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DANISH CENTRE ON ENDOCRINE DISRUPTERS

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GROWTH REPRODUCTION

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 tebuconazole, triclosan, methylparaben and ethylparaben and their endocrine disrupting properties.

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 on Endocrine Disrupters to conduct a project which is to evaluate tebuconazole, triclosan, methyl paraben and ethyl paraben. The overall aim is to categorize the 4 substances on the basis of the Danish proposal for criteria for endocrine disrupters which is scientifically justified by a report (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, the 4 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.

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

Substance	Use(s)
Tebuconazole	A triazole fungicide used agriculturally to treat against plant pathogenic fungi and as biocide (film, wood, masonry fibre, leather, rubber and polymerised material preservation).
Triclosan	A biocide used as an antibacterial and antifungal agent.
Methylparaben	Antifungal agent often used in a variety of cosmetics and personal-care products. As a food additive, it has E number E218
Ethylparaben	An antifungal preservative used in personal care-products and as a food additive. As a food additive, it has E number E214

1.1 Danish Criteria for identification of ED

The Danish proposal for criteria for EDs (Endocrine Disrupters) 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, categorization 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 ED 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[1] Repeated Exposure (RE) to determine whether or not the hazardous property of "endocrine disruption" should be identified for regulatory purposes.

[1] 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[2] and GHS[3] 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 Regulations for chronic studies, but it is proposed here that they should be half the sub chronic study values (by applying the sub chronic 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 when it satisfies 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):

[2] 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

[3] 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 & 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 ethylparaben the following search criteria were used:
((ethylparaben or ethyl and paraben*) and (endocrine*))
((ethylparaben or ethyl and paraben*) and (estrogen* or oestrogen*))
((ethylparaben or ethyl and paraben*) and (androgen*))
((ethylparaben or ethyl and paraben*) and (aromatase*))
((ethylparaben or ethyl and paraben*) and (thyroid*))

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 registered 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 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 (datasheets are 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 reproduction toxicity 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.

3. Results

3.1 Evaluation based on DK criteria for identification of ED

Among the 4 substances, tebuconazole and triclosan are categorized as EDs in category 1 and methyl- and ethylparaben as suspected ED in category 2a. An overview of the separate evaluations based on epidemiological data, *in vitro* data plus animal data, and ecotoxicity data as well as the overall evaluations is shown in table 4.

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

Chemical	Tox	Hum	Eco	Overall
Tebuconazole	1	No data	2a	1
Triclosan	1	Too limited data	2a	1
Methylparaben	2b	No evidence	2a	2a
Ethylparaben	2b	Weak associations	2a	2a

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

The detailed datasheets with evaluations, study summaries and references for each compound are shown in the Appendix. Below substance summaries are given based on these datasheets.

Tebuconazole, CAS 107534-96-3

No relevant human data was found.

Studies show that tebuconazole has an anti-estrogenic and anti-androgenic mode of action *in vitro*.

Tebuconazole induced adverse effects on reproductive development in the offspring after exposure *in utero, i.e.* virilised the female offspring, and caused feminizing effects in male offspring. Moreover tebuconazole increased gestational length and increased progesterone levels.
Tebuconazole cause vitellogenin induction in fish.
Evaluation: Endocrine disrupter in category 1.

Triclosan, CAS 3380-34-5
Epidemiological data are too limited for an evaluation of endocrine disrupting effects of triclosan. Triclosan has been shown to have multiple modes of action *in vitro*, incl. estrogenic, androgenic and anti-androgenic effects, and can also depress synthesis of testosterone. Data on the estrogenic modes of action of triclosan are conflicting, whereas there is some evidence of the anti-androgenic mode of action of triclosan *in vitro*. Furthermore, triclosan affects metabolism processes in the liver, which may be the mechanism behind the thyroid hormone lowering effects seen *in vivo*.
In vivo, there is some evidence that triclosan may act as an estrogen, as lowered age of sexual maturation and increased uterine weights were seen in young adult females, and furthermore triclosan potentiated the effects of estradiol treatment. In adult male rats, triclosan treatment has in a single study been shown to result in decreased weights of several reproductive organs, histopathological changes in these organs, and decreased levels of reproductive hormones. These effects could be caused by both estrogenic and anti-androgenic modes of action. There is also strong evidence that triclosan affects the thyroid hormone system *in vivo* , as triclosan exposure has lead to a very marked decreases in serum thyroxine levels in weanling rats exposed for varying periods of time, as well as in rat dams exposed during gestation and lactation.
Triclosan caused vitellogenin induction in fish.
Evaluation: Endocrine disrupters in category 1

Methylparaben, CAS 99-76-3
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.
Methylparaben has weak estrogenic and weak anti-androgenic effects *in vitro*, but data are conflicting.
Methylparaben may have weak estrogenic effects *in vivo*, but data are conflicting.
A study in young male rats found no adverse effects and another study aimed to repeat this study similarly concluded no reproductive effects of methylparaben. However, an increase in abnormal sperm and a decrease in normal sperm number were observed, although no change in total sperm count was seen. Some evidence of thyroid toxicity has been shown as T4 levels were decreased and relative thyroid weight decreased in a study in peripubertal rats.
Methylparaben caused vitellogenin induction in fish as well as testicular tissue changes in fish.
Evaluation: Suspected endocrine disrupter in category 2a.

Ethyl paraben CAS: 120-47-8
A few human studies have indicated weak associations between increased exposure to other parabens (see methyl-, propyl- and butylparaben) and markers for human reproductive health. However, our knowledge in this area is very limited.
Ethylparaben has weak estrogenic and weak anti-androgenic effects *in vitro*.
In vivo studies indicate that ethylparaben has estrogenic effects *in vivo* in immature and ovariotectomized mice. A study on fetal exposure to ethylparaben revealed no changes in male anogenital distance and no changes of fetal testosterone production, but changes in gene expression levels pointed to subtle endocrine disrupting effects. A limited study in young male rats found no adverse effects, although tendencies to lower sperm counts are seen.
Ethylparaben causes vitellogenin induction in fish.
Evaluation: suspected endocrine disrupter in category 2a.

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 no substances considered as EDs or suspected EDs of very high regulatory concern (table 5).

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

Substance	DK criteria	LOAEL below 10 mg/kg
Tebuconazole	1	No
Triclosan	1	No
Methylparaben	2a	No
Ethylparaben	2a	No

4. Discussion

4.1 Evaluations based on the Danish proposal for ED criteria and the potency cut-off in the DE-UK criteria

Based on the Danish proposal for ED criteria, tebuconazole and triclosan are evaluated as EDs in category 1 and methyl- and ethylparaben as suspected ED in category 2a.

Inclusion of the potency cut-off in the DE-UK criteria means that none of the two substances (tebuconazole and triclosan) evaluated as EDs in category 1 is likely to be considered as endocrine disrupters of very high regulatory concern (table 4). 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.

None of the 2 substances (methyl- and ethylparaben) evaluated as suspected EDs in category 2a appear likely to fulfil the potency criteria in the DE-UK proposal. This is not very surprising as these substances were allocated to this category mainly based on mode of action data *in vivo* whereas there are limited data on adverse effects and the DE-UK potency criteria is a cut-off in relation to adverse effects.

4.2 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 methyl- and ethylparaben *in vivo* data showed endocrine mode of action, but there was a lack of robust *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.

Human studies

For 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 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

The two substances evaluated as suspected EDs in category 2a, i.e. methyl- and ethylparaben have an expected estrogenic mode of action shown as effect in the Uterotrophic assay and/or in vitellogenin (VTG) assays. However, also other modes of endocrine actions are indicated based on *in vitro* studies. If further data are needed, the substances are proposed to be tested in the Extended one-generation reproductive toxicity study (OECD TG 443) and not in the Two-generation study (OECD TG 416), because both the design and the endpoints included into TG 443 in contrast to TG 416 has been enhanced with focus on detection of effects of endocrine disrupters. Moreover, 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

Methyl-and ethylparaben have an expected estrogenic mode of action. The substances have shown vitellogenin (VTG) induction in one or more species but lack the connection to an adverse effect. Both substances are proposed to be tested in Fish Sexual Development test (TG 234) where the VTG concentration can be linked to an adverse effect on the sex ratio.

5 Summary and conclusions

DK-EPA has asked the Danish Centre for Endocrine Disrupters to provide a science based evaluation of tebuconazole, triclosan, methylparaben and ethylparaben and their endocrine disrupting properties. The overall aim is to categorize the 4 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, the 4 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.

Using the Danish proposal for ED criteria tebuconazole and triclosan were evaluated as EDs in category 1, whereas methyl- and ethylparaben were categorized as suspected EDs in category 2a.

Inclusion of the potency cut-off in the DE-UK criteria leads to none of the substances considered as endocrine disrupters of very high regulatory concern. This indicates that use of the potency criteria may lead to inadequate protection for some endocrine disrupting substances. The Danish proposal

for ED criteria does 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 (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

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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
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
SIN:	Substitute It Now
STOT:	Specific Target Organ Toxicity
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
Datasheets with substance evaluations

Tebuconazole[4], CAS 107534-96-3
Synonyms: LYNX; ELITE; RAXIL; MATADOR;LYNX(R); Lynx 1.2

Human data
No relevant data

***In vitro* data**
Azole fungicides were developed to inhibit sterol biosynthesis in fungi and they inhibit steroidogenesis in mammals.
In a study the H295R cell line was used to screen a number of pesticides including tebuconazole for potential effects on the catalytic activity and mRNA expression of aromatase. Tebuconazole decrease aromatase activity close to cytotoxic concentrations in H295R cells (Sanderson et al 2002).
The critical mechanism for tebuconazole (and other azole fungicides) seems to be disturbance of steroid biosynthesis.
In another study with the H295R cell assay, tebuconazole and other conazoles enhanced production of progesterone and reduced production of testosterone and estradiol, indicating inhibition of enzymes involved in the conversion of progesterone to testosterone (Kjaerstad et al 2010b). In the MCF- cell proliferation assay, tebuconazole showed anti-estrogenic effect, including aromatase inhibition, since they inhibited the response induced by both 17β-estradiol and testosterone. Moreover, the triazoles were anti- androgenic in an androgen receptor reporter gene assay (Kjaerstad et al 2010b).
Overall these studies show that tebuconazole has anti-estrogenic and anti-androgenic mode of action *in vitro*.

In vivo, human health
Overall, tebuconazole showed effects on reproductive development in the offspring after exposure *in utero*. Tebuconazole virilised the female offspring, and caused feminizing effects in male offspring. Moreover tebuconazole increased gestational length and increased progesterone levels.
In the Hershberger assay, used for investigating anti-androgenic effects *in vivo*, tebuconazole (50, 100, or 150 mg/kg bw/day) had no effect on reproductive organ weights or on hormone levels (Taxvig et al 2008).
Pregnant rats were dosed with tebuconazole (50 or 100 mg/kg bw/day) from GD (gestation day) 7 to GD 21. Some dams were chosen for caesarean section at gestational day 21 (GD21) to evaluate effects on sexual differentiation in the foetuses whereas others gave birth (Taxvig et al 2008). Tebuconazole caused an increased gestational length at 100 mg/kg bw/day. This effect is probably caused by a marked increase in plasma concentrations of progesterone in the mothers as this was seen at GD 21 in other dams exposed to the same dose level (100 mg/kg bw/day) (Taxvig et al 2007). The anogenital distance (AGD) was increased in the female pups in the highest tebuconazole dose group indicating a virilising effect on the females. No effect on AGD was seen in the newborn male pups. The testosterone level in testis from the male foetuses was decreased by tebuconazole, while progesterone and 17α-hydroxyprogesterone levels were increased at both doses. Furthermore, tebuconazole caused a significant increase in the number of nipples in the male pups and a tendency towards decreased plasma testosterone concentration in male pups. Thus, the overall picture is that tebuconazole virilises the females and feminizes the male pups (Taxvig et al 2008).
In another study pregnant dams were exposed from GD 7 to PND 16 to 50 or 100 mg/kg bw/day. The results showed an increase in gestation length and pup mortality and furthermore, tebuconazole virilised female

[4] For substances which are registered 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 these data would influence the proposed categorisation.

pups, (increase AGD) and a demasculinised the male pups (increase in number of retained nipples) and affected steroid hormone levels in dams (Taxvig et al., 2007).
Another study with the same study design has shown an impaired parturition (and other effects with a mixture of procymidone, prochloraz, tebuconazole, epoxiconazole and mancozeb (Jacobsen et al 2010).

Toxicity studies, environment
Studies have shown vitellogenin induction in fish but no effect on stress response in the teleost fish *Rhamdia quelen* after tebuconazole exposure. Summarized effects and evaluation:
Vitellogenin induction in fish (level 3) with LOEC of 230 µg/l. (The 96 h LC_{50} in fish is above 5 mg/l).

See study summaries below:
Cericato et al. 2008. No effect on cortisol stress response to three concentations of tebuconazole (NOEC = 2.65 mg/l) after 96 h exposure in the teleost fish *Rhamdia quelen*. Also, no effect on behaviour was seen.
Sancho et al. 2010. The fish were exposed to a sub-lethal fungicide concentration of 230 µg/L for 7 or 14 days and allowed to recover for 7 or 14 more days, respectively. Whole-body levels of vitellogenins, triglycerides, cholesterol, glucose, lactate and proteins as well as the activities γ-glutamiltranspeptidase (γ-GT), alaninaminotransferase (AlAT), alkalinephosphatase (AP) and lactatedehydrogenase (LDH) were assayed; corpulence factor (k) was also calculated. Fish exhibited significant increase of vitellogenin (Vtg), which continued to increase after 14 days of recovery. Levels of glucose, lactate, cholesterol and triglycerides increased after 7 and 14 days of exposure. Finally, cholesterol and glucose recovered after 14 days of recovery whereas triglycerides and lactate continued to be elevated. Proteins and k remained unaltered the entire experiments. AAT, AlAT and AP enhanced during exposure and did not recover at the end (except AlAT). A longer recovery period should be necessary to re-establish fish physiology. These results alert about the multiple disruptive physiological actions that tebuconazole may have on fish.

Weight of evidence for ED and category
When evaluating the combined results from the *in vitro* and *in vivo* data on human health, tebuconazole fulfils the criteria of being an endocrine disrupter, as *in vivo* studies have shown adverse effects (e.g. virilised female pups, increased gestation length) compatible with the endocrine modes of action. This is supported by the *in vitro* findings of clear endocrine disrupting modes of action. These findings lead to category 1.

Based on *in vivo* ecotoxicity studies, tebuconazole is evaluated as suspected ED category 2a because Vtg induction indicate estrogenic mode of action.

Overall, tebuconazole 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. For tebuconazole adverse endocrine disrupting effects was seen from 50 mg/kg, and tebuconazole can therefore not be classified as an endocrine disrupter of very high regulatory concern according to DE-UK potency criteria.

References, epidemiology
No references

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Triclosan (TCS), CAS 3380-34-5
Synonyms: 5-chloro-2-(2,4-dichlorophenoxy)phenol, Irgasan.

Human data
The body of literature on endocrine related human health effects of triclosan exposure is limited. Seven studies have been identified as potentially relevant for inclusion in an evaluation of the endocrine disrupting potential of triclosan. The intervention study by Allmyr et al. found no association with thyroid hormones (1), but the lack of effects may be due to the small sample size and the short exposure period, and therefore the study cannot be used to exclude an adverse effect on thyroid hormones. Likewise, in the two small studies by Wollf et al., 2007 and Chevrier et al., endocrine disrupting effects cannot be ruled out on the basis of these studies due to the small sample sizes. Two studies found no association between prenatal exposure and size at birth (3;4), except for a statistically non-significant association between triclosan level and reduced birth length among boys. In both studies TCS exposure classification is based on only one spot urine sample, and exposure may therefore be subject to non-differential misclassification, which likely would bias the estimates towards the null. Finally, in the study by Wolf et al. 2010 a weak, non-monotonic inverse association between triclosan and pubic hair development was found (2), which could be suggestive of a modest endocrine disrupting effect of triclosan, but it may also be the result of residual confounding or a chance finding.

Study summaries are provided below:
(1) Allmyr et al., 2009: This is an intervention study, where 12 healthy adults were exposed to triclosan containing toothpaste for a period of 14 days. Participants were instructed to brush their teeth for 3 minutes with a 2 cm strain of toothpaste containing 0.3% (w/w) triclosan twice a day for 14 days. Two blood samples were collected; one the day before the start of exposure and one the day after the end of exposure. Plasma triclosan concentrations were significantly higher after 14 days of exposure to triclosan containing toothpaste (median 54 ng/g) compared with before exposure (median 0.12 ng/g). There were, however, no significant differences in plasma concentrations of the analysed outcome variables 4β-hydroxycholesterol, free tri-iodothyronine (FT3), free thyroxine (FT4) or thyroid stimulating hormone (TSH) before and after exposure. One participant had for unknown reasons unexpectedly high plasma triclosan level after 14 days, but showed no major changes in the outcome variables.
Comments: A severe limitation to this study is the small study population and short exposure time, which limits the conclusions that can be drawn from this study. The exposure time of 14 days may be too short to impact circulating levels of thyroid hormones as humans have reserve storage normally lasting for around a month and the small sample size of only 12 persons may not provide sufficient statistical power.
(2) Wolf et al. 2010: The study population consisted of 1,239 girls enrolled at the age of 6-9 years (median age 7.50 years) and were followed up approximately one year later (median age 8.50 years). At each visit, breast (B1-B5) and pubic hair (PH1-PH5) stages were assessed by inspection and palpation. Urine samples were collected at the first visit. There were 985 girls with biomarkers and visit 2 breast stage and 967 girls with visit 2 pubic hair stages. Concentrations were divided into quintiles and adjustments for creatinine were made. One year after urine samples were collected breast development was present in 30% and pubic hair in 22% of the girls.
Creatinine-corrected triclosan concentration showed a weak inverse association with pubic hair development (PH2+), which was statistically significant. The prevalence ratios (PR) and CIs were similar in the four upper quintiles compared with the first quintile (reference group), e.g. adjusted PR of 0.89 (95% CI 0.83 to 0.94) in

the fourth versus first quintile. No statistically significant association between TCS and breast development was found.
Comment: Exposure was measured one year before outcome assessments and it can be questioned whether this is the relevant time window. Residual confounding cannot be excluded as a possible explanation for the results, and in addition the results could be due to chance findings, as more than 100 comparisons were made between different chemical exposures and the pubertal outcomes.
(3) Wolff et al. 2008: The study group consisted of a birth cohort of 404 mother-infant pairs. Maternal urine samples were obtained primarily during third trimester. Urinary concentrations of triclosan were divided into tertiles using the creatinine corrected values and were natural log transformed. Predictors of birth weight, length, head circumference, and gestational age at delivery were analysed using generalized linear models. Median TCS concentration was 11 µg/L. Maternal urinary triclosan concentrations were not associated with any birth outcomes. Interaction terms between infant sex and TCS showed a possible sex-specific association between TCS and birth length, although statistically insignificant: birth length among boys was reduced with 0.26 cm (95% CI -0.51 to 0.001) per 1 unit ln-TCS (p-value of interaction term < 0.1).
Comment: exposure is measured in a single spot urine sample. Due to the intra-individual variation some exposure misclassification occurs, which likely leads to bias towards the null, i.e. underestimation of the effect.
(4) Philippat et al., 2011: This study asses the relationship between prenatal exposure to phthalates and phenols and fetal growth among male newborns. It is a case-control study on male malformations of the genitalia nested in two French mother-child cohorts. Cases were male newborns with undescended testis or hypospadias. Each of the cases was matched with 3 male newborns without congenital malformation of the genitalia. Phenols, including triclosan were measured in a sub cohort of 191 mother-newborn pairs (48 cases and 143 controls). Maternal urine samples were collected between 24 and 30 gestational weeks. Biomarker concentrations were standardized for sampling conditions. The associations between standardized TCS concentration and offspring size at birth were estimated using weighted linear regression, taking into account the overrepresentation of congenital malformations. TCS concentration was not statistically associated with measures of size at birth (birth weight, birth length, head circumference). Sensitivity analyses excluding the 48 cases of male malformations of the genitalia showed similar results.
Comment: A limitation to the study is that only a single measure of exposure has been made. As for the study by Wolf et al, 2008 this involves some degree of exposure misclassification, which can lead to an underestimation of a potential association.
(5) **Chevrier et al, 2012:** In the same study population as above mentioned in Phillipat et al, 2011 the research group have examined the association between prenatal levels of triclosan and the occurrence of hypospadias and undescended testes. It is a case control study with 21 cases of hypospadias and 50 cases of undescended testes. Cases were matched with three controls per case. No association between triclosan and undescended testes was found. Analyses of association between triclosan and hypospadias could not be made due to too small a sample size. Comment: The finding of no association may very likely be due to the small sample size.
(8) Wolff et al 2007 : This is a pilot study including 90 girls aged 6-8 years enrolled in a study of female pubertal development (see study by Wollf et al, 2010). Triclosan was measured in a single spot urine sample or in a early morning void. Body Mass Index (BMI) were calculated and participants were classified as < 85^{th} national percentile, age- and sex-specific, or ≥ 85^{th} percentile. No statistical significant difference in adjusted geometric means of triclosan (µg/g creatinine) between the two BMI groups were found (15.8 µg/g creatinine among the girls with BMI < 85^{th} percentile vs. 11.7 µg/g creatinine among the girls with BMI ≥ 85^{th} percentile).

Comment: The lack of statistical significant differences in triclosan levels between the two BMI groups may likely be due to the small sample size and it is therefore not possible to draw conclusions on the association between triclosan and BMI based on this study.

(9) **You et al, 2011:** The aim of the study is to evaluate the hypothesis that urinary excretion of BPA and alkylphenols, including triclosan may be reduced among people with insufficient renal function, as measured by estimated glomerular filtration rate (estimated by means of serum creatinine levels). The study population consist of participants from the NHANES study 2003-2006 (n=2,573), which is a cross sectional survey. The study showed that urinary excretion of triclosan decreased with decreasing renal function among participants below age 65 years. No other outcomes were assessed.
As the study do not include outcomes related to endocrine function, this study is unsuitable for inclusion in an evaluation of the endocrine disrupting potential of triclosan.

***In vitro* data**
Triclosan affects many test systems *in vitro*, including some which indicate endocrine disrupting activity. *In vitro* data show some evidence of estrogenic activity, while the antiandrogenic mode of action *in vitro* is better documented. In an *in vitro* study using human liver fractions, triclosan has been shown to act as selective inhibitor of the glucuronidation and sulfonation of phenolic xenobiotics (Wang et al 2004), and in human hepatoma cells exposed to different concentrations of triclosan, the substances proved to be a moderate inducer of hPXR activity (Jacobs et al 2005), studies which indicate that triclosan can affect metabolism processes in the liver, a common mode of action for thyroid hormone disrupting chemicals. In a study by Gee et al (2008) the estrogenic and androgenic activity of triclosan in breast cancer cells was examined, and the authors fond that triclosan was capable of producing both estrogenic and androgenic effects, as it displaced radiolabeled estradiol from estrogen receptors of MCF7 human breast cancer cells, whilst also inhibiting testosterone from binding to the rat androgen receptor. Antagonistic activity in both ER- and AR-responsive bioassays was also seen by Ahn et al (2008), and triclosan has also been shown to bind to the androgen receptor in studies by Chen et al. (2007) and Tamura et al. (2006). Recently Christen et al (2010) also found triclosan to acts as a partial androgen receptor agonist and as an AR antagonist, whereas Svobodova et al (2009) did not find estrogenic effects of triclosan when tested in a β-galactosidase assay and no androgen activity in the bioluminescent androgen assay. Kumar et al (2008) showed that triclosan decreases synthesis of cAMP, and that this decrease resulted in the disruption of entire steroidogenic cascade, causing a depressed synthesis of testosterone. Transcription and translational of four steroidogenic proteins (P450scc, 3β-HSD, 17β-HSD, StAR) also decreased in a dose-dependent manner in triclosan-treated Leydig cells. This study further confirmed the anti-androgenic activity of triclosan in Leydig cells.
Triclosan has also shown weak agonistic activity in the aryl hydrocarbon receptor (AhR)-responsive bioassay (Ahn et al 2008), indicating that it affects receptors, which are implicated in various toxic and biological responses, in the body, and triclosan has also been shown to alter thyroid homeostasis *in vitro* (Veldhoen et al., 2006).
Lastly, triclosan is a powerful inhibitor of estrogen sulfonation in sheep placental tissue, and as such, could have deleterious effects on the ability of female mammals to maintain a full term pregnancy (James et al. 2010). This was seen in a study where triclosan proved to be a powerful inhibitor of estradiol sulfonation in the placental tissue of sheep. As the majority of estrogen secreted by the placenta is sulfoconjugated and estrogen sulfonation has been linked to pregnancy loss, triclosan could potentially have a negative impact on fetal environment and pregnancy maintenance (James et al. 2010).

***In vivo*, human health**

In vivo, the antibacterial compound triclosan has been shown to have adverse effects on reproductive endpoints in two studies where either young adult female rats (Stoker et al 2010) or adult male rats (Kumar et al 2009) have been exposed for varying time periods. In the females triclosan lowered age of sexual maturation, increased uterine weight and potentiated the effects of estradiol treatment (Stoker et al 2010), while findings in males included decreased weights of several reproductive organs, histopathological changes in these, and decreased levels of reproductive hormones (Kumar et al 2009). On the other hand no significant effects on timing of sexual maturation or reproductive organ weight were seen in a study where male rats were exposed in the post-weaning period (Zorilla et al 2009). LOAELs for the studies investigating reproductive endpoints were between 20 -150 mg/kg/day and NOAELS between 10-75 mg/kg/day.

Effects on the thyroid system *in vivo* have also been reported in several studies, as triclosan exposure has lead to a very marked decreases in serum thyroxine levels in both weanling rats exposed for varying periods of time (Crofton et al. 2007; Zorilla et al. 2009; Stoker et al. 2010, Paul et al 2010a), as well as in rat dams exposed during gestation and lactation (Paul et al. 2010b; Rodriguez & Sanchez 2010). For thyroid disruption LOAELs were between 10-300 mg/kg/day and NOAELs values between 1-100 mg/kg/day (30, 3, 19, 30, 100 and 1 in the six studies respectively). The observed differences probably mostly reflect which strains of rats have been used, as studies where higher doses of triclosan were needed to see an effect on T4 were all performed in Long Evans rats, while the other studies used Wistar rats.

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

Crofton et al 2007: Young Long-Evans female rats (n=8-16) were dosed by daily gavage with 0, 10, 30, 100, 300, 1000 mg/kg/day, from PND 28-31. The only measured endocrine sensitive endpoint was serum levels of T4, and the three highest doses caused reductions in this hormone of 28, 34 and 53% respectively. The benchmark dose BMD20 (20% reduction in T4) was 69.7 mg/kg/day. At the highest does increases in liver weights were seen. Evidence that PXR is activated by triclosan suggests that decreases in T4 may result from increases in the sulfonation or glucuronidation activity via PXR-linked genes.

Kumar et al 2009: Adult male Wistar rats (n=8) were dosed with triclosan for 60 days. The dose levels were 5, 10 and 20 mg/kg/day, and the dose of 20 caused decreased weights of several reproductive organs and histopathological changes in these, together with decreased levels of FSH, LH and testosterone. Furthermore decreased sperm production and altered gene expression of androgen regulated genes was seen. The decreases in testosterone levels and spermatogenesis were likely the result of decreases in serum levels of LH and FSH, thereby implicating the pituitary–gonadal axis, as a target for endocrine disruption by triclosan.

Zorilla et al 2009: Young Wistar males (n=8-25) were dosed daily with 3, 30, 100, 200, 300 mg/kg/day by gavage, from PND 23-58 (31 days). This caused T4 levels to decrease by approximately 50% at 30 and 100 mg/kg/day and by approximately 80% at 200 and 300 mg/kg. In this study the BMD20 was 14.51 mg/kg/day. Reduced T3 and testosterone levels were seen at 200 but not at 300 mg/kg, and TSH levels were unaffected. An increase in liver weights was seen in the highest dose group, probably caused by induction of liver enzymes, as PROD activity in the liver was increased at 300 mg/kg. Thyroid histology was affected at 300, while no effect on body weight, sexual maturation or weight of reproductive organs was seen. Based on this study, testicular function did not appear to be affected by triclosan exposure during the postweaning period.

Paul et al 2010a: Data from Crofton et al 2007 was used, and an extra block of animals was added. 28 day old Long-Evans females were dosed by gavage with 0, 10, 30, 100, 300, 1000 mg/kg/day from PND 28-31 (n=8-24). The three highest dose levels caused T4 levels in serum to decrease with 26, 35 and 57%

respectively. BMD20 was 99.4 mg/kg/day. Reduced T3 levels were seen at 300 and 1000mg/kg. TSH increases were not significant. Increased liver weights were observed in the highest dose group. Triclosan upregulated both mRNA expression and activity of some phase I and phase II hepatic enzymes, while no effects were seen on the measured hepatic cellular transporters. The upregulated phase II enzymes and the observed increased catabolism may be partially responsible for the observed decrease in T4 levels, but other mechanisms may also contribute.

Paul et al 2010b: A developmental reproductive study in Long Evans rats (n=10) where dosed with 30, 100 or 300 mg/kg/day by oral gavage from GD 6 to PND 21. A dose of 300 mg/kg/day caused decreased T4 levels in dams on PND 22 and in offspring on PND 4, but not on PND 14. Serum thyroxine (T4) was reduced 31% in dams on PND22. In pups, a unique pattern of hypothyroxinemia was observed; serum T4 decreased 27% in PND4 pups with no significant reduction observed on PND14 or PND21. Comparable reductions of approximately 30% in serum T4 at 300 mg/kg/d for dams and PND4 neonates and a lack of effect at PND14 and PND21 suggest that toxicokinetic or toxicodynamic factors may have contributed to a reduced exposure or a reduced toxicological response during the lactation period.

Stoker et al 2010: Two studies were performed. A uterotrophic assay where immature Wistar rat were dosed for 3 day, and a pubertal assay were young females were dosed from PND 22-43 (21 days). The dose levels were 1.2, 2.4, 4.7, 9.4, 18.8, 37.5. 75, 150 mg/kg. In the pubertal assay the highest dose advanced vaginal opening and increased uterine weight, whereas triclosan in itself did not affect uterine weight in the 3-day uterotrophic assay. However, dose of 4.7 and above potentiated the effects of estradiol. Furthermore, doses of 37.5 mg/kg and above resulted in a decrease of total serum T4 levels.

Rodrizuez et al 2010: Adult Wistar dams (n=8-9) were exposed daily to triclosan (0, 1, 10, or 50 mg/kg/d) administered via drinking water from 8 days before mating until weaning of offspring, and dosing was continued in offspring after weaning. Dam blood samples were taken, and decreased serum T4 and T3 levels were seen in the 10 and 50 mg/kg groups throughout gestation and lactation. No differences in mean numbers of implantation sites were observed in treated rats, but the highest dose of triclosan significantly reduced the live birth index and 6-day survival index. Triclosan treatment lowered pup body weights on postnatal day (PND) 20, which probably caused the delayed sexual maturation seen in female offspring.

***In vivo*, ecotoxicity**

Triclosan caused vitellogenin induction in fish. Decreased sperm-counts at 101 µg/l in fish (NOEC 57.9 µg/l). Thyroid gland hypertrophy (LOEC 0.3 µg/l) and decreased follicle cell height (LOEC 1.3 µg/l, NOEC 0.3 µg/l) was observed in frog. Increased egg production (shelled and unshelled) in freshwater snail. The LOEC was 0.2 µg/L, the NOEC was 0.05 µg/L.

Detailed study summaries are provided below:

Fort et al 2011b: A standard metamorphosis anuran model to assess potential effect of the antibacterial agent triclosan (TCS) on normal prometamorphic *Xenopus laevis* was used. Results indicated that environmentally relevant TCS concentrations did not alter the normal course of thyroid mediated metamorphosis in this standard anuran model. However, to examine potential effects of TCS exposure during premetamorphosis and to distinguish between effects on metamorphosis and effects on growth, a longer term TCS exposure study was conducted. Standard Nieuwkoop and Faber (NF) stage 47 *X. laevis* larvae were exposed for 32 days (ca. NF stage 59–60) via flow-through to four different concentrations of TCS: < 0.2 (control), 0.8, 3.1, 12.5, or 50.0 µg TCS/l. Primary endpoints were survival, hind limb length, body length (whole; snout-to-vent), developmental stage, wet whole body weight, thyroid histology, plasma thyroid hormone (TH) concentrations, TH receptor beta (TRb), and type II and III deiodinase (DI-2 and DI-

3) expression. Endpoints measured to evaluate effects on thyroid-mediated metamorphosis including developmental stage, thyroid histology, TRb expression, DI-2 and DI-3 expression, and thyroid gland 3,5,3',5'-tetraiodothyronine (T4) and plasma T4 and 3,5,3'- triiodothyronine (T3) levels were not affected by TCS exposure. However, increased larval growth based on whole body length (0.78, 12.5, and 50 mg TCS/l), snout-vent length (3.1 and 12.5 mg TCS/l), and whole body weight (0.8, 12.5, and 50.0 mg TCS/l) was observed following 32-day TCS exposure. These results indicated that TCS exposure during pre- and prometamorphosis increased larval growth but did not alter the normal course of metamorphosis in *X. laevis*. The increased growth associated with TCS exposure was not unexpected and is generally consistent with the presence of reduced bacterial stressors in culture.

Helbing et al 2011a, comments to Fort et al 2011: The statements regarding no effect of TCS on the thyroid axis misrepresent the data presented in the article. In fact, three thyroid endpoints measured by (Fort et al. 2011b) showed evidence of thyroid axis disruption. These include thyroid histology (thyroid gland hypertrophy and follicle cell height) and plasma T4 levels. The authors found a ''TCS-concentration–related increase'' in the occurrence of ''minimal'' thyroid gland hypertrophy and vascular congestion at day 32. As the TCS concentrations increased, so did the incidence of pathology such that at the highest TCS concentration, 8 of 10 animals showed thyroid gland hypertrophy and 6 of 10 showed increased vascular congestion. Increased vascularity is consistent with larger gland size to accommodate gas, nutrient, and molecule exchange. Follicle count and area, mean colloid area/animal, and mean colloid area/follicle were not significantly different from controls. However, thyroid gland area was significantly increased at three (0.3, 5.9, 29.6 µg/l) of the four TCS concentrations compared to controls. The 1.3 µg/l concentration had a p value of 0.053. Follicle cell height was significantly decreased compared with the control at the three higher concentrations tested (1.3, 5.9, and 29.6 µg/l). The OECD test guideline TG 231 includes thyroid gland hypertrophy and follicular cell height as diagnostic of thyroid axis disruption (OECD TG 231, 2009). Therefore, the observations presented in the article show that thyroid histology is significantly affected by TCS exposure.

Fort et al. and Helbing et al. continue to discuss the thyroidal effects of triclosan in the following articles, comments and letters to editor: (Fort et al. 2010;Fort et al. 2011c;Fort et al. 2011a;Fort et al. 2011d;Helbing et al. 2011c;Helbing et al. 2011b).

Ishibashi et al 2004: The effects of TCS on the early life stages and reproduction of medaka (*Oryzias latipes*) were investigated. The 96-h median lethal concentration value of TCS for 24-h-old larvae was 602 µg/l. The hatchability and time to hatching in fertilized eggs exposed to 313 µg/l TCS for 14 days were significantly decreased and delayed, respectively. An assessment of the effects of a TCS 21-day exposure period on the reproduction of paired medaka showed no significant differences in the number of eggs produced and fertility among the control and 20, 100 and 200 µg/l TCS treatment groups. Hepatic vitellogenin was increased significantly in males treated with TCS at 20 and 100 µg/l. In the F1 generations, although the hatching of embryos in the 20 µg/l treatment showed adverse effects, there was no dose–response relationship between hatchability and TCS treatment levels. These results suggest that TCS has high toxicity on the early life stages of medaka, and that the metabolite of TCS may be a weak estrogenic compound with the potential to induce vitellogenin in male medaka but with no adverse effect on reproductive success and offspring.

Foran et al 2000: Japanese medaka fry (*Oryzias latipes*) were exposed for 14 days beginning 2 days post-hatch to triclosan (100, 10, 1 µg/l), 17β-estradiol (E2; 1 µg/l), or a solvent control (ethanol). Two months post-exposure, the phenotypic sex of each adult was assessed visually using sexually dimorphic fin shape and size. The proportion of females in each group was similar for triclosan-exposed animals and solvent-treated controls (ethanol 53%, 1 ppb 58%, 10 ppb 45%, 100 ppb 36%) although E2 treatment did produce 92% female adults. Sexually dimorphic fin traits were quantified to look for potential effects of triclosan and

E2 on the development of secondary sexual characters. A slight increase in the length of the dorsal fin and anal fin could indicate weak anti-estrogenic or androgenic effect.
Matsumura et al 2005: The study investigated the effects of nonylphenol (NP) and triclosan (TCS) on production of vitellogenin (Vg), testosterone (T), and hepatic cytochrome P450 1A and 2B activities in male South African clawed frogs (*Xenopus laevis*). In a 14-d waterborne exposure test, no significant differences in the level of plasma Vg synthesis in male frogs were observed among the control, 10, 50, and 100 µg/l NP and 20, 100, and 200 µg/l TCS treatment groups. Intraperitoneal injection of male frogs with 2, 20, and 200 µg/g body weight NP resulted in no significant differences in plasma Vg levels among the control and all treatment groups. However, the levels of plasma Vg in all TCS treatment groups (intraperitoneal injection of 4, 40, and 400 µg/g body weight) were lower than that in the solvent control group, and male frogs injected with high doses of NP or TCS had lower T levels than the control group. No significant differences in hepatic cytochrome P450 1A and 2B activities were observed among the all treatment groups. Male frogs injected with 20 µg/g body weight of estradiol-17β had significantly higher plasma Vg levels than the control group. These results suggest that profiles of plasma Vg and T production in male *Xenopus laevis* could be useful biomarkers for detecting hormonally active agents.
Raut et al 2010: Previous studies have demonstrated that TCS has the potential to act as an endocrine disruptor. The present study tested the hypothesis that TCS acts as an endocrine-disrupting agent in fish. Mature male western mosquitofish, Gambusia affinis, were exposed to TCS concentrations of 100, 200, and 350 nM (29.0, 57.9, and 101.3 µg/L) for 35 d by the static renewal method. Induction of vitellogenin gene expression and reduction in sperm count were quantified as biomarkers of endocrine disruption. Vitellogenin mRNA expression was significantly elevated in the 350 nM TCS treatment. Sperm counts in the same treatment group were significantly decreased. The mean hepatosomatic index in the 350 nM treatment group was significantly increased. This study demonstrates that TCS has the potential to act as an endocrine disruptor in male mosquitofish.
Orvos et al 2002: The 48-h *Daphnia magna* median effective concentration (EC50) was 390 µg/L and the 96-h median lethal concentration values for *Pimephales promelas* and *Lepomis macrochirus* were 260 and 370 µg/L, respectively. A NOEC and LOEC of 34.1 µg/L and 71.3 µg/L, respectively, were determined with an early life-stage toxicity test with *Oncorhynchus mykiss*. During a 96-h Scenedesmus study, the 96-h biomass EC50 was 1.4 µg/L and the 96-h NOEC was 0.69 µg/L. Other algae and Lemna were also investigated. Bioconcentration was assessed with *Danio rerio*. The average TCS accumulation factor over the five-week test period was 4,157 at 3 µg/L and 2,532 at 30 µg/L. Algae were determined to be the most susceptible organisms. Toxicity of a TCS-containing wastewater secondary effluent to *P. promelas* and Ceriodaphnia was evaluated and no observed differences in toxicity between control and TCS-treated laboratory units were detected. The neutral form of TCS was determined to be associated with toxic effects. Ionization and sorption will mitigate those effects in the aquatic compartment.

Weight of evidence for ED and group
The available epidemiological studies are too limited for an evaluation of endocrine disrupting effects of triclosan.

In vitro data show some evidence of estrogenic activity, while the antiandrogenic mode of action *in vitro* is better documented. *In vivo* there is also some evidence that triclosan may act as an endocrine disrupter as it lowered age of sexual maturation, increased uterine weight and potentiated the effects of estradiol treatment in female rats in one study, and causes decreased weights of several reproductive organs, histopathological changes in these and decreased levels of reproductive hormones in a study in male rats. Also a number of

studies exist which show that triclosan lowered thyroid hormone levels in rats after different exposure scenarios. Induction of liver enzymes may be the mode of action behind the reduced thyroid hormone levels, however the exact endocrine mode of action of triclosan are not yet fully understood. When evaluating the combined results from the *in vitro* and *in vivo* data on human health, triclosan fulfils the criteria of being an endocrine disrupter, as *in vivo* studies have shown adverse effects compatible with the thyroid disrupting and estrogenic modes of action seen *in vitro*. These findings lead to ED category 1 according to the Danish criteria.

In the ecotoxicologal studies, triclosan caused vitellogenin induction in fish. Decreased sperm-counts in fish has been found. Thyroid gland hypertrophy and decreased follicle cell height was observed in frog. Increased egg production (shelled and unshelled) was seen in freshwater snail. Based on these findings triclosan is evaluated as suspected ED category 2a because Vtg induction show estrogenic mode of action *in vivo*. It is, at the moment, not known whether the histological changes in frog and changed reproduction in snails are adverse effects.

Based on the combined evidence from the ecotoxicological studies and the *in vitro*, *in vivo* and epidemiological studies, triclosan 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 triclosan the dose levels causing adverse effects are higher (LOAELs between 20 -150 mg/kg/day and NOAELS between 10-75 mg/kg/day), and triclosan can therefore not be classified as an endocrine disrupter of very high regulatory concern according to DE-UK criteria.

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Methylparaben, CAS 99-76-3
S**ynonyms**: Methyl 4-hydroxybenzoate

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 at 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. 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, the widespread use of parabens especially as antimicrobial preservatives in cosmetic products causes exposure of several parabens to most of the population. 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 methylparaben show evidence that this compound has weak estrogenic and weak antiandrogenic effects *in vitro* (Table 1)

The literature review for methylparaben 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. Modified from 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) -ve (yeast + receptor binding) (Terasaki 2009) +ve (human MCF7) (Byford 2002, Pugazhendhi 2005, Schultis and Metzger 2004, Vanapyris 2006) +ve (rat uterus receptor binding) (Blair 2000) -ve (rat uterus receptor binding) (Lemini 2003) -ve (human HeLa overexpressing ER) (Gomez 2005)	-ve (recombinant hAR) (Sato 2005) +ve (transfected CHO-K1 cells) (Sato 2005) +ve (transfected HEK 293 cells) (Chen 2007)

+ve = effect; -ve = no effect

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, although some of these assays were negative for methylparaben indicating that this compound has weaker estrogenic actions than parabens with longer chain lengths. Cell based studies show induction of estrogen responsive genes for methylparaben as well as for parabens of longer chain lengths (Vo et al., 2011).

Toxicity studies, human health

The available data for methylparaben show some evidence that this compound has weak estrogenic effects *in vivo* (see Table 2). The literature review for methylparaben 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	No effect up to 80, sc No effect up to 800, oral		
Hossaini 2000		No effect at 100 No effect at 1, 10 and 100 (oral)	
Lemini 2003	LOEL 55 NOEL 16.5	LOEL 16.5 NOEL 5.5	LOEL 165 NOEL 55
Lemini 2004			LOEL 55

Studies in male rats do not show clear adverse effects, and no studies on perinatal exposure to methylparaben including endocrine sensitive endpoints have been published. Only a few studies in the same laboratory have found positive responses in Uterotrophic assays (Lemini et al., 2003, Lemini et al., 2004), whereas other uterotrophic studies on methylparaben were negative (Hossaini et al., 2000, Routledge et al., 1998, Vo et al.,

2010). A study in young male rats found no adverse effects at 100 and 1000 mg/kg bw/day (Oishi 2004). As for other paraben studies by Oishi this study has some shortcomings including very low values for sperm counts. Another study by Hoberman et al., 2008, aimed to repeat this study and similarly concluded no reproductive effects of methylparaben. However, an increase in abnormal sperm and decrease in normal sperm number were observed, although no change in total sperm count was seen. Some evidence of thyroid toxicity has been shown as T4 levels were decreased and relative thyroid weight decreased in a study in peripubertal rats (Vo et al., 2010).

Detailed study summaries of the most important studies are provided below:
Hoberman et al., 2008. performed a repeat study of the study by Oishi from 2001 and 2004 on butyl- and methyl paraben by exposing 3-week old male Wistar rats to dietary doses of 10, 100 and 1000 mg/kg bw/day for 8 weeks. This study was performed under GLP conditions and included a higher number of animals than the Oishi studies. The authors reported "no adverse effects" at all dose levels concluding a NOAEL of 1000 mg/kg bw/day. No hormone measurements were reported although the study protocol mentions that this is measured. A statistically significant increase in the number of abnormal sperm was reported in the two highest dose groups and the testicular spermatid concentration appeared dose-dependently decreased (to 77% of control level), although this was not statistically significant. Further investigation of the study report revealed that also the number of normal sperm is reduced, but raw data were not available for analysis. The SCCP have evaluated the study report on this methylparaben study and conclude that due to several shortcomings the study "cannot be considered as scientifically valid" and may "undermine the decision taken earlier for methyl paraben" of an ADI of 10 mg/kg bw/day based on a NOAEL of 1000 mg/kg bw/day (SCCP 2006). This study may indicate that adverse reproductive effects (increase in abnormal sperm number and reduction of normal sperm number) can be seen for methylparaben.
Oishi 2004. Young rats (25-27 days old) were exposed to 0.1% and 1.0% of methylparaben in the diet for 8 weeks. No effects on male reproductive organ weights or sperm counts in epididymis or testes were observed, although a tendency to reduced sperm counts were noted. No changes in serum concentrations of testosterone, LH or FSH were observed. As for other paraben studies by Oishi this study has some shortcomings and extremely low epididymal sperm counts are reported for control rats.
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 methylparaben 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 methylparaben, and no changes in uterine epithelium thickness was observed as was seen for isobutylparaben, butylparaben and isopropylparaben. Histological changes in the ovary were seen at the middle dose only (decrease in corpora lutea numbers and increase in cycstic follicle numbers). Serum T4 levels were significantly reduced at the highest dose only, and similar reductions were seen for some other parabens indicating thyroid toxicity. Relative thyroid, adrenal and liver weights were increased at the highest dose level compared to controls. This study indicates methylparaben does not have similar estrogenic effects *in vivo* as parabens with longer chain lengths.

Toxicity studies, environment
Two relevant ecotoxicology studies have been performed. Methylparaben caused vitellogenin induction in fish as well as testicular tissue changes in fish. Furthermore, reproductive toxicity effects in invertebrates (apical endpoint but not endocrine specific) have been seen. This indicates endocrine disrupting activity of methylparaben.

Study summaries are provided below:

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. 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 whereas reproduction was not affected.
Ten day, static-renewal exposures were conducted with *D. magna* following U.S. EPA methods. Ten replicates with one daphnia in 80 ml of each concentration of test solution (plus a RHW and 0.1% acetone solvent control) were prepared and renewed every other day. Total neonates produced per daphnid were calculated and compared to controls.
For methylparaben, the LOEC for *D. magna* reproduction was 1.5 mg/l. Effect on growth was seen at 6.0 mg/l. Reproduction is an apical endpoint but not endocrine specific.
Barse et al 2010: Effects were evaluated in adult male common carp (*Cyprinus carpio*) by exposing them to fractions ($1/143^{rd}$ to $1/29^{th}$) of the LC_{50} dose with every change of water for 28 days. Vitellogenin induction, metabolic enzymes, somatic indices and bioaccumulation were studied at weekly intervals. The 96^{th} h LC_{50} of MP in fingerlings was 120 mg/L. Compared to the control, except for increases ($p<0.01$) in alkaline phosphatase, alanine aminotransferase and liver size, there were decreases in activity of acid phosphatase, aspartate aminotransferase, and testiculosomatic index following exposure to any dose of MP. Vitellogenin induction was significantly higher ($p<0.01$) in exposed than control fish (LOEC 0.82 mg/l). Fish exposed to 4.2 mg/l became lethargic after the 26^{th} d. Histology revealed degeneration, vacuolization and focal necrotic changes in the liver. Fibrosis-like changes in testicular tissue were noted from 0.82 mg/l with infiltration of inflammatory cells, narrower interstitium and less spermatozoa. The observed vitellogenin induction demonstrates endocrine disrupting activity of methylparaben.

Weight of evidence for ED and category
Based on *in vivo* and *in vitro* data alone, methylparaben can be placed in category 2b as an indicated endocrine disrupter as there is evidence of an estrogenic mode of action *in vitro* and *in vivo* in the uterotrophic assay, but data are conflicting.

Methylparaben is evaluated as 2a (suspected ED) based on ecotoxicity data showing estrogenic mode of action in fish. A link between adverse effects and endocrine mode of action is missing in invertebrates and it is not clear whether the testicular changes observed in fish would cause adverse reproductive effects.

Based on the combined evidence from the ecotoxicological studies and the *in vitro*, *in vivo* and epidemiological studies, methylparaben is evaluated as an 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 methylparaben there are no clear adverse *in vivo* effects and methylparaben cannot be categorized as an endocrine disrupter of very high regulatory concern.

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Ethyl paraben CAS: 120-47-8
S**ynonyms**: Ethyl 4-hydroxybenzoate

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).
In conclusion, the widespread use of parabens especially as antimicrobial preservatives in cosmetic products causes exposure of several parabens to most of the population. A few human studies have indicated weak associations between increased exposure to other parabens (see methyl-, propyl- and butylpraben) and markers for human reproductive health. However our knowledge in this area is very limited.

***In vitro* data**
The available data for ethylparaben show evidence that this compound has weak estrogenic and weak anti-androgenic effects *in vitro* (Table 1). The literature review for ethylparaben 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 estrogenic and androgenic activity *in vitro*.

Table 1 *In vitro* studies published on the estrogenic and antiandrogenic 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) -ve (yeast + receptor binding) (Terasaki 2009) +ve (human MCF7) (Okubo 2001, Byford 2002, Schultis and Metzger 2004, Vanapyris 2006) +ve (rat uterus receptor binding) (Blair 2000, Lemini 2003) +ve (human HeLa overexpressing ER) (Gomez 2005)	-ve (recombinant hAR) (Sato 2005) +ve (transfected CHO-K1 cells) (Sato 2005)

+ve = effect; -ve = no effect

The available data for ethylparaben show evidence that this compound has estrogenic effects *in vitro*, and there are also reports of weak anti-androgenic effects *in vitro* (see Table 1). 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, although one yeast screen assay was negative for ethylparaben

(Table 1). Cell based studies show induction of estrogen responsive genes for ethylparaben as well as for parabens of longer chain lengths (Vo et al., 2011).

Toxicity studies, human health

The available data for ethylparaben show evidence that this compound has estrogenic effects *in vivo* (see Table 2). The literature review for ethylparaben 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)
Hossaini 2000		No effect at 100 No effect at 1000 (oral)	
Lemini 2003	LOEL 180 NOEL 60	LOEL 60 NOEL 18	LOEL 18 NOEL 6
Lemini 2004			LOEL 60

The evidence of estrogenic effects is based on a few studies that have found positive responses in uterotrophic assays (Lemini et al., 2003, Lemini et al., 2004), whereas other uterotrophic studies on ethylparaben were negative (Hossaini et al., 2000, Vo et al., 2010). A study on fetal exposure to ethylparaben revealed no changes in male anogenital distance and no changes of fetal testosterone production, but changes in gene expression levels pointed to subtle endocrine disrupting effects (Taxvig et al., 2008). A study in young male rats found no adverse effects at 100 and 1000 mg/kg bw/day, although tendencies to lower sperm counts are seen (Oishi 2004). As for other paraben studies by Oishi this study has some shortcomings and extremely low sperm counts are reported also for control rats.

Detailed study summaries of the most relevant studies are provided below:

Oishi 2004: Young rats (25-27 days old) were exposed to 0.1% and 1.0% of ethylparaben in the diet for 8 weeks. No effects on male reproductive organ weights or sperm counts in epididymis or testes were observed; although a tendency to reduced sperm counts were noted. No changes in serum concentrations of testosterone, LH or FSH were observed. As for other paraben studies by Oishi this study has some shortcomings and extremely low epididymal sperm counts are reported for control rats.

Taxvig et al., 2008: Pregnant Wistar rats were orally exposed to 400 mg/kg bw/day of ethylparaben from gestation day 7 to 21. No changes in anogenital distance or serum levels of testosterone or progesterone were observed in foetuses, and no changes in plasma T3, T4, 17alpha-hyroxyprogesterone or progesterone levels were detected. Fetal adrenal expression of mRNA for proteins involved in steroid synthesis was down regulated, and ovarian ERbeta mRNA expression was down regulated. 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. Ethylparaben did not alter growth in the T-screen assay indicating no interference with thyroid receptors.

Overall, no anti-androgenic effects were observed but this study shows some indications of endocrine disrupting effects at gene expression level.
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 ethylparaben 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 ethylparaben, and no changes in uterine epithelium thickness was observed as was seen for isobutylparaben, butylparaben and isopropylparaben. Serum estradiol was significantly reduced at the highest dose only, and similar reductions were seen for isobutylparaben, but were not statistically significant for the other parabens. This study indicates ethylparaben does not have similar estrogenic effects as parabens with longer chain lengths.

Toxicity studies, environment
Two relevant ecotoxicological studies have been performed, which have shown ethylparaben to cause vitellogenin induction in fish, and reproductive toxicity effect in invertebrates.

Study summaries are provided below:
Pedersen et al 2000: Yolk protein induction in sexually immature rainbow trout was used as an oestrogen-specific endpoint after two injections (day 0 and 6) of the compounds. All tested parabens were oestrogenic in doses between 100 and 300 mg/kg measured by ELISA at day 12. Ethylparaben caused a 60 fold elevation of vitellogenin at a dose of 300 mg/kg (approximately sixty times weaker than propyl- and butylparaben). NOEC was 100 mg/kg. The study confirms *in vivo* oestrogenicity of ethylparaben in fish. Vitellogenin induction is not an adverse apical effect but a biomarker for estrogenic exposure.
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. 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 whereas reproduction was not affected. Ten day, static-renewal exposures were conducted with *D. magna* following U.S. EPA methods. Ten replicates with one daphnia in 80 ml of each concentration of test solution (plus a RHW and 0.1% acetone solvent control) were prepared and renewed every other day. Total neonates produced per daphnid were calculated and compared to controls. For ethylparaben, the LOEC for *D. magna* reproduction was 2.3 mg/l. Effect on growth was seen at 9.0 mg/l. Reproduction is an apical endpoint but not endocrine specific.

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 are very limited and further studies are needed to clarify possible effects of parabens on human reproductive health.

Based on *in vivo* data, ethylparaben can be placed in category 2b as an indicated endocrine disrupter as there is some evidence of an estrogenic mode of action *in vitro* and conflicting data on uterotrophic effects.

In ecotoxicological studies, ethylparaben caused vitellogenin induction in fish showing estrogenic mode of action. Reproductive toxicity effect was seen in invertebrates, but these effects were on apical endpoint, not

endocrine specific. Thus, a link between adverse effects and endocrine mode of action is missing and ethylparaben is evaluated as 2a (suspected ED) based on the ecotoxicity data.

When the evidence from *in vivo*, *in vitro*, ecotoxicological and human studies are evaluated together, ethylparaben is grouped 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 ethylparaben there are no clear adverse *in vivo* effects and ethylparaben cannot be categorized as an endocrine disrupter of very high regulatory concern.

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