mg SMD/kg/day and an increase in spleen weight at 200 mg/kg/day and decrease in thymus weight at 300 mg/kg/day was observed. Body weight decreased dose dependently and was significant at 300 mg/kg/day. The group concludes that most of the immunological effects of SMD are not secondary to generalized toxicity as only high doses significantly decreased body weight. Humoral immune responses are not the major target for the acute effects of SMD. Surviving lymphocytes are able to proliferate and differentiate in response to mitogen or antigen. NK cell function is sensitive to SMD but whether it is due to elimination or dysfunction is not known.

**Pruett 2001**. Summary and review regarding toxicology of metam sodium.

Immune system toxicity: Allergic hypersensitivity due to occupational exposure has been reported in humans, with the characteristics typical of a specific delayed hypersensitivity response (Richter 1980). MITC is a known skin and respiratory irritant (Richter 1980). Immunosuppressive effects after oral exposure in mice with targeting of lymphocytes in spleen, peripheral blood and thymus. Route of exposure is not a critical factor in targeting immune function, same effect with oral and dermal exposure. Effects on immunological parameters differ between mice and rats. Comparable decreases in thymus weight and cellularity occurred in rats and mice when rats received approximately one third of the SMD dosage given to mice (Keil et al. 1996). Suppressed NK activity and increased spleen weight was not observed in rats. Cytotoxic effect of dithiocarbamates *in vitro* does not correlate with immunological effects *in vivo*.

Developmental toxicity: MITC produce decrease in fetal body weight and size and dosages below those resulting in maternal toxicity produces pre- and post-implantation loss of embryos (Alexeeff et al. 1994). A greater than expected rate of unconfirmed spontaneous abortions in humans exposed during the third trimester in relation to the Sacramento River spill has been reported.

**Pruett 2005**. In this study 50, 100, 200 or 300 mg SMD/kg administered by oral gavage to 8-12 week old mice (B6C3F1) resulted in a dose dependent decrease in IL-12 production and increased IL-10 production. Dermal application of 300 mg SMD/kg induces similar cytokine and stress response. Both oral and dermal application affects a LPS-induced cytokine response, and oral administration of 200 and 300 mg SMD/kg decreases resistance to E. Coli peritonitis.
**Pruett 2009**. In this study, mice 8-14 weeks old received 50-300 mg SMD/kg which resulted in a decreased concentration of glutathione, suggesting oxidative stress, in peritoneal cells (mainly macrophages). It is demonstrated that this modulates the effects of SMD on IL-6 and IL-12 production and the LPS-induced IL-6 production.
**Thompson 2002.** Oral exposure to adult male rats showed hepatotoxic effects of SMD (2 mmol/kg).

***In vivo*, ecotoxicity**
Overall, sodium metam shows teratogenic effects in embryonic zebrafish. *In vivo* ecotoxicity studies on endocrine related endpoints are needed for evaluation of the endocrine disrupting potential of sodium metam in wildlife.

Study summaries are reported below.
***Haendel et al. 2004***: Embryonic zebrafish were exposed to sodium metam from 4 hours post-fertilization (hpf) to 24 hpf and allowed to develop in clean water. The lowest observed adverse effect level (LOAEL) for both notochord defects and decreased hatching rate was 0.2 µM (26 µg/L). At 120 hpf, 98% of the control animals hatched from their chorions, compared to 5% at 0.8 µM.
This study shows adverse effects of sodium metam on embryonic zebrafish (notochord defects and decreased hatching). However, it is not known if these effects are endocrine related.
***Tilton and Tanguay 2008:*** Embryonic zebrafish were exposed to 1.0 µM (129 µg/L) sodium metam from 4 hours post-fertilization (hpf) to 24 hpf and total RNA was isolated at 11, 14, 18 and 24 hpf. Abnormal somitogenesis; muscle and neuronal gene expression were affected at 18 hpf, when notochord distortion started. Primary motor neurons were irregular and slightly malformed, secondary motor neurons in areas of distorted notochord did not reach the developing muscle and exhibited stunted neuronal tracts and irregular branching.
This study shows effects of sodium metam on embryonic zebrafish (distortion of notochord, muscular system and nervous system). However, it is not known if these effects are endocrine related.
***Tilton et al. 2008:*** In this study it was investigated whether cell death plays a role in the manifestation of sodium metam-induced notochord distortions in the developing zebrafish and if thiol-containing compounds or antioxidants could modify this developmental toxicity.
The results show that induction of apoptotic pathways and widespread cell death are not involved in the manifestation of the adverse developmental outcomes following sodium metam exposure. However, cellular thiol status or critical sulfhydryl moieties are important considerations in the mechanisms of sodium metam developmental toxicity.
This study shows notochord distortion after sodium metam exposure of embryonic zebrafish. The mechanism of this distortion is investigated. However, endocrine related mechanisms are not considered.
***van Boxtel et al. 2010:*** In this study, it was shown that exposure to sodium metam from 3 hpf until 6.5 dpf leads to craniofacial abnormalities in developing zebrafish. The effects observed on craniofacial development were dose dependent with an EC50 value around 1µM (129 µg/L).
Like the other studies, this study shows teratogenic effects of sodium metam on embryonic zebrafish.

**Weight of evidence for ED and Category**

*In vivo* studies in rats have shown adverse effects, e.g. blocked ovulation and persistent dioestrus, as well as relevant endocrine modes of action such as rise in LH and a dose related decrease in serum prolactin. Thus, metam natrium fulfil the criteria of being an endocrine disrupter in Category 1. The substance is a dithiocarbamate and some of the effects are like the effects of thiram, so read across to thiram also support this evaluation.

Based on the combined evidence from all studies, metam natirum is evaluated as an ED in **Category 1**.

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which adverse effects are observed, i.e. effects need to be observed at an oral dose of 10 mg/kg/day or lower. Adverse effects of metam natrium has been found at 100 mg/kg or higher and consequently metam natrium can not be considered an endocrine disrupter of very high regulatory concern according to the DE-UK potency criteria.

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**Methyl tertiary butyl ether (MTBE), CAS 1634-04-4**
**Synonyms:** 2-Methoxy-2-methylpropane.

**Human data**
Three human studies have been found on MTBE, they are, however, unsuitable for inclusion in an evaluation of the endocrine disrupting potential of MTBE as none of them include endpoints related to endocrine function.

Study summaries are provided below.
**Zhou et al, 1999**: The potential health effects of MTBE were investigated among 96 occupationally exposed workers from Chinese petroleum factories. Controls constituted 102 unexposed subjects working in the same factories. Data from questionnaires showed a higher incidence of various health complaints (eye irritation, dizziness, insomnia, nausea or vomiting etc.) among the exposed group as compared to the unexposed group. Data does not deal with endpoints related to endocrine function. No direct exposure data are available. Thus, the study is unsuitable for inclusion in an evaluation of the endocrine disrupting potential of MTBE.
**Ahmed, 2001**: A part of this review deals with studies on human effects of MTBE exposure. The original studies referred to focuses on health effects like headache, eye irritation, nose/throat burning, nausea, dizziness etc. Human data referred to in the review does not deal with endocrine related endpoints. Thus, the studies referred to as well as the review itself are unsuitable for inclusion in an evaluation of the human data on endocrine disrupting potential of MTBE.
**McGregor, 2006**: A part of this review deals with studies on human effects of MTBE exposure. The original studies referred to focuses on health effects (seven key symptoms) like headache, eye irritation, nose/throat burning, cough, nausea, dizziness and disorientation. No studies deal with testicular or thyroid effects, which have been dealt with in animal studies. One study dealing with immunological effects provided negative results. Human data referred to in the review does not deal with endocrine related endpoints. Thus, the studies referred to as well as the review itself are unsuitable for inclusion in an evaluation of the human data on endocrine disrupting potential of MTBE.

***In vitro***
Li et al (2006) investigated the toxicity of methyl tert-butyl ether (MTBE) on mouse spermatogenic cells *in vitro*. The results suggest that a high dose MTBE could exert a direct toxic effect on Sertoli cells that would impair their function and subsequently impair spermatogenesis or even cause cell death. The Sertoli cell is a terminally differentiated testicular cell in the adult that is required to maintain the process of spermatogenesis. Sertoli cells have multiple functions such as providing the cytoarchitectural support and microenvironment for developing spermatogenic cells.

***In vivo***
The effects observed in animal studies of adult rats include: decreased relative ovary and pituitary weights and increased oestrous cycle length (Moser et al 1998), decreased serum testosterone, DHT, LH, prolactin and T3 levels (Williams et al 2009), increased abnormal sperm percent and irregular histopathology of testes and altered levels of testosterone, LH, FSH (Li et al 2008). Thus the effects observed in adult rats include both adverse effects (increased abnormal sperm percent, irregular histopathology of testes) and relevant mode of action data (altered levels of testosterone, LH and FSH).

Detailed study summaries are provided below:

**Moser et al 1998**: After female B6C3F1 mice (12 per group) were exposed through vapour to 8,000 ppm MTBE for either 3 or 21 days or 4 or 8 months, their body weight gain and ovary and uterine weights significantly decreased, and the number of uterine glands and epithelial layers in their cervix and vagina also decreased and the oestrus cycle length was increased (Moser *et al.*, 1998). Authors note that effects were not mediated through the oestrogen receptor (ER). The dose used in this study is very high. The general US public is routinely exposed to a maximum of 2.6 ppm in the area where customers or attendants pump gas and 0.2 ppm at the perimeters of gas stations (Anderson et al, 1993 in Moser et al 1998).

**Williams et al 2000a**: 9 week old males SD rats, dosed for 15 or 28 days with 250, 500, 1000, 1500 mg/kg/day. The relative organ weights increased at various doses for the adrenal gland, pituitary gland, kidneys, liver and testes; decreased serum testosterone, DHT, LH, prolactin and T3 at highest dose after either 15 or 28 days. For instance the testicular weights of these rats increased significantly following oral administration of 1500 mg/kg bw MTBE for 28 days.

**Williams et al 2000b**: 9 week old males SD rats, dosed for 15 or 28 days with 250, 500, 1000, 1500 mg/kg/day. Hepatic microsomal cytochrome P450(CYP), such as CYP2B1/2 CYP1A1/2, CYP2A1 and CYP2E1,increased in 15 day-treated and 28 day-treated rats orally administered with 1,500 mg MTBE/kg/day, indicating that the decrease in serum testosterone with MTBE may be the result of enhanced testosterone metabolism and subsequent clearance (Williams *et al.*, 2000b).

**Li et al 2008**: Mild hormonal level changes occurred in Sprague–Dawley rats orally administered with high MTBE doses (400, 800, 1600 mg/kg/day) for 15 or 28 days (Williams *et al.*, 2000a; Li *et al.*, 2008). Serum testosterone levels were decreased in rats treated for 15 days but not 28 days, and increased luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels were commonly observed following exposure to chemicals for 15 days. This was linked with oxidative stress and an enzyme related to DNA repair. In conclusion, the data indicates that relatively high doses of MTBE can exert reproductive system toxicity of male rats. The authors report that MTBE could disturb the secretions of testosterone, LH and FSH; influence the production of sperm; damage the seminiferous tubule and Sertoli cells; and induce oxidative stress

**Li et al 2009a**: In subchronic studies by gavage, the relative testicular weights of male Sprague–Dawley rats decreased significantly after exposure to 400, 800 and 1600 mg/kg body weight MTBE for 14 days. In addition, their testicular histology changed significantly only at 1600 mg/kg body weight MTBE after treatment for 28 days.

**Zheng et al 2009:** Adult Sprague–Dawley rats had 2 or 4 μL, injected into the brain. The rats in both low and high dose groups displayed an impaired ability for spatial learning in the Morris water maze task, as shown by finding the platform. At both doses: increased expression of a subunit of the GABA(A) receptor and decreased phosphorylated ERK1/2 (extra cellular signal regulated kinase) in the hippocampus.

In a **review by Li and Han** (2011) it was reported that in mice, rats and rabbits, no significant reproductive and developmental toxicity or teratogenicity responses were reported after MTBE exposure (Conaway *et al.*, 1985; Biles *et al.*, 1987; Bevan *et al.*, 1997a,b). In a single generation reproduction study, prior to mating, male and female rats were exposed to target concentrations of MTBE of 300, 1,300 and 3,400 ppm 6 hours/day and 5 days/week for 12 and 3 weeks, respectively (Biles *et al.*, 1987). The results showed that MTBE did not induce significant or specific toxicity to reproduction in Sprague–Dawley rats. In a two-generation study, no reproductive toxicity was found in two generations of Sprague–Dawley rats exposed to MTBE vapors even in the presence of parental toxicity at concentrations of 3,000 and 8,000 ppm. Moreover, they conclude that the mouse model seems to be more appropriate to compare with humans than the rat model, but no significant effects to mouse testes or other reproductive organs have been induced by MTBE. Therefore, MTBE unlikely poses human reproductive or developmental hazards (Li and Han 2011).

***In vivo*, ecotoxicity,**
Decreased reproduction in *Daphnia magna* with NOEC/LOEC of 51/100 mg/l. Mysids exposed to 50 mg MTBE/l had also statistically significant reductions in reproduction. In zebrafish, the LOEC for Vtg-induction was 110 µg/l after three weeks exposure of adult males and significant effect on sperm parameters were observed from 440 µg/l.

Detailed study summaries are provided below:
**Wong et al. 2001**: Chronic toxicity data for a fish and an invertebrate were developed in order to complete the freshwater database. The toxicity tests followed U.S. EPA and American Society for Testing and Materials (ASTM, Philadelphia, PA, USA) procedures and were conducted in accordance with U.S. EPA Good Laboratory Practice guidelines. Based on measured exposure concentrations, acute toxicity endpoints ranged from 472 to 1742 mg MTBE/L, while chronic endpoints (IC25) were 57 to 308 mg MTBE/L. Aquatic invertebrates were generally more sensitive than fish to MTBE in both acute and chronic exposures. Reproduction and growth (length and dry weight) of *Daphnia magna* were the most sensitive responses during a life cycle exposure to MTBE and a significant reduction ($p < 0.05$) in daphnid reproduction and growth was found at 100 mg MTBE/L, thereby establishing the NOEC at 51 mg MTBE/L. Daphnids in the control produced an average of 77.1 young per surviving individually exposed adult. Reproduction in the 26-, 51-, 100-, and 195-mg-MTBE/L treatments was 87.1, 65.9, 29.8, and 13.5 young per adult, respectively.
**Rausina et al. 2002**: The *Americamysis* chronic test was conducted as a flow-through exposure. The measured endpoints were *Americamysis* survival, reproduction, and growth (length and dry weight). Statistical analyses of the test data were performed on the survival of first generation mysids, number of young released per reproductive day, and length and dry weight of each surviving first generation mysid. The number of young released per adult reproductive day was calculated by dividing the total number of young released by the number of adult reproductive days for each replicate. The number of reproductive days is the number of days that a female was alive from the first brood release to the end of the test. There were no statistically significant effects on survival, reproduction, or growth of *Americamysis* exposed to 626 mg MTBE/l after 28 d. The survival of mysids exposed to 103 and 207 mg MTBE/l was significantly reduced from test initiation to pairing (day 13) and from pairing to test termination. The 7, 14, 21, and 28 d LC50 values were 90, 58, 49, and 44 mg MTBE/l, respectively (Table 4). All surviving mysids were normal, thus, the EC50 values were similar to the LC50 values. Reproduction and growth of *Americamysis* were the most sensitive endpoints measured in the 28 d test. Mysids exposed to 50 mg MTBE/l had statistically significant reductions in reproduction, length, and dry weight. The mean number of young produced per reproductive day was 0.3, 0.3, and 0.0 in the 16, 26, and 50 mg MTBE/l treatments compared to 0.3 young per reproductive day in the controls.
**Moreels et al. 2006:** Methyl-tert-butyl ether (MTBE), an anthropogenic chemical used as a gasoline additive, is being detected at an increasing frequency in the environment. The acute lethal concentration and the chronic effects of exposure to MTBE were investigated in the zebrafish (*Danio rerio*). Chronic exposure over three weeks to effective MTBE concentrations as low as 0.11 mg/L induced a significant increase in the vitellogenin concentration of male fish. The impact of a chronic, eight-week exposure at effective concentrations ranging from 0.44 to 220 mg/L had no significant effect on fecundity, fertilization, or hatch rate but highly significant impacts on sperm motility. Spermatozoa of all MTBE-exposure groups showed a significantly lower straight-line velocity and lower average path velocity compared to those of the non-exposed group. These results suggest that chronic exposure to MTBE negatively affects fish sperm motility at concentrations that are environmentally relevant and several orders of magnitude lower than concentrations inducing acute effects.

**Weight of evidence for ED and Category**
The available human studies are unsuitable for inclusion in an evaluation of the endocrine disrupting potential of MTBE due to lack of relevant endpoints.

The effects observed in adult rats include both adverse effects (increased abnormal sperm percent, irregular histopathology of testes) and relevant mode of action data (altered levels of testosterone, LH and FSH) and therefore MTBE is evaluated as ED Category 1.

Decreased reproduction was seen in *Daphnia magna* and Mysids. After three weeks of exposure of adult male zebrafish, Vtg-induction and significant effect on sperm parameters were observed.
The Vtg induction in male zebrafish is a biomarker for estrogenicity and the reproductive effects in the crustaceans are adverse but the link between endocrine specific and adverse effects has yet to be established. MTBE is therefore evaluated as a suspected ED in Category 2a.

Based on the combined evidence from the ecotoxicological studies and the *in vitro*, *in vivo* and epidemiological studies, MTBE is evaluated as an ED in **Category 1**.

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which adverse effects are observed, i.e. effects need to be observed at an oral dose of 10 mg/kg/day or lower. Adverse effects of MTBE have been found at much higher doses (400-1200 mg/kg) and consequently MTBE can not be considered an endocrine disrupter of very high regulatory concern according to the DE-UK potency criteria.

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**Pentachlorophenol, CAS 87-86-5**
Synonyms: PCP

**Human data**
The available human data are relatively limited. However, two studies indicate associations to adverse effects on thyroid system in newborns of women with higher levels of PCP. In addition, there are hypothesis generation studies indicating effects on female reproduction and miscarriages.

Study summaries are reported below:
***Gerhard et al, 1998***: Small study of 45 pregnant women with 16 miscarriages. One of 7 (14%) had PCP concentration below 6 microgram per L, and 11 of 22 with concentrations above 12. Confounding not considered. No control group. It is a hypothesis generation study suggesting that PCP may be associated with miscarriage, without significant conclusion.
***Gerhard et al 1999***: 171 women, refereed to gynaecological dept for infertility or endocrinopathy in whom PCP was measured. They were divided into 2 groups: a group of 65 women where PCP levels were above 20 microgram per L and those below this level, 106 women, named as 'control group'. Small differences were found with regard to ACTH stimulated cortisol levels, stimulated androgen levels and some adrenal metabolites. No consistency in the results. Also some suspicion of effect on thyroid hormone levels, which however were within normal reference levels.
Hypothesis generations study.
***Dallaire et al 2009***: A study on thyroid hormone levels in > 100 Inuit women and their infants. Blood was taken from women at delivery, from cord blood, and from children at 7 months of age. In a small subgroup, a significant inverse relationship was found between maternal PCP levels and T4 in umbilical cords. This was interpreted as inhibition of binding of T4 to TTR (transthyretin). It is an interesting study, which, however, needs to be repeated in a bigger controlled study, i.e. it is evaluated as a hypothesis generating study.
***Glynn et al 2011***: A study limited to generating exposure levels of PCP. Not considered relevant for this evaluation.
***Roze et al 2009***: PCP was one of the compounds which were measured in mother's blood in 35 week of pregnancy. A total of 62 of 69 invited children were followed up in Dutch CoMPARE study. Significant correlations between PCP and lower T3 concentrations in cord blood were seen. At follow up PCP correlated with coordination, sensory integrity, visiomotor integration and attention. The study is evaluated as a convincing study showing effects on thyroid.
***Guvenius et al 2003***: Exposure study, not considered relevant for this evaluation.

***In vitro* data**
The endocrine disrupting properties of PCP *in vitro* have been investigated in a number of test systems. PCP has been shown to act as an anti-estrogen and anti-androgen by inhibiting 5alpha-DHT binding to rat AR, rat androgen binding protein (ABP) and human sex hormone binding globulin (hSHBG) by more than 30% (Danzo et al 1997) and in a recombinant yeast screen assay receptor mediated (anti-) estrogenic and (anti-) androgenic activity (Orton et al 2009). Furthermore, a study used *xenopus oocytes* to measure effects on the ovulatory response and ovarian steroidogenesis. In this assay PCP had clear effect on both ovulatory response and hormone production, as progesterone, testosterone, E2 and ovulation was decreased (Orton et al 2009). PCP also acted as a thyroid disruptor by inhibiting T3 binding to quail transthyretin (TTR) (Ishihara

et al 2003) and by inhibition of sulfation of T2 through interference with deiodinase, an enzyme essential for removal of one iodide ion from T4 (inactive) to T3 (active) (Schuur AG et al 1999).
Overall the *in vitro* results indicate that PCP shows anti-androgen, anti- estrogen and thyroid disrupting modes of action and can interfere with sex hormones.

***In vivo*, human health**
Overall, *in vivo* studies with PCP in rats have shown decreased T4 in rat dams which in the long term could be associated with long-lasting impairment, including impaired brain function. Also, adverse effect, i,e, decreased spermatid counts, in offspring has been found. Several ED modes of action related to this adverse effect are plausible with anti-androgen mode and interference with sex hormones being the most plausible ones.

The different studies are presented below.
A study examined the effects of developmental exposure of rats to PCP on various aspects of brain development, male reproductive function, and adrenal function in a (Wistar) rat developmental study (Kawaguchi et al 2008). PCP was administered (6.6 mg/L) in drinking water from gestation through lactation (GD 0 to PND 21). Dams were decapitated at weaning and tissues taken from F1 females at 3 and 12 weeks and from F1 males at 12 weeks. Gene expression of T4 (Thyroxine) receptor beta1 and synapsin1 was increased in cerebral cortex of 3-weeks old F1 females. Total T4 was decreased in dams and 3-week old pups while TSH was increased in 3-week old males. Corticosterone was decreased in the 12-week old females and testis weight was increased in the 12-week old males. The study shows that PCP exposure during gestation and lactation causes moderate thyroid function vulnerability, adult testicular hypertrophy, and aberrations in gene expression in the cortex (may affect growth of the brain).
In conclusion, although pentachlorophenol treatment had no adverse effects on litter size and body and organ weight (liver and adrenal) of the dams, pentachlorophenol disrupted the thyroid hormones, which in the long term could be associated with long-lasting impairment, including impaired brain function, in their offspring.
A two-generation reproduction toxicity study, with Crl Sprague-Dawley rats, was performed to evaluate the potential for pentachlorophenol (PCP) to induce general and reproductive/ developmental toxicity (Bernard et al 2002). PCP was administered by gavage at doses of 0, 10, 30, and 60 mg/kg/day. In both generations, the parental animals (30/sex/group) were intubated daily for 10 weeks before cohabitation and continuing through cohabitation, gestation, and lactation periods. Toxicity, in the form of liver pathology (single cell necrosis), reduced body weights and associated reductions in organ weights, and reduced feed consumption were noted in both generations at the 30- and 60-mg/kg/day doses. Developmental toxicity associated with these doses included reduced pup weights and viability. The 60-mg/kg/day dose also resulted in delayed sexual maturation, decreased spermatid counts, small prostates and testes, decreased implantations, reduced fertility, and increased resorptions of embryos. Based on these results, it was concluded that 30 mg/kg/day is the lowest-observable-adverse-effect level (LOAEL) and 10 mg/kg/day is the no-observable-adverse-effect level (NOAEL) for both reproductive and general toxicity. Generally, the absolute feed consumption values were significantly reduced and relative feed consumption values were significantly increased, observations associated with the reduced body weights in the 30- and 60-mg/kg/day dose groups. The reductions in body weights were also associated with significant changes in multiple organ weights and the ratios of the affected organ's weight to body or brain weight. The current study also identified that the type of reproductive effects produced (reduced pup body weights, viability, and organ weights at 30 and 60 mg/kg/day and retarded sexual maturation and reduced testicular spermatid count at 60 mg/kg/day) are sequelae of developmental toxicity (i.e., in utero exposure of the offspring) occurring at severely toxic parental doses.

In summary, this study finds a LOAEL of 30 mg/kg/day and a NOAEL of 10 mg/kg/day for both reproductive and general toxicity. The study does not evaluate thyroid disrupting endpoints (hormone measures, thyroid gland weight or DNT).

***In vivo*, ecotoxicity**

14 relevant ecotoxicological studies were found in the open literature. A number of studies examine the effect of pentachlorophenol in mammals, i.e. mink and sheep. Pentachlorophenol affects reproduction in some of the experimental setups but not in others. Furthermore, the studies show thyroid function disrupting effects of pentachlorophenol. Likewise, pentachlorophenol interferes with the thyroid system of *Xenopus laevis* and multiple hormone systems in fish. Moreover, pentachlorophenol affects reproduction and steroid hormone biotransformation in daphnia.

Study descriptions of each of the ecotoxicology studies:

***Parks and LeBlanc 1996:*** Daphnia magna were exposed to pentachlorophenol and the effects on reproduction and steroid hormone biotransformation/elimination were examined.

Reproduction of daphnids was significantly compromised from chronic exposure to pentachlorophenol (LOEC: 0.25 mg/L, NOEC: 0.12 mg/L). I an acute exposure test (48 h), pentachlorophenol significantly inhibited the elimination of glucose conjugates of testosterone (LOEC: 0.062 mg/L).

These results demonstrate that pentachlorophenol alters steroid hormone biotransformation and affects reproduction in daphnia. However, general toxic effects cannot be excluded since mortality occurred.

***Beard et al. 1997:*** Mink were fed a diet treated with pentachlorophenol (1 mg $kg^{-1}$ $day^{-1}$) from before breeding until weaning. Mink were mated twice, at 7-8 day intervals.

Pentachlorophenol had no effect on the proportion of mink accepting the first mating; however, pentachlorophenol caused a decrease in the percentage of females accepting the second mating. The proportion of mated mink that whelped subsequently was reduced by pentachlorophenol treatment. Mink that mated only once had a lower whelping rate than mink that mated twice; therefore, it could not be determined whether the decreased whelping rates were due to the lack of a second mating or to increased embryo loss.

In conclusion, pentachlorophenol decreased fertility in mink. This is an adverse apical effect but not endocrine specific.

***Beard and Rawlings 1998*:** In this study, reproductive function in second and third generation male and female mink exposed to pentachlorophenol (1 mg $kg^{-1}$ $day^{-1}$) from conception to maturity was examined.

No overt signs of toxicity were seen. Pentachlorophenol did not affect the percentage of mink mated. Serum concentrations of cortisol, testosterone and oestradiol were not affected by pentachlorophenol treatment; however, thyroxine concentration was significantly reduced by pentachlorophenol.

In this study, pentachlorophenol caused no adverse effects after long-term exposure. However, pentachlorophenol caused a decrease in serum thyroxine concentration suggesting endocrine effects.

***Rawlings et al. 1998:*** Ewes (female sheep) received orally into their rumen gelatin capsules pentachlorophenol. (2 mg/kg) 2 times per week for 43 d. After 36 d of treatment, blood samples were taken every 12 min for 6 h for hormone analysis. Ewes were euthanized at the end of the study for necropsy and histopathology.

No overt signs of toxicity were seen, and body weight was not affected by treatment. Pentachlorophenol resulted in a marked decrease in thyroxine concentrations. Concentrations of insulin in serum were markedly increased in ewes given pentachlorophenol, but no effects were seen on concentrations of estradiol. Pentachlorophenol caused a significant increase in severity of oviductal intraepithelial cysts in ewes.

These results show that pentachlorophenol influences serum concentrations of thyroxine. Pentachlorophenol caused an increase in oviductal intraepithelial cysts, but did not produce endocrine changes that could be specifically related to the incidence of intraepithelial cysts.

***Beard and Rawlings 1999:*** In this study, ewe lambs were exposed to pentachlorophenol from conception to necropsy at 67 wk of age. The ewe lambs (and their mothers) were given feed treated with 1 mg/ kg body weight/day of pentachlorophenol. Oestrus was synchronized at 32 wk of age, and ewe lambs were then exposed to rams during the following two natural oestrous periods and subsequent reproductive performance was monitored. Serum was collected every 2 wk during development, daily during the synchronized cycle and frequently (every 15–60 min) for 6–18 h either with or without stimulation with thyroid-stimulating hormone (TSH) during the synchronized luteal phase or TSH/ thyroid-releasing hormone (TRH) at 65–66 wk of age.

Ewe lambs fed a pentachlorophenol-treated diet had a significantly reduced serum concentration of both T4 and free T4, and a reduction in the magnitude and duration of the T4 and free T4 response to TSH, despite normal endogenous levels of TSH and a normal TSH response to TRH. The T3 response to TSH was markedly reduced in pentachlorophenol-treated ewe lambs. Detrimental effects on reproductive function were only seen following oestrous synchronization when pentachlorophenol reduced the number of corpora lutea (CL) and total CL volume and increased luteinizing hormone (LH) pulse frequency. No marked effects of pentachlorophenol were seen on fertility following mating during natural oestrous periods.

In conclusion, pentachlorophenol affected reproduction only after oestrous synchronization, whereas pentachlorophenol consistently disrupted thyroid function, most likely through a direct effect on the thyroid gland.

***Beard et al. 1999:*** The effects of pentachlorophenol on reproduction and general endocrine function were examined in breeding ewes. Animals were fed a diet treated with pentachlorophenol (1 mg/kg/d) during the 5 wk prior to mating and throughout pregnancy and lactation. Mating response, ovulation rate, follicle and corpus luteum size, gestation length, pregnancy rate, lambing rate, and lamb birth weight were recorded. Pentachlorophenol did not markedly affect any of the aspects of reproductive function studied. In pentachlorophenol-treated ewes, serum concentrations of T4 were significantly reduced compared to control ewes; however, the T4 response to TSH was not altered by pentachlorophenol treatment. No other measured endocrine parameters were consistently affected by pentachlorophenol. Thyroid follicle size was significantly increased in the pentachlorophenol treated ewes compared to the control ewes. Low serum concentrations of T4 in the pentachlorophenol treated ewes may have resulted in increased TSH secretion and increased thyroid follicle size.

In conclusion, although pentachlorophenol treatment had no adverse effects on reproductive function in breeding ewes, pentachlorophenol reduced T4 concentration, which in the long term could influence reproductive and general performance.

***Beard et al. 1999a:*** In this study, ram (male) lambs were exposed to pentachlorophenol from conception to necropsy at 28 weeks of age. The rams (and their mothers) were given feed treated with pentachlorophenol (1 mg $kg^{-1}$ $day^{-1}$).

Pentachlorophenol did not affect body weight and ejaculate characteristics, or cause overt toxicity. In pentachlorophenol-treated rams, scrotal circumference was increased. Seminiferous tubule atrophy was more severe and epididymal sperm density was reduced in comparison with untreated rams at necropsy. Thyroxine concentrations were lower in pentachlorophenol-treated rams than in untreated rams.

In summary, the effects of pentachlorophenol on the testis may be linked to a decrease in thyroxine concentrations thus providing information on the endocrine mode of action.

***Blaise et al. 1999:*** In this study, the ALP assay is used as an indirect method of determining vitellogenin-like proteins in clam hemolymph. Injection of clams with pentachlorophenol significantly induced hemolymph ALP levels. The authors therefore conclude, that their data indicate that pentachlorophenol is estrogenic.
***Preston et al. 2000***: In this study, a 96-h reproductive assay using the freshwater rotifer *Brachionus calyciflorus* to screen for potential endocrine disruptors was developed. Pentachlorophenol had no significant effect in this assay at the tested concentration (10 mg/L).
***Sanchez et al. 2005:*** A water/sediment system was utilized using two invertebrates: *Chironomus prasinus* a benthic detritivore invertebrate and *Daphnia magna* a pelagic filtering invertebrate. Sediments were spiked with sodium pentachlorophenol at nominal concentrations of 1.25, 2.5, 5, 10 and 20 mg/kg, respectively. Each system contained caged and free (unrestrained) *Daphnia magna* organisms to consider differences in the exposure route (through sediment and/or water column) and *Chironomus prasinus* organisms. Acute (lethality) and chronic effects (reproduction) for *Daphnia magna* were monitored after 48 h and 16 days of exposure, respectively. Adult emergence and oviposition success of *Chironomus prasinus* were monitored at the end of test (16 days).
Mortality and reproduction inhibition of *Daphnia magna* occurred at the two higher doses, corresponding to maximum measured pentachlorophenol water concentrations of 1.95 and 0.746 mg/l, respectively. *Chironomus prasinus* was less sensitive to pentachlorophenol than *Daphnia magna*. Concentrations inhibiting reproduction and provoking almost 100% adult mortality in *D. magna* only reduced slightly the emergence and reproduction of chironomids. Statistical analyses were performed with data on both reproduction and adult emergence of *Chironomus prasinus* to compare controls versus treatments by combining all of the treatment data. Numbers of egg ropes were lower for treated than for control groups. In addition, the male/female ratio was statistically greater for treated than for control groups. However, no clear dose-response relationships were observed for these endpoints.
The study shows that pentachlorophenol affects reproduction of *Daphnia magna*, which is an adverse apical effect. However, general toxic effects cannot be excluded since mortality occurred. For *Chironomus prasinus*, the male/female ratio was statistically greater for treated than for control groups indicating an endocrine related mode of action of pentachlorophenol. However, a clear dose-response relationship was not observed.
***Sugiyama et al. 2005:*** This study investigates whether pentachlorophenol interferes with the thyroid system of *Xenopus laevis.*
Pentachlorophenol showed $T_3$-antagonist activity in an *in vivo* metamorphosis-based assay.
The study shows that pentachlorophenol exhibits $T_3$-antagonistic activity in *Xenopus laevis* which provides information on the endocrine mode of action of pentachlorophenol.
***Zha et al. 2006:*** In the present work, Japanese medaka (*Oryzias latipes*) was exposed to pentachlorophenol for 28 days (F0 generation) with subsequent measurements of vitellogenin, hepatic 7-ethoxyresorufin-*O*-deethylase (EROD), and reproductive endpoints. Plasma vitellogenin significantly increased in male fish treated with pentachlorophenol concentrations lower than 200µg/l and decreased in male and female animals exposed to 200µg/l. Hepatic EROD from female fish increased when pentachlorophenol exposure concentrations exceeded 20µg/l, but decreased in the 200 µg/l pentachlorophenol treatment group. Fecundity and mean fertility of female medaka decreased significantly in the second and third week following exposure concentrations greater than 100 µg/l, and testis-ova of male medaka was observed at pentachlorophenol concentrations greater than 50 µg/l. Histological lesions of liver and kidney occurred when exposure concentrations exceeded 50 µg/l. In F1 generations, the hatching rates and time to hatch of offspring were significantly affected in fish exposed to 200µg/l.

These results indicated that pentachlorophenol exposure caused responses consistent with estrogen and aryl hydrocarbon receptor activation as well as reproductive impairment in fish. However, effects were also observed on growth and mortality suggesting general toxic effects of pentachlorophenol on fish.
***Zhang et al. 2008:*** In this study, serum testosterone concentration, activity of liver microsome ethoxyresorufin O-deethylase (EROD) and glutathione S-tranferases (GST) of crucian carp (*Carassius carassius*) exposed to pentachlorophenol for 7 and 15 d, respectively, were examined. The results showed that testosterone concentration was increased remarkably after 7 d, and the testosterone concentrations in 15 d treatment crucian carp were higher than those in 7 d treatment. It was found that there were significant effects on activities of EROD and GST after crucian carp were exposed to pentachlorophenol for 7 and 15 d, compared to the controls. EROD and GST activities increased with increase in pentachlorophenol concentration and also with increase in time of exposure.
The study shows that pentachlorophenol increases testosterone concentrations in fish. This provides information about the endocrine mode of action of pentachlorophenol.
***Orton et al. 2009:*** A short-term exposure (6 days) of adult female *Xenopus* to low concentrations (0.1 or 1 µg/L) resulted in slight elevations in plasma progesterone levels (which were only significant when the treated groups were pooled) and degenerative ovarian features.

**Weight of evidence for ED and Category**
The available human data are relatively limited. However, two studies indicate associations to adverse effects on thyroid system in newborns of women with higher levels of PCP. In addition, there are hypothesis generation studies indicating effects on female reproduction and miscarriages.

*In vivo* studies in rats have shown decreased T4 in rat dams which in the long term could be associated with long-lasting impairment, including impaired brain function. Also, adverse effects, i,e, decreased spermatid counts, in offspring has been found. Several ED modes of action are plausible with anti-androgen mode and interference with sex hormones being the most plausible ones (*in vitro* data show both thyroid-, anti-estrogenic and anti-androgenic modes of action). PCP could therefore in principle fulfil the criteria of being an endocrine disrupter in Cat 1. However, the relatively few number of studies investigating *in vivo* effects, and the fact that the 2- generation study also showed clear reduction in body weights and associated reductions in organ weights means that general toxic effects cannot be excluded. This indicates that the evidence from the experimental animals might not be sufficiently convincing to place the substance in Category 1, but rather in Category 2a.

A number of studies examine the effect of pentachlorophenol in mammals, i.e. mink and sheep. Pentachlorophenol affects reproduction in some of the experimental setups but not in others. Furthermore, the studies show thyroid function disrupting effects of pentachlorophenol. Likewise, pentachlorophenol interferes with the thyroid system of *Xenopus laevis* and multiple hormone systems in fish. Moreover, pentachlorophenol affects reproduction and steroid hormone biotransformation in daphnia. Based on the effects on hormone levels in multiple studies and the adverse effect on daphnia and mammalian reproduction, pentachlorophenol could be validated as an ED (Category 1). However, the results are rather equivocal and general toxic effects cannot be excluded. Therefore, based on *in vivo* ecotoxicity data, pentachlorophenol is categorized as a suspected ED (Category 2a).

The evidence from the human data, *in vitro* studies, *in vivo* studies and ecotoxicological studies all point in the same direction, i.e. towards adverse effects on thyroid function of pentachlorophenol, and based on the combined evidence, pentachlorophenol is evaluated as an ED in **Category 1**.

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which effects are observed, i.e. effects need to be observed at an oral dose of 10 mg/kg/day or lower. For Pentachlorophenol in the 2-generation study a LOAEL of 30 mg/kg/day and a NOAEL of 10 mg/kg/day for both reproductive and general toxicity was found. On that basis, PCP cannot be considered an endocrine disrupter of very high regulatory concern according to the DE-UK potency criteria.

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**Perchlorethylene, CAS: 127-18-4**
**Synonyms**: Tetrachloroethene, PCE, 1,1,2,2-tetrachloroethene

**Human data**
The available literature on human data does not substantiate endocrine disruptive effects of PCE in humans. The section in italic style below is taken from SCOEL/SUM/133 of June 2009: *Recommendation of the Scientific Committee on Occupational Exposure Limits for Tetrachloroethylene (Perchloroethylene)* available at: http://www.ser.nl/documents/72963.pdf. This report evaluates animal and human toxicological data on PCE and section 5.8.1 specifically deals with human data in relation to reproductive and developmental toxicity where possible endocrine disruptive effect(s) of PCE would appear. The findings of the SCOEL report are in line with the findings of the current evaluation of the available literature.
*5.8 Reproductive and developmental toxicity*
*5.8.1 Human data*
*The studies reviewed by NEG-DECOS (2003) show some evidence for an association between maternal occupation of dry cleaning and increased risk of spontaneous abortion and more limited information to suggest an association more specifically with tetrachloroethylene (Table 7). There is also very limited evidence of an association between tetrachloroethylene and disruption of the menstrual cycle. There is no evidence of an association between tetrachloroethylene and congenital malformations or between paternal exposure to tetrachloroethylene and increased risk of spontaneous abortion. There is no clear evidence of an effect on male fertility, although the available data do not exclude the possibility of a small effect. There have been no epidemiological investigations of the effects of infant exposure to tetrachloroethylene in breast milk but obstructive jaundice and hepatomegaly have been described in the breast fed child of a mother exposed to tetrachloroethylene (Bagnell et al, 1977). No new studies appear to have been published since the NEG-DECOS review in 2003.*
Following the SCOEL report in June 2009, two review articles have been published: Dzubow et al., 2010 and Bukowski, 2011. Furthermore, a review by Ruder (2006) which was not included in the SCOEL report has been evaluated.
Two regular articles have been published following the SCOEL report: Tucker et al. (2011) investigated chromosome damages in 18 laundry workers and 18 dry cleaners (all women), but this study is not linked to endocrine disruptive effects. Aschengrau et al. (2009) analysed congenital malformations among children whose mothers were exposed to PCE via drinking water. This study reported a slightly increased risk of genitourinary malformations (particularly hypospadias) but as stated by Dzubow et al. (2011) the ability to examine congenital malformations and PCE exposure in the Aschengrau study is quite limited and so the conclusions are of limited value.
The only study examining semen parameters following PCE exposure (Eskenazi et al. 1991) reported that sperm of dry cleaners "tended to swim with greater amplitude of lateral head displacement than those of laundry workers". Furthermore, sperm of dry cleaners displayed less linearity in swimming paths than sperm of laundry workers. On the other hand, sperm concentration was positively correlated to the PCE concentration in expired air of dry cleaners and dry cleaners on average had semen within normal clinical limits (as described by the authors). The subtle findings and the very limited number of dry cleaners examined (n=34) makes the study of limited value with regards to possible correlations between PCE and endocrine disruptive effects.

***In vitro* data**
No relevant data on endocrine disruption were found.

***In vivo*, human health**

Three animal studies have been performed in which rats have been exposed to PCE for varying periods of time. Reproductive effects as well as effects on the rat brain have been seen, however no effects pointing to endocrine disruption have been found.

Study summaries are provided below:

**Honma et al 1980**. Male SD rats exposed to 200, 400, 800 ppm via inhalation for one month thereafter brains were dissected and homogenized. Acetylcholine was significantly decreased in striatum at 800 ppm.

**Fredriksson et al 1993**. This study evaluated effects of exposure to tetrachloroethylene on the contents of acetylcholine, dopamine, norepinephrine and serotonin in the mice brain. Male MNRI mice dosed at 5 and 320 mg/kg/d orally with a PVC tube on PND 10-16. Tested on PND 17, no change in activity; on PND 60 motor activity that included locomotion, rearing and total activity was decreased.

**Carney et al 2006**. Developmental toxicity studies in rats following inhalation exposure to perchloroethylene. Pregnant SD rats were exposed to 65, 250, 600 ppm inhalation, 6 hours/day, 7 days/week, from GD 6-19. At 250 and 600 ppm reduced weight of placenta and foetuses was seen and at 600 ppm gravid uterine weight was decreased. These effects are general developmental toxicity effects indicating retarded growth of the foetuses and do as such not point towards endocrine disruption as the mode of action.

***In vivo*, ecotoxicity**

No endocrine specific endpoints or mechanistic data have been tested for PCE in wildlife. Deformities in amphibians could be via some kind of thyroid disruption but this needs to be further investigated. Study summaries are provided below:

**Reimschuessel et al. 1993**: Environmental exposure of rainbow trout, *Oncorhynchus mykiss*, to tetrachloroethylene was associated with massive mortalities (about 50%) with acute hepatic and renal toxicity. The liver changes consisted of diffuse hepatocellular necrosis. The kidney changes were manifested as degeneration and necrosis of epithelial cells lining the first segment of the proximal tubule. Intensely basophilic newly developing nephrons were observed in surviving fish sampled 10 days to 3 wk following exposure. The presence of developing nephrons in adult fish may serve as a biomarker for nephrotoxic pollutant exposure.

**Spencer et al. 2002**: Exposure to sub lethal concentrations of PCE resulted in a significant reduction in hatching, as well as the development of a significant number of morphological and physiological abnormalities in the Japanese medaka fish including scoliosis, pericardial edema, enlarged heart, hemostasis, and deformed fin. Therefore, the major developmental defects noted were mainly of the cardiovascular and circulatory systems. Other lesions such as enlarged spleen, colourless blood, eye convergence, enlarged gall bladder, and gaped mouth were also observed to a lesser extent. A strong concentration-response relationship was found between the level of PCE exposure and the severity of morphological and developmental lesions in the Japanese medaka. Due to the large abdomen produced by the yolk-sac edema, the larvae ability to swim was also affected. The lowest tested concentration (1.5 mg/L) produced a significant number of developmental effects on the Japanese medaka.

**Spencer et al. 2006**: The former demonstrated that tetrachloroethylene (TCE) is acutely toxic to Japanese medaka (Oryzias latipes) larvae with a 96 hr-LC50 of 18 (17-19) mg/mL (Spencer et al., 2002). In the present study it was hypothesized that TCE exposure induces a developmental effect in Japanese medaka. Growth and age specific sensitivity of Japanese medaka larvae were studied with four age groups (7, 14, 21 and 28 days old) to determine tetrachloroethylene effects on these parameters. The medaka larvae were exposed for 96 hours in a single concentration (10 mg/mL) of TCE. The toxic endpoints evaluated were

larvae weight, length, water content and protein concentration. The study revealed that exposure of medaka larvae to this sub-acute concentration of TCE significantly reduced length and weight in the treated group. The difference in growth between control and treated groups was more obvious in age versus length, than in age versus weight. The dry weight-fresh weight ratio (dw/fw) was shown to be higher in the control group. Water content in TCE-treated medaka was higher than in the control group, and younger fry had more water content than older ones. A higher protein concentration was also observed in TCE-treated medaka compared to the control group. These results indicate that TCE has a profound effect on the growth and development of Japanese medaka larvae.

**McDaniel et al. 2004:** Acute (96-h static renewal) exposures to PCE and its major degradation products, trichloroethylene and *cis*- and *trans*-dichloroethylene were conducted on embryos of four North American amphibian species: Wood frog *Rana sylvatica*, green frog (*R. clamitans*), American toads (*Bufo americanus*) and spotted salamander (*Ambystoma maculatum*). Median $EC_{50}$ for developmental deformities for wood frog and green frog for PCE and TCE were 12 and 40 mg/l respectively. Besides, a dose dependent increase in deformities was observed for all species.

No endocrine specific endpoints or mechanistic data have been tested for PCE in wildlife.

**Weight of evidence for ED and Category**

The available literature on human data does not substantiate endocrine disruptive effects of PCE in humans.

Based on *in vitro* and *in vivo* literature, no effects pointing to endocrine disruption have been found.

No endocrine specific endpoints or mechanistic data have been tested for PCE in wildlife.

PCE can therefore not be placed in Category 1, 2a or 2b as there is no available literature pointing in the direction of endocrine disruption.

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**Propylparaben, CA S 94-13-3**
**Synonyms:** propyl-4-hydroxybenzoate, E216

**Human data**
Humans are exposed to several different parabens. In urine samples from 60 young Danish men four common parabens were measured in nearly all samples: methyl paraben (MP) in 98% of the samples (median level = 18 ng/ml ); propyl paraben (PB) in 98% of the samples (median level = 3.6 ng/ml ); ethyl paraben (EP) in 80% of the samples (median level = 2.0 ng/ml ); butyl paraben (BP) in 83% of the samples (median level = 0.2 ng/ml ) (Frederiksen et al., 2011). Similar trend was observed in an U.S. population (NHANES 2005-2006), although MP and PB were observed in about a threefold higher level in the U.S. samples compared to the Danish samples and furthermore EP and BP were detected less frequently than in Danish samples, 42% and 47%, respectively (Calafat et al., 2010).

Only few human studies have investigated possible endocrine disrupting effects of parabens. One study analysed the association between urinary concentration of MP, PP and BP and markers for male reproduction health (Meeker et al., 2011). No associations were observed between the three parabens and serum hormone levels or semen quality parameters. Another study found no associations between the urinary concentration of parabens (MP, PP and BP) and pubertal stage; breast development and pubarche in U.S. girls (Wolff et al., 2010). Finally two unpublished studies "Urinary paraben concentrations and *in vitro* ferterlization (IVF) outcomes" (Sabatini et al,. 2011) and "The association of urinary paraben concentrations with measures of ovarian reserve among patients from a fertility canter" (Smith et al., 2011), both presented as poster abstracts indicated that 1) increased urinary MP and PP were associated with increased incidence of poor embryo quality and 2) that there was a suggestive evidence for an association between PP and higher serum follicle stimulating hormone and lower antral follicle count on day three of the menstrual cycle.

In conclusion, a few human studies have indicated weak associations between increased paraben exposure and markers for human reproductive health. However, the knowledge in this area is very limited.

***In vitro* data**
The available data for propylparaben show strong evidence that this compound has estrogenic effects *in vitro*. In general, references to these statements can be found in the review by Boberg et al., 2010. There are also reports of weak antiandrogenic effects *in vitro* (Chen et al., 2007, Kjaerstad et al.2010). The following table from Boberg et al., 2010, collects information from studies on estrogenic and anti-androgenic activity *in vitro*.

**Table 1** *In vitro* studies published on the estrogenic and anti-androgenic activity of parabens. Boberg et al., 2010.

| **Estrogenic effect *in vitro*** | **Anti-androgenic effect *in vitro*** |
|---|---|
| +ve (yeast + receptor binding) (Routledge 1998, Miller 2001, Schultis and Metzger 2004, Morohoshi 2005)<br>+ve (human MCF7) (Okubo 2001, Byford 2002, Schultis and Metzger 2004, Vanapyris 2006)<br>+ve (rat uterus receptor binding) (Blair 2000, Lemini 2003)<br>+ve (human HeLa overexpressing ER) (Gomez 2005) | +ve (recombinant hAR) (Sato 2005)<br>+ve (transfected CHO-K1 cells) (Sato 2005)<br>+ve (transfected HEK 293 cells) (Chen 2007) |

The evidence of estrogenic effects is based on several reports of weak estrogenic effects *in vitro* using MCF-7 assay, reporter gene assay, or recombinant yeast screen assay. Anti-androgenic effects have also been shown in a few *in vitro* studies (for references see Table 1).

***In vivo*, human health**

The available data for propylparaben show strong evidence that this compound has estrogenic effects *in vivo*. In general, references to these statements can be found in the review by Boberg et al., 2010. One study shows effect of propylparaben on sperm count/quality following exposure of young males (Oishi et al., 2002), but some doubt has been raised on the quality of this study (SCCS 2006).

The evidence of estrogenic effects is based on *in vivo* using uterotrophic assays or exposure of immature females (for references see Table 2 below). Other studies have found no effects in uterotrophic assays (Hossaini et al., 2000). One study by Vo et al., 2010, showing estrogenic effects of propylparaben is presented in detail below, as this was performed in immature female rats and thus a relevant model for exposure of developing humans, i.e. more relevant than uterotrophic assays in ovariectomized animals. No studies with perinatal exposure to propylparaben have been published. The study by Oishi, 2002, is not considered sufficient to reach conclusions on whether propylparaben can affect male rat reproduction following peripubertal exposure.

**Table 2**. Summary of results of uterotrophic assays. Modified from Boberg et al., 2010. Route: subcutaneous (SC) unless otherwise stated.

| Study | Response in immature rats (effective doses in mg/kg) | Response in immature mice (effective doses in mg/kg) | Response in ovariectomized mice (effective doses in mg/kg) |
|---|---|---|---|
| Hossaini 2000 | | No effect at 100<br>No effect at 1, 10 and 100 (oral) | |
| Lemini 2003 | LOEL 65<br>NOEL 20 | LOEL 20<br>NOEL 6.5 | LOEL 20<br>NOEL 6.5 |
| Lemini 2004 | | | LOEL 65 |
| Vo 2010 | LOEL 1000<br>NOEL 250 | | |

**Oishi et al., 2002**. In a study in young male rats exposed to propylparaben for 4 weeks, daily sperm production (testis sperm production) was reduced in all three dose levels of approximately 10, 100 and 1000 mg/kg bw/day. At 100 mg/kg bw/day, epididymal sperm count was affected in a dose-related manner. Serum

testosterone levels were reduced in a dose-related manner in all dose groups, but only statistically significant at 1000 mg/kg bw/day. Body weight was reduced at 1000 mg/kg bw/day. This indicates a lowest-observed adverse effect level (LOAEL) of 10 mg/kg bw/day for propylparaben. However, this study has some shortcomings, and as the raw data were not available for review by the European Union Scientific Committee on Consumer Products (SCCP), they acknowledge some doubt about the result of this study.
**Vo et al., 2010.** Effects of 6 parabens were compared in a female pubertal assay in rats. Female SD rats were orally exposed to 62.5, 250 or 1000 mg/kg bw/day of propylparaben from postnatal day 21 to 40 (4 weeks), and reproductive endpoints were examined at postnatal day 40. No changes in age at vaginal opening or changes in estrous cycles were observed for propylparaben, but the uterine epithelium was thickened at the highest dose level. This was also seen for isobutylparaben, butylparaben and isopropylparaben but not for the short-chain parabens methyl- and ethylparaben. Adrenal weights were increased at the highest dose, and serum T4 was significantly reduced at the middle dose only. This study shows estrogenic effects of propylparaben at higher doses than for butylparaben exposure.

***In vivo*, ecotoxicity**
A number of ecotoxicological studies of propylparaben have been performed. Rainbow trout Vtg induction after injection of propylparaben had a LOEC of 100 mg/kg and oral exposure to 33 mg propylparaben/kg/2 day also induced Vtg. So did 225 µg/l via water. Vtg gene 1 and 2 was induced at 9.9 mg/l in Japanese medaka. Daphnia magna reproduction declined with a LOEC of 6 mg/l.

Detailed study summaries are provided below:
**Pedersen et al 2000**: The widely used phenolic preservative propylparaben was tested for the ability to evoke an oestrogenic response *in vivo*. Vitellogenin induction in sexually immature rainbow trout (*Oncorhynchus mykiss*) was used as an oestrogen-specific endpoint after two injections of the compound (day 0 and 6). Vitellogenin was measured in blood at day 0, 6 and 12. Propylparaben had oestrogenic potency comparable to bisphenol A with a LOEC of 100 mg/kg.
**Bjerregaard et al 2003**: The estrogenic effect of propylparaben was investigated in a rainbow trout *Oncorhynchus mykiss* test system. Propylparaben was administered **orally** to sexually immature rainbow trout every second day for up to 10 days in doses between 7 and 1830 mg/kg/2 day and in the water at 50 and 225 µg/l for 12 days. Plasma vitellogenin was measured before and during the exposures and the concentrations of propylparaben in liver and muscle were determined at the end of experiments. Increases in average plasma vitellogenin levels were seen at oral exposure to 33 mg propylparaben/kg/2 day; the most sensitive fish responded to 7 mg/kg. The ED values for increase in vitellogenin 50 synthesis were 35, 31 and 22 mg/kg/2 day at day 3, 6 and 11, respectively. Exposure to 225 µg propylparaben/l increased vitellogenin synthesis, but exposure to 50 µg/l did not. Propylparaben showed little tendency to bioaccumulation in rainbow trout; less than 1‰ of the total amount of propylparaben administered orally at 1830 mg kg/2 day over the 10-d experimental period was retained in muscle and liver 24 h after the end of the experiment. Exposure to 225 µg propylparaben/l for 12 days led to concentrations of 6700 and 870 µg propylparaben/ kg liver and muscle, respectively. Half lives for propylparaben were 8.6 h in liver and 1.5 h in muscle.
**Inui et al 2003**: In the present study, the estrogenicity of propyl paraben (n-propyl-p-hydroxy-benzoate; PP) was examined using male medaka (*Oryzias latipes*), in regard to production of vitellogenin (VTG) and choriogenin (CHG) during water exposure. First, using a VTG ELISA system, an increase in VTG plasma concentration in medaka due to exposure to PP was determined. Next, increases in mRNA expression levels of VTG subtypes VTG-1 and VTG-2, and CHG subtypes CHG-L and CHG-H, in liver were found. In addition, increased mRNA expression levels of estrogen receptor (ER) α, among sex hormone receptors was

also found in the liver. In this study, it was shown that PP has estrogenic activity in fish. LOEC – 0.0055 mM (9.9 mg/l) (VTG-1 and 2).
**Dobbins et al 2009**: Standardized acute and subchronic endpoints in larval fish (*Pimephales promelas*) and cladoceran (*Daphnia magna*) models were examined for seven different parabens (methyl-, ethyl-, isopropyl-, propyl-, isobutyl-, butyl-, benzylparaben), which encompassed a range of log P values. Paraben 48 h median lethal concentration values (LC50) ranged from 4.0 to 24.6 mg/L in D. magna and 3.3 to .160.0 mg/L in fathead minnow. Growth and reproduction in D. magna had lowest-observed-effect concentrations (LOECs) ranging from 0.12 to 9.0 mg/L and 1.5 to 6.0 mg/L, respectively. Fathead minnow growth was adversely affected at levels ranging from 1.0 to 25.0 mg/L. Aquatic toxicity of the parabens was inversely related to lipophilicity, suggesting that responses using standardized endpoints resulted from narcosis. Utilizing toxicity benchmark concentrations (e.g., LC50s, LOECs) for each compound, chemical toxicity distributions, a probabilistic hazard assessment technique, were developed to assess the probabilities of detecting parabens that elicit a response at or below a given concentration. For the responses assessed in the present study, the 5th centile values (the concentration at which 5% of parabens elicit a response) ranged from 15 mg/L to 2.43 mg/L, with D. magna growth eliciting the lowest 5th centile value and acute D. magna mortality eliciting the highest.

**Weight of evidence for ED and Category**
A few human studies have indicated weak associations between increased paraben exposure and markers for human reproductive health. However, our knowledge in this area is very limited.

Based on *in vivo* and *in vitro* studies, propylparaben can be placed in Category 2a, as a suspected endocrine disrupter, as there is evidence of an estrogenic mode of action *in vivo* that is suspected to be linked to adverse effects *in vivo*. The *in vivo* data on adverse estrogenic effects *in vivo* are conflicting and therefore propylparaben cannot be placed in Category 1 using the available data.

In ecotoxicological studies, based on the confirmed Vtg induction in multiple studies and the adverse effect on daphnia reproduction, propylparaben could be evaluated as an ED (Category 1) but as reproduction is not endocrine specific, a test with an endocrine specific adverse endpoint is needed (e.g. sex ratio) before this paraben could be evaluated as an ED. Therefore, propylparaben is evaluated as suspected ED (Category 2A).

Based on the combined evidence from *in vivo*, *in vitro*, ecotoxicological and human studies, propylparaben is evaluated as a suspected ED in **Category 2a.**

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which severe adverse effects are observed. For subchronic studies with oral dosing, effects at 10 mg/kg bw/day and below leads to a classification as an endocrine disrupter of very high regulatory concern. No NOAEL can be determined for propylparaben, and knowledge on adverse effects of propylparaben is very limited. A study by Oishi et al., 2001, indicated effects on semen quality at 10 mg/kg bw/day after 4 weeks dosing, but uncertainties on this study makes it impossible to judge whether this LOAEL can be used as an overall LOAEL. Overall, it is evaluated as unclear whether propyl parabens can be considered as an endocrine disrupter of high regulatory concern based on the DE-UK potency criteria.

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**Quadrosilan, CAS 33204-76-1**
**Synonyms:** 2,6-cis-diphenylhexamethylcyclotetrasiloxane

**Note on uses and comparable substances**
The SIN list reports use of Quadrosilan in bearing grease and breast implants. This use is not documented. However, a report on identification of potential endocrine disrupters lists a usage level of 10 tonnes/year and a potential for high human exposures (Petersen et al., 2007). It is possible that quadrosilan is also in use for certain personal care products. Internet searches reveal that a data sheet for an "over-the-counter" drug (zink oxide cream) use the name "quadrosilan" as a synonym to "cyclotetrasiloxane" and it is unclear if the ingredient used for that specific product is in fact quadrosilan (also called z-cyclotetrasiloxane) or the comparable substance cyclotetrasiloxane D4 (Daily med online 2010). Cyclotetrasiloxane D4 (Octamethylcyclotetrasiloxane (CAS: 556-67-2) is a commonly used ingredient in personal care products, which is classified as a reproductive toxicant and can be considered an endocrine disrupter based on estrogenic effects and fertility effects (SCCS opinion 2008).

**Human data**
Due to the estrogenic and gonadotrophin suppressing activity Quadrosilan has been tested as an antigonadotropic drug (under the name Cisobitan®) for treatment of prostate cancer patients.
In a study of Krarup et al. 1978 on 13 patients with stage III and IV carcinoma of the prostate it is stated in the abstract that "the drug proved to be a strong antiandrogen and exerted all the known effects of estrogens, including feminization and cardiovascular complications"
In a subsequent prospective controlled multicentre study on the use of Cisobitan in treatment of prostate cancer in 140 patients received an oral dose of 300 mg Cisobitan three times daily for three months (Alfthan et al., 1983). Unfortunately FSH and LH determinations were omitted in many patients before start of therapy, but it was noted that during the course of treatment subnormal values of FSH and LH were observed in 42/44 patients in the Cisobitan group, while prolactin levels were normal. No information on testosterone levels was given (but the whole idea of the treatment is to reduce androgen activity).
In the paper, by Alfthan et al. 1983 it is stated with reference to Sundwall 1980 that post mortem examinations indicate that Cisobitan accumulates in fat and prostate tissues. The concentration in fat tissue was about 1000 time higher than in serum. This may also explain the observed long elimination time. Two patients, in whom therapy had been discontinued, had still measurable serum levels of Cisobitan six months after therapy had been stopped.

***In vitro* data**
No relevant data found.

**Toxicity studies, human health**
Quadrosilan is clearly a potent reproductive toxicant. Older references describe quadrosilan as a candidate drug for use as contraception, i.e. male or female antifertility agent. The compound has also been tested for effects in prostate cancer treatment. The effects are likely due to the strong estrogenic effects shown in uterotrophic assays.

Detailed study summaries are provided below:
**Le vier and Jankowiak 1971**. Study on quadrosilan at 0.33 mg/kg bw/day in female rats administered GD 1-5 and at 3 mg/kg administered at GD 1. Used as postcoital antifertility agent, and inducing ovum distruction. Estrogenic activity.

**Le vier and Jankowiak 1972**. Study on male rats showing decrease of testosterone, decreased weight of prostate and seminal vesicle, altered pituitary structur eand function in male rats exposed to quadrosilane at 33 mg/kg bw/day. This study shows endocrine disruption suggesting an anti-gonadotropic action.
**Le vier and Jankowiak 1975a**. Similar studies to those presented by Le vier and Jankowiak 1972. Quadrosilan is considered an estrogenic compound acting as an antigonadotropin in the male rat.
**Le vier and Jankowiak 1975b**. Ovariectomized rats were exposed to quadrosilan in concentrations from 0.001 to 33.3 mg/kg bw/day and also at these doses together with 0,005 mg/kg bw/day of estradiol benzoate for 3 days. When administered alone all doses increased uterine wet weight. No anti-estrogenic effects were seen in the experiment with concomitant estradiol benzoate exposure. In studies on pregnant rats, antifertility effects were observed. These studies show clear estrogenic effects of quadrosilan.
**Tarasek et al., 1978**. Adult SD rats were exposed to quadrosilan at doses of 1, 2, and 5 mg/kg bw/day and mated with control females with 2 weeks interval. The number of implantations and corpora lutea were determined 10 days after mating. The number of implantations was reduced at all doses and returned to normal after the end of dosing. In rabbits exposed for 3 weeks complete inhibition of fertility was obtained at 1 mg/kg bw/day, whereas fertility was decreased at 0.5 mg/kg but not affected at 0.1 mg/kg bw/day. Semen quality was affected at all doses in rabbits. Thus, a LOAEL of 0.1 mg/kg bw/day can be determined based on fertility effects in the rabbit.
**Nicander 1972** describes a study on quadrosilan as an antifertility compound in rabbits and dogs. In rabbits, exposure to 2 mg/kg bw/day for 5 to 30 days resulted in spermatogenic arrest and altered Leydig cell structure. In dogs, exposure to 10 mg/kg bw/day or 250 mg/kg bw/day quadrosilan for 40 days resulted in spermatogenic arrest and complete degeneration of germ cells, atrophy of interstitial cells, atrophy of epididymis and prostate. The authors find that these changes are due to an estrogenic or antiandrogenic action of quadrosilan.
**Albanus et al., 1975.** A study in dogs showed clear effects on sperm production and uterus structure at 10 or 250 mg/kg bw/day.
**Aire et a., 1979**. A study in mice shows reproductive effects at 20 and 40 mg/kg bw/day of quadrosilane. Adult mice were exposed orally for 12, 21 or 42 days. Severe testicular and epididymal effects and reduced cholesterol levels were observed indicating effects on steroidogenesis (antiandrogenic effect).

***In vivo*, ecotoxicity**
No relevant data found.

**Weight of evidence for ED and Category**
Based on human data, there is no doubt that Quadrosilan shows *in vivo* estrogenic, antigonadotropic and thereby anti-androgenic effects in humans following oral exposure to pharmacological doses. Based on human data, Quadrosilan is considered an endocrine disrupters in Category 1.

No relevant *in vitro* or ecotoxicity data were found.

Based on *in vivo* studies, quadrosilan is a potent reproductive toxicant with effects at 0.1 mg/kg bw/day in the rabbit (LOAEL, Tarasek et al., 1978). As these effects are likely related to an anti-androgenic or estrogenic mode of action, this compound can be considered an endocrine disrupter in Category 1.

Based on the combined evidence from the human studies and the *in vivo* studies, Quadrosilan is evaluated as an endocrine disrupter in **Category 1.**

The adverse effects of Quadrosilan have been seen in humans and in experimental animals at 0.1 mg/kg/day, and Quadrosilan is therefore also as an endocrine disrupter of very high regulatory concern according to DE-UK potency criteria.

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**Resorcinol, CAS 108-46-3**
**Synonyms**: 1,3- benzenediol, m-hydroxybenzene, Resorcin.

**Human data**
According to human case reports, resorcinol indeed exerts antithyroid functions. Data are old (all from before 1973), but quite clear: long-term administration of resorcinol to permeable (damaged) skin can cause myxoedema (reduced thyroid function). Cessation of exposure causes the myxoedema to disappear.

Below detailed study summaries are provided.
**Bull and Fraser 1950.** The study is a report of 3 cases of women developing myxoedema after long-term topical administration of ulcer ointments containing 4-12 % resorcinol. Ointments containing resorcinol were administered to large leg ulcers daily / several times daily. In two of the women, the myxoedema disappeared / diminished when resorcinol treatment was suspended. The third women died from cardiac failure, and therefore no treatment suspension data was available from her (cardiac failure did not relate to resorcinol). After this initial report, more case reports with similar conclusions appeared. Some of them are listed below. The most recent one is from 1973. Thus, no case reports of thyroid function effects have been published within the last 30 years.
**Quentin et al, 1951**. A clear case report of a man developing myxoedema and goitre after use of ointments containing 4 and 12% resorcinol against ulcerations of the legs. Upon ointment withdrawal, symptoms disappeared.
**Berthezene et al, 1973**. This is a report written in French and describing two cases of resorcinol-induced hypothyroidism. Due to the language, the report has not been read. However, other studies refer to this one as a clear example of resorcinol-induced hypothyroidism.
**Katin et al, 1977.** A case report of a patient on chronic hemodialysis, who developed hypothyroidism while using large amounts of a cream containing 2% resorcinol against pruritus (itching skin). The hypothyroidism disappeared when skin cream was removed. The patient also received replacement thyroid hormones for a period while the resorcinol-containing cream was removed. When replacement therapy was withdrawn (and resorcinol use still avoided) the patient continued to be euthyroid. Because of the combination of resorcinol withdrawal and replacement therapy, at least for a period, no clear cut causal relationship can be concluded. However, the study supports the hypothesis, that resorcinol inhibits thyroid function.
**Yeung et al, 1983.** The study investigated resorcinol uptake across intact skin in a few healthy human volunteers. A solution containing 2 % resorcinol was applied twice daily to three volunteers on 30 % of their total body surface. The daily doses were 60 times higher than during typical acne treatment (resorcinol is used in anti-acne ointments). Exposures lasted up to 4 weeks, and blood samples were collected at 1, 2, 3, and 4 weeks. No free resorcinol was detected in any blood samples taken. TSH, $T_3$, and $T_4$ levels were unchanged throughout the study. Less than 3% of the applied dose was detectable in urine samples. According to the authors, the study demonstrated a good safety of topical ointments containing 2 % resorcinol.
**Roberts et al, 1990**. This study investigates the occurrence of hypothyroidism in textile workers at a certain factory. The workers are exposed to resorcinol and thiourea, both of which have antithyroid activity. Due to the concurrent exposure to more compounds (and even a potent antithyroid agent like thiourea), this study cannot be used to conclude anything about resorcinol exposure and thyroid function.

***In vitro* data**

Resorcinol, and some of its derivates, has been shown to be very potent inhibitors of the enzyme thyroid peroxide *in vitro*, and to inhibit uptake of radioactive iodide (Lindsey et al 1992). Irreversible loss of thyroid peroxidase activity was also shown in a study by Divi & Doerge (1994), and more recently resorcinol has also been shown to disrupt the thyroid hormone system in the T-screen, by proliferation of the TH-dependent rat pituitary GH3 cell (Ghisari and Bonefeld-Jorgensen 2009). Furhtermore, resorcinol has been shown to affect both the aryl hydrocarbon receptor (AhR) and the androgen receptor (AR) *in vitro* (Krüger et al 2008), and to inhibit prostaglinding production (Alanko et al 1993) and affect glucose metabolism by inhibiting phosphorylase (Aiston et al 1999).

**Toxicity studies, human health**

Rat studies performed in the 1950s quite unambiguously show effects on the thyroid hormone system of rats treated with resorcinol, shown as decreased uptake levels of radioactive iodine (Doniach & Fraser 1950, Arnott & Donich 1952) and increased thyroid weight and altered thyroid histopathology (Samuel 1955, Doniach and Logothetopoulos 1953). In their conclusions Doniach and Logothetopoulos 1953 stress the importance of maintaining a continuous high antithyroid drug level in the blood stream. Since resorcinol is rapidly cleared from the plasma through urinary excretion a mode of exposure that allows for a slower and more continuous release of resorcinol to the systemic circulation is likely required to produce histological evidence of goiter in rats i.e., resorcinol administered by gavage or subcutaneously in an aqueous vehicle is rapidly cleared from circulation and, therefore, resorcinol is not present systemically for a sufficient time to inhibit thyroid hormone synthesis. In two more recent studies, effects of resorcinol exposure in rats have been seen at a very low dose levels (5mg/kg/day), however both studies used only one dose level. In the first study resorcinol caused decreases in T3 and T4 levels and increased size of the thyroid after 30 days of dosing (Cooksey et al 1985) while altered thyroid hiostopathology was seen after 12 weeks of dosing in the other study (Seffner et al 1995). In both studies resorcinol was added to the drinking water.

In 1992, the National toxicology Program of the US EPA tested the effects of resorcinol given to rats by gavage for 13 weeks and no significant effects on T4 levels were seen (NOAEL 130 mg/kg/day). In a more recent two-generation study examining the effects of resorcinol dosing through the drinking water on the thyroid system in rats, the only significant effect was histopathological changes in the thyroid of males from the parental generation, while no effects on thyroid hormone levels or thyroid gland weight were seen at any time point in the parental or offspring generations (Welsch et al 2008). The LOAEL from this study was 233 mg/kg/day in males and 340-660 mg/kg/day in females.

The discrepancy between available data is most likely due to different administration routes and forms. In the animal studies reporting negative results, resorcinol has been administered via gavage or drinking water. Free resorcinol is extremely efficiently metabolized (possibly by first pass through the liver) and effectively removed from the body via the urine, which may explain the lack of thyroid effects seen in some of these studies.

Study summaries are provided below:

**Doniach & Fraser (1950**).Hooded Lister rats were injected subcutaneously with 1 mg/kg or more, which caused decreased uptake levels of radioactive iodine.

**Arnott & Donich 1952**. Subcutaneous (sc) injection of resorcinol at doses of 70 and 180 mg/kg caused decreased radioactive iodine uptake, at levels that were 24% and 14 % of controls, respectively

**Doniach and Logothetopoulos (1953)** Different exposure routes of resorcinol in rats were found to affect thyroid function in different ways. Treating of shaved rats with resorcinol containing ointment for three weeks (300 mg/kg/day) had no effect on thyroid weight, whereas twice daily sc injections of the same dose

of resorcinol dissolved in oil resulted in increased thyroid weight and histopathological changes. The authors also tested the effects of sc injections with resorcinol diacetate (1500 mg/kg/day), which after 12 days of treatment also led to increased thyroid weights and histopathological changes. A single injection of resorcinol in water had no effect on iodine intake. In their conclusions the authors stress the importance of maintaining a continuous high antithyroid drug level in the blood stream, in order for thyroid hyperplasia to occur. Even a few hours 'break' from the antithyroid action, would be associated with temporary thyroxine formation (probably the effect seen after injection of resorcinol in water, owing to its rapid elimination), which would prevent or delay goitrogenesis. They further conclude that the sc exposure to resorcinol in oil caused slow release of resorcinol at the oil-tissue interspace and, while resorcinol diacetate slowly hydrolysed, releasing the drug into the circulation during the hours between injections at a rate sufficient to maintain a continuous antithyroid level over the period of the experiment.

**Samuel et al 1955.** Paper reviewed in Lynch et al 2002. Rats treated sc for periods between 21-78 days at doses of 300 and 400 mg/kg/day dissolve in peanut oil, showed increased thyroid weights and histological changes in the thyroid.

**Cooksey et al 1985.**. Rats treated with resorcinol in the drinking water for 1 month at 5 mg/kg/day showed thyroid enlargement and decreases in T3 and T4.

**Seffner et al 1995** WELS/Fohm rats treated with resorcinol in the drinking water for 3 month at a dose of approximately 5-10 mg/kg/day showed histomorphometric changes of the thyroid.

**NTP 1992** National toxicology Program of the US EPA tested the effects of resorcinol given to F344/N rats (n=10) by gavage for 13 weeks (5 days a week) at doses of 32, 65, 130, 260 and 520 mg/kg/day in deionized water vehicle (high dose animals died). Thyroid hormone levels were measured in the dose group receiving 130 mg/kg/day, however no significant effects were seen and no other signficicant thyroid histology effects were seen at any dose level.

**Welsch et al 2008**. Doses of 120, 360, 1000, 3000 mg/L were given to SD rats (n=30) in the drinking water, starting 10 weeks before mating and continuing throughout lactation of the second generation, in a two-generation study examining the effects of resorcinol on the thyroid system. The only effect on the thyroid hormone system of the rats seen in this study were histopathological changes in the thyroid of males from the parental generation, while no effects on thyroid hormone levels or thyroid gland weight were seen at any time point in parents or offspring (PND 4, 21 and adulthood). Also, no reproductive effects of resorcinol exposure were seen. The NOAEL in this study was therefore 1000 mg/L which for males corresponded to 86 mg/kg/day and in females was 126-225, while the LOAEL from this study was 3000 mg/L which in males was 233 mg/kg/day and in females was 304-660 mg/kg.

***In vivo*, ecotoxicity**

Thienpont et al (2011) did a screening for the thyroid gland function disrupting activity of drugs, environmental pollutants, and naturally occurring substances in zebrafish eleutheroembryos using intrafollicular T4-content (IT4C) as the end point. Zebrafish eleutheroembryos were exposed to resorcinol at the maximum tolerated concentrations from 48 to 120 hpf and IT4C was measured using TIQDT. Resorcinol lowered IT4C to less than 20% of control level and was defined as thyroid gland function disruptor in zebrafish. EC50 was around 120 µM resorcinol.

**Weight of evidence for ED and Category**

According to human case reports, resorcinol exerts antithyroid functions. Data are old, but quite clear: long-term administration of resorcinol to permeable (damaged) skin can cause myxoedema (reduced thyroid function). Cessation of exposure causes the myxoedema to disappear. In the human case reports, exposure has occurred via damaged skin, directly to the blood stream, thereby escaping first pass metabolism. In the

human study investigating dermal uptake in healthy individuals (Yeung 1983) the dermal barrier avoids uptake of resorcinol. These data alone can place resorcinol in Category 1.

*In vitro*, resorcinol has been shown to be a very potent inhibitor of the enzyme thyroid peroxide and to inhibit uptake of radioactive iodide, which are both effects that *in vivo* could lead to decreased thyroid hormone levels. The results from the *in vivo* studies are however somewhat inconsistent. A number of older studies using relatively few animals per dose group found adverse effects on the thyroid hormone system if dosing was performed in a way that allowed for a slow and continuous release of resorcinol to the systemic circulation (i.e. sc injections in oily solution). However, all these studies only investigate one dose level. Some more recent extensive rat studies investigating the effects on the thyroid hormone system using more animals and several dose levels have also been performed. Here rats have been dosed by gavage or in the drinking water but no or only very few effects on the thyroid system have been found, indicating that route of exposure is very important. This is most probably because rapid metabolism in most cases prevents resorcinol from reaching concentrations which are toxic for the thyroid gland. Based on the evaluation of all of these studies taken together, resorcinol can be placed in Category 2a.

In the ecotoxicology study, anti-thyroid effect in zebrafish embryos was seen. It should be noted that this study could be viewed as an *in vitro* study instead of *in vivo* due to the EU legislation about fish embryos. Resorcinol is evaluated as indicated ED in Category 2b, because the only relevant study cannot be viewed as real "*in vivo*".

Mainly based on human case studies showing antithyroid effects, but also supported by some *in vivo* and *in vitro* studies showing that resorcinol can affect the thyroide hormone system, resorcinol is evaluated as an ED in **Category 1.**

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which effects are observed i.e. effects need to be observed at an oral dose of 10 mg/kg/day. For resorcinol, adverse effects were seen at lower dose levels in some studies and not seen at higher dose levels in other studies, and therefore, it is unclear whether resorcinol can be considered as an endocrine disrupter of very high regulatory concern according to DE-UK potency criteria.

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**Tert-butylhydroxyanisole (BHA), CAS 25013-16-5**
**Synonyms:** 2-tert-butyl-4-methoxyphenol; 3-tert-butyl-4-methoxyphenol, butylated hydroxanisole, E320, BHA

**Human**
No relevant data found.

***In vitro* studies**
Several studies have shown (weak) estrogenic effect of BHA *in vitro*, by causing decreased binding of 17beta-estradiol to trout ER, proliferation of MCF-7 cells (Jobling et al 1995), and by showing competitive binding affinity with 17β-estradiol (Okubo and Kano 2003). The latter is paper written in Japanese, so results are from abstract only. Furthermore, a study has shown estrogenic effect in ERα and ERβ reporter gene cell lines (Ter veld et al. 2006). BHA has also shown to be estrogenic in the E-screen assay (Soto et al. 1995). Overall the *in vitro* results show that BHA has estrogenic mode of action.

***In vivo* studies**
One Uterotrophic assay indicates that BHA has anti-estrogenic mode of action. No effect was seen in the Hershberger assay.
In rats dosed with BHA a long list of adverse reproductive effects has been found. Several of these effects points toward endocrine disruption, i.e. altered oestrous stages and cycles, decreased epididymis weights, altered sperm head morphometry, decreased caudal sperm number and abnormal thyroid histology in both femla and male offspring. Also, reduced levels of testosterone and thyroid hormones were found.

Summaries of the *in vivo* studies are provided below:

**Kang et al 2005**: Evaluation of estrogenic and androgenic activity of butylated hydroxyanisole in immature female and castrated rats. SD rats were dosed with 50, 100, 250, 500 mg/kg/day in the Uterotrophic assay: BHA alone decreased absolute and relative uterine weight at 50, 100, 250, 500 mg/kg. BHA also decreased 17beta-estradiol-stimulated absolute and relative uterine and vaginal weights at 500 mg/kg. No effect of androgen-dependent accessory sex organ weights was found in a Hershberger assay. However, the decrease in uterine weight indicates that the compound possesses anti-estrogenic activity *in vivo*.
**Wurtzen G, Olsen P (1986).** BHA study in pigs (SPF Danish Landrace) dosed with 50, 200, 400 mg/kg/day in the feed (from day of artificial insemination to GD 110). In dams, there was decreased weight gain at 400 mg/kg, highly significant increases in actual and relative liver and thyroid weights and thyroid histology indicated a reduced activity. Iodine analyses indicated the effect observed in the thyroid could not be due to any interference with iodine uptake. Neither effect on reproduction nor on gross birth defects in the piglets were seen.
**Jeong et al 2005**: In a generation study, effects of butylated hydroxyanisole on the development and functions of reproductive system in rats were explored. SD rats were, dosed with 10, 100 or 500 mg/kg/day by oral gavage in F0 generation 2-4 weeks pre-breeding; exposure continued for a total of 7 weeks (males) or 10 weeks (females). F0 males: weight increased in liver (100, 500 mg/kg), adrenal and thyroid (500 mg/kg); weight decreased in spleen and ventral prostate (500 mg/kg); testosterone decreased (100, 500 mg/kg), T4 decreased (500 mg/kg); days to copulation increased (500 mg/kg). F0 females: increased liver weight (500 mg/kg). F1 progeny were then exposed from PND 21 until 13 weeks of age. At PND 21: in F1 males body weight and brain weight decreased (500 mg/kg) and AGD was slightly shortened (500 mg/kg, note this effect

was not found at 10 weeks); in F1 females body and liver weight decreased (500 mg/kg). Also vaginal opening and preputial separation were delayed (500 mg/kg). At 13 weeks: in F1 males increased liver weight (500 mg/kg), decreased body (500 mg/kg), spleen (100, 500 mg/kg), testes, ventral prostate (500 mg/kg), and epididymis (100 mg/kg) weights, altered sperm head morphometry (10, 100, 500 mg/kg), decreased caudal sperm number, sperm curvilinear velocity, straight line velocity, and velocity of average path (500 mg/kg), and abnormal thyroid histology (100, 500 mg/kg); in F1 females increased adrenal and liver weight (500), decreased spleen (10, 100, 500) and vaginal weight (100, 500 mg/kg), altered oestrous stages and cycles (500 mg/kg), increased cholesterol (500 mg/kg), decreased T4 (500 mg/kg) and abnormal thyroid histology (100, 500 mg/kg). In conclusion, this study show BHA induce dysfunction and underdevelopment of reproductive system of male and female rats with the change of T4 and testosterone levels, sex organ weights and sexual maturation and histological lesions of thyroid gland.

**Ecotoxicology**
No relevant ecotoxicity data.

**Weight of evidence for ED and Category**
No relevant epidemiological or ecotoxicological studies were found.

Based on *in vivo* and *in vitro* data, there is evidence of estrogenic mode of action *in vitro*, evidence of anti-estrogenic activity *in vivo* and adverse reproductive effects such as altered oestrous cycles, altered sperm morphology and decreased sperm number in developmental *in vivo* studies. The adverse reproductive effects could be caused by general reproductive toxicity, but since decreased levels of testosterone are also observed there is evidence pointing towards endocrine disrupting mode of action. Also, abnormal thyroid histology and reduced levels of thyroid hormones were found providing both evidence for ED mode of action and adverse effects, respectively. Therefore BHA is evaluated as an ED in **Category 1.**

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which adverse effects are observed, i.e. effects have to be observed at 10 mg/kg or lower. For BHA effects on sperm morphology are seen from doses of 10 mg/kg/kg and based on this study BHA may also be evaluated as an as an endocrine disrupter of very high regulatory concern based on the DE-UK potency criteria.

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**Thiram[5], CAS 137-26-8**
Synonyms: tetramethylthiuram disulphide

**Human data**
No relevant human data was found.

***In vitro* data**
It is unclear whether thiram is a thyroid disruptor *in vitro*. The mechanisms of action of thiram are not completely understood but may be similar to other thyroid peroxidase (TPO) inhibitors. Chinese hamster ovary cells transfected with the human TPO gene showed no effect of thiram on TPO inhibition when using iodination of a Glu-Tyr-Glu peptide and oxidation of guaiacol as endpoints (Marinovich et al 1997). However, TPO is a complex enzyme that functions beyond iodination, such as the coupling of monoiodinated tyrosine/diiodinated tyrosine to make T3 and T4 (Taurog et al 1996). Thus, although thiram may not inhibit iodination or oxidation of guiacol *in vitro*, it may inhibit those processes *in vivo* or inhibit other functions of this enzyme that are involved in TH synthesis. Other data indicate more similarities between thiram (and pronamide) and other TPO inhibitors than thyroid hormone disruptors that induce UGTs or inhibit deiodination. Therefore, thiram may still be a TH synthesis inhibitor (Flippin et al 2009).

***In vivo*, human health**
Thiram is a dialkyldithiocarbamate (together with ferbam, metam natrium and ziram) and these pesticides are metabolised via different mechanisms than the group of ethylenbisdithioracbamates (EBDC) which include maneb, mancozeb and zineb, and are not metabolised to ETU (IPCS 1978). In the literature search of both published and unpublished studies, no data showing adverse effects of thiram on the thyroid hormone axis were found. However, some evidence of endocrine activity of thiram exists, as the pesticide has been shown to block ovulation and LH and thereby reduce pregnancy rate and increase number of resorptions. These effects have been reported in both unpublished studies (as reviewed by IPCS 1978) and in the published literature.

See below for study descriptions.
**3 studies by Stoker et al:** In a study of the effects of thiram on hormonal control of ovulation in Long-Evans rats, intact, prooestrous females were i.p. administered one dose of 0, 12, 25 or 50 mg/kg thiram. Ovulation was blocked in all rats by 25 and 50 mg/kg and the luteinizing hormone (LH) surge was blocked in all rats at 50 mg/kg and in 60% of the females at 25 mg/kg (Stoker et al 1993). The group sizes were 5-6 animals per group. To characterize pregnancy outcome following thiram-induced ovulatory delay, regularly cycling female rats were injected with thiram (0 or 50 mg/kg, i.p.) on prooestrous and mated (Stoker et al 1996). There were 12 animals in the control group and 30 animals in the thiram group. The number of females in the thiram-group that became pregnant was reduced to 46% and the number of live foetuses on GD 11 was reduced, but number of implantation sites was not different from controls. Also, signs of delayed development of live embryos were seen on GD 11. A follow-up study in similarly dosed and mated females showed that a thiram-induced 24-h delay of ovulation altered the fertilizability of the released oocytes

[5] For substances which are registrered as plant protection products, biocides or pharmaceuticals within the EU, there may be additional documentation for the toxicity of the substances. This documentation has not been a part of this assessment, The authors are aware of this documentation and find it unlikely that it would influence the proposed categorisation.

(Stoker et al 2003). The changes observed included a significant decrease in the percentage of fertilized oocytes and a significant increase in polyspermic zygotes.
Thiram was administered to adult male rats via gavage at dose 0, 5, 10, and 25 mg/kg for 30, 60 and 90 days (Mishra et al 1993). A significant increase in relative testes weight and pathomorphological changes (incl. degeneration of few seminiferous tubules) was found at 25 mg/kg after 90 days exposure. The dosing with thiram also affected testicular enzymes and induced signs of toxicity such as diarrhoea, salivation, nasal bleeding and mild nasal bleeding in a dose dependent matter. In the group dosed with 25 mg/kg, 8 of 25 rats died and the body weight gain during the 90 days appeared reduced (132 g compared to 165 g in controls). The results suggest that thiram at 25 mg/kg may induce testicular dysfunction, but the effects seen on the testes and testicular enzymes may also be indirect effects of the general toxicity seen as decreased body weight gain, mortality and clinical symptoms.

***In vivo*, ecotoxicity**
Thiram have been shown to cause a down regulation of sox9a during zebrafish development (LOEC 24 µg/l) and disrupting corticosterone action on the glucocorticoid receptor in zebra finches.

Study descriptions of the ecotoxicolocy studies:
**Katz et al. 2008.** The effects of glucocorticoids were tested on ventricular zone cell proliferation in adult zebra finches where neurons are produced that migrate to and incorporate within the neural circuits controlling song learning and performance. Adult male zebra finches sing and have an enlarged song circuitry; females do not sing and the song circuit is poorly developed. Freshly prepared slices from adult males and females containing the lateral ventricles were incubated with the mitotic marker BrdU with or without steroid treatments. BrdU-labeled cells were revealed immunocytochemically and all labeled cells within the ventricular zone were counted. Significantly higher rates of proliferation along the ventricular zone of males than in females were found. Moreover, acute administration of corticosterone significantly reduced proliferation in males with no effects in females. The corticosterone effect was reversed by thiram, which disrupts corticosterone action on the glucocorticoid receptor.
**van Boxtel et al. 2010.** Down regulates sox9a during zebrafish development after exposure from 0-5 dph to 100 nM thiram (24µg/l).

**Weight of evidence for ED and Category**
Thiram down regulates sox9a during zebrafish development (LOEC 24 µg/l) and disrupts corticosterone action on the glucocorticoid receptor in zebra finches.Thiram is evaluated as 2b (indicated ED) because the described effects may possibly be caused by endocrine mechanisms.

As *in vivo* studies in rats have shown adverse effects e.g. blocked ovulation and decreased number of live foetuses, where an ED mode of action is highly plausible (block of LH surge *in vivo)*, thiram could fulfil the criteria of being an endocrine disrupter in Cat 1.The alternative route of exposure (i.p.), however, means that the evidence from the experimental animals might not be sufficiently convincing to place the substance in Category 1, but rather in a Category 2a. However, the substance is a dialkyldithiocarbamate and the observed adverse effects on reproduction are similar to the effects of metam natrium, so read across to metam natrium has been included in the evaluation. Overall, thiram is therefore evaluated as an ED in **Category 1**.

According to the DE-UK potency criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which effects are observed, i.e. effects need to be observed at an oral dose of 10 mg/kg/day or lower. For thiram in the 2-generation study the report concluded that the NOAEL

was greater than 180 ppm equal to 8.9 and 14 mg/kg bw/day in males and females, respectively. On that basis, it could be discussed whether thiram would be considered an endocrine disrupter of very high regulatory concern according to the DE-UK potency criteria.

**References, epidemiology**

No references

**References, *in vitro* and *in vivo***

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**Zineb[6], CAS 12122-67-7**
**Synonyms:** Zinc ethylenbis (dithiocarbamate)

**Human data**
Overall, the available human studies are unsuitable for inclusion in an evaluation of the endocrine disrupting potential of zineb. The different studies are reported below but no relevant data have been found.

Study descriptions are provided below:
**Israeli et al, 1983**: This is a case study of a 42-year old man, who experienced acute intoxication after exposure to maneb and zineb. The intoxication occurred after spraying with a high concentration of maneb/zineb, and subsequently walking through the sprayed area. Symptoms were linked to the central nervous system and included loss of consciousness, convulsions and right hemiparesis – and disappeared after few days.
The study looked at a combination of maneb and zineb, reported a case of high exposure, and reported symptoms unrelated to the endocrine system. Thus, the study is unsuitable for inclusion in an evaluation of the endocrine disrupting potential of zineb.
**Steerenberg et al, 2008**: This European study investigated the association between urine levels of ethylenethiourea (ETU, metabolite of ethylenbisdithiocarbamate, and used as general marker of pesticide exposure in the study) and biomarkers of immunotoxicity. Occupationally exposed individuals from five European field studies were included, of which one was a Bulgarian zineb factory. These were compared with non-occupationally exposed persons. There were no associations between ETU and immune parameters.
The study did not look at or measure zineb in specific. Biological endpoints were not relevant to endocrine disruption. Thus, the study is unsuitable for inclusion in an evaluation of the endocrine disrupting potential of zineb.

***In vitro* data**
Chinese hamster ovary (CHO) cells transfected by hTPO gene have been used to examine the effect of zineb (and other dithiocarbamates) on peroxidase activity in CHO cells. Thyroid peroxidase is catalysing the transfer of iodine to thyroglobulin. The results from this study showed that the CHO cells transfected with human thyroid peroxidase (TPO) gene (exposed to 5 and 10 μM zineb) decreased TPO iodination activity which is a sign of thyrotoxicity (Marinovich et al 1997).
Overall, zineb seems to show similar mechanism of action as mancozeb, where inhibition of thyroid peroxidase (TPO) leads to decreased thyroidal synthesis of T3 and T4.

***In vivo*, human health**
Zineb is a dithiocarbamate that has the same thyroid hormone disrupting effects as the other dithiocarbamates, including reduction of thyroide hormone levels, increase of Thyroid Stimulating Hormone (TSH), enlargement of thyroid size and histopathological changes in the thyroid (unpublished studies reviewed in IPCS INCHEM 1978). All published *in vivo* studies of zineb, where rats have been exposed to Zineb, have also shown a tendency to or a significant increase in thyroid weight (hyperplasia) and/or changes in thyroid function indicating thyroid disrupting activity (Raizada et al 1979, Ivanova-C L et al 1974, Nebbia

[6] For substances which are registrered as plant protection products, biocides or pharmaceuticals within the EU, there may be additional documentation for the toxicity of the substances. This documentation has not been a part of this assessment, the authors are aware of this documentation and do not find it likely that it would influence the proposed categorisation.

et al 1996). In one of the studies, where adult male rats were exposed to either vehicle or 1000 mg/kg day for a period of 30 days, pathological changes in the testis (damaged tubules) were also reported (Raizada et al 1979). This paper refers to an older study that has shown that zineb has caused sterility in rats (Korte 1972 in Raizada et al 1979). A post-implantation study (using pseudo pregnant and pregnant rats) was conducted to look at reproductive effects after zineb exposure (Sing et al 1981). The study concluded that zineb in this system had low toxic effects on the foetus and maternal reproduction system.
A 90 days study in rabbits was performed with 0.3-0.6% zineb in the feed (Nebbia et al 1995). The results showed that T3 and liver triglycerides decreased, testicular glutathione S-tranferase and thyroid absolute and relative weights was increased, and there were histological changes in liver, thyroid, and spleen. At 0.6%, at 75 days, body weight decreased while at 90 days, T4, liver lipids and testes absolute weight decreased, liver relative weight and serum total lipids and serum cholesterol increased, and there were decreases in hematocrit, hemoglobin and erythrocytes. Moreover, white blood cell counts decreased by about 50% (Nebbia et al 1995, only from abstract).
In a reproduction study, rats were given zineb at doses of 50 or 100 mg/kg bw/day orally for 2-6 months. Sterility, resorption of foetuses and anomalous tails in newborn rats were observed at the high levels. The lower doses did not cause any significant changes, compared with a control group (Rjazanova, 1967 at www.inchem.org ).
Moreover to examine if exposure to zineb (and other pesticides) during critical periods of postnatal development could result in neuronal dysfunction and enhance the impact of these pesticides during exposure as adults C57BL/6 mice was injected at PND 5-19 with 5 mg/kg i.p., and then challenged 8 months later with 50 mg/kg. Dopamine and DOPAC (its metabolite) decreased in striatum (only after challenge). Authors note that in a preliminary study without early postnatal exposure, they did not find this effect using only 50 mg/kg at 8 months. The results indicate that exposure to zineb during critical periods of postnatal development contributes to neurotransmitter changes upon re-challenge in adulthood (Jia et al. 2007).
No NOAELs or LOAELS was found for Zineb (see weight of evidence for read across below). Overall from the *in vivo* studies it was found that Zineb is disrupting the thyroid hormone system in rats and could also be involved in neuronal dysfunction in mice and pathological changes in the testis in rats.

***In vivo*, ecotoxicity**
No endocrine related endpoints have been investigated. No relevant data found.

**Weight of evidence for ED and Category**
No relevant human studies have been found.

Only few *in vitro* studies have evaluated endocrine disrupting properties of Zineb. *In vivo* studies in rats have shown some evidence for endocrine disrupting effects, e.g. increase in thyroid weight and decrease in T3 and T4. Furthermore read across from other dithiocarbamates has been used in the assessment of zineb. Dithiocarbamates are degraded to the known thyroid hormone disrupting substances ethylenethiourea (ETU) and propylthiouracil (PTU), which inhibit the formation of T4 in the thyroid. The JMPR (Joint Meeting on pesticide residues) evaluated the toxicity of the four ethylenebisdithiocarbamates (EBDCs) mancozeb, maneb, metiram and zineb, as well as ethylenethiourea (ETU), the major common metabolite, degradation product and contaminant of the EBDCs. Since no differentiation can be made between the parent EBDCs, it was decided to establish a group ADI for the EBDCs, and an ADI was allocated to ETU (FAO/WHO, 1993). The meeting concluded that the database for zineb was inadequate to determine its own ADI. A NOAEL of 4.8 mg Mancozeb/kg bw/day has been reported in a two-year toxicity/carcinogenicity feeding study (dietary concentrations of 0, 20, 60, 125 or 750 ppm, the NOAEL was 125 ppm) with findings of decreased body-

weight gain, histological changes in the thyroid (thyroid follicular cell hypertrophy, hyperplasia, and nodular hyperplasia at 750 ppm), increased absolute and relative thyroid weight and decreased T3 and T4 levels and increased TSH in rats exposed for 2 years (Stadler, 1990, as reviewed in (FAO/WHO, 1993)). This NOAEL was used in the determination of the group ADI for Maneb, Mancozeb and Zineb. Thus Zineb can disrupt the thyroid hormone system in rats and could also be involved in pathological changes in the testis in rats. However, the relatively low number of studies investigating adverse effects during the developmental period indicates that the evidence from the experimental animals might not be sufficiently convincing to place the substance in Category 1.

Overall, zineb is evaluated as an **ED in Category 1** based on disruption of the thyroid hormone system in rats seen in both published and unpublished Zineb studies and which is supported by read-across from the other similar dithiocarbamates (Maneb and Mancozeb) and their metabolite (ETU) which are all known to disrupt the thyroid hormone system.

According to the DE-UK criteria, categorization as an endocrine disrupter of very high regulatory concern is based on the dose level at which effects are observed i.e. effects need to be observed at an oral dose of 10 mg/kg/day. For zineb, adverse effects were seen at much higher dose levels in a reproduction study in rats. Therefore, zineb can not be considered as an endocrine disrupter of very high regulatory concern according to DE-UK potency criteria.

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