



List of Endocrine Disrupting Chemicals

Hass, Ulla; Christiansen, Sofie; Andersen, Mille Dahl; Abildgaard Rosenberg, Sine; Egebjerg, Karen Mandrup; Brandt, Sidsel; Nikolov, Nikolai Georgiev; Holbech, Henrik; Ebsen Morthorst, Jane

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Appendix 1

Deltamethrin, CAS no. 52918-63-5

Synonyms: the IUPAC name is alpha-cyano-3-phenoxybenzyl (1R,3R)-cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate. Trade names include Decis, Decasyn, Butox, K-Othrine, Kordon, Sadethrin, and many more.

Deltamethrin (Figure 1) is a pyrethroid ester insecticide used in pest control. It is highly toxic to aquatic life, and in humans deltamethrin has been found to be neurotoxic and an allergen that may cause asthma in some people.

Since no registration dossiers on deltamethrin are available, it is assumed that it is produced and/or imported to EU in tonnages less than 100 tpa.

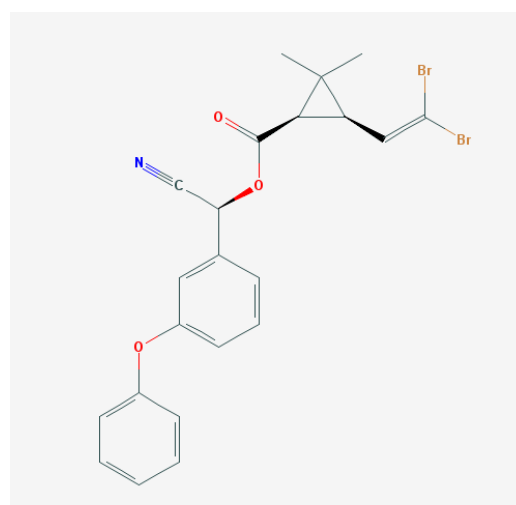


Figure 1. 2D structure from PubChem

4. Human health hazard assessment

4.10.3 Endocrine disruption

4.10.3.1 General approach – human health

4.10.3.2 *In vitro* information indicative of endocrine activity

Christen et al. (2014)

Summary: Part of this study aimed at assessing androgen receptor (AR) antagonistic activity of 6 pyrethroids, including deltamethrin (CAS no. and purity not reported) and compare the results from data from Du et al. (2010). MDA-kb2 cells were used for the AR reporter gene assay in an antagonistic mode with co-exposure to dihydrotestosterone (DHT) (1 nM). Deltamethrin was only tested in a single dose of 10 μ M (number of replicated experiments not reported), which was below the cytotoxicity concentration. Deltamethrin at 10 μ M resulted in a lower anti-androgenic activity than was previously reported by Du et al. (2010).

Study quality and assessment: The study does not report information such as CAS no., purity, number of replicated experiments, and only one dose has been tested. Based on this the study is assessed to be of low quality. The study provides moderate evidence for a weak anti-androgenic MoA of deltamethrin.

Abdallah et al. (2010a)

Summary: The purpose of the present study was to investigate the *in vitro* effects of deltamethrin (CAS no. and purity not reported) on rat spermatozoa. Spermatozoa from adult male rats were obtained and dispersed to a final concentration of $10 \cdot 10^6$ spermatozoa/ml and incubated for 3 h with 0, 10, 50, 100 or 200 μM deltamethrin (n=8 rats/group, read from the tables and figures). Sperm viability was assessed as was sperm motility and morphology. The activities of superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation (LP) were estimated.

Spermatozoa viability decreased significantly at all concentrations and motility decreased at concentrations of 50 μM and above. An increase the number of morphologically abnormal spermatozoa was seen at 100 and 200 μM deltamethrin. A concentration-dependent increase in SOD, CAT and LP activities was seen starting from the lowest concentration of 10 μM deltamethrin indicating oxidative stress.

Study quality and assessment: The study is well-described although information on the CAS no. and purity is not reported. Overall the study is assessed to be of high quality. The study provides weak evidence for an ED MoA underlying the effects on sperm parameters *in vitro* as these effects are more likely due to an oxidative stress MoA.

Du et al. (2010)

Summary: The purpose of this study was to evaluate the activities of 9 pyrethroids, including deltamethrin (CAS no. 52918-63-5, 99% purity), on estrogen receptor (ER), androgen receptor (AR) and the thyroid hormone receptor (TR). Deltamethrin was tested in concentrations of 10^{-9} - 10^{-4} M for ER α activity with or without 10^{-9} M 17 β -estradiol (E2) as well as for TR activity with or without $5 \cdot 10^{-9}$ M T3 using transfected CV-1 cells. Deltamethrin was tested at the same concentrations for AR activity with or without 10^{-9} M DHT in transfected MDA-kb2 cells. Cytotoxicity of deltamethrin with or without E2 or triiodothyronine (T3) in CV-1 cells, and of deltamethrin with or without dihydrotestosterone (DHT) in MDA-kb2 cells was evaluated. All experiments were done in triplicate. No effects of the tested concentrations of deltamethrin were seen on cell viability or proliferation in both CV-1 cells and MDA-kb2 cells. Deltamethrin exhibited weak ER activity and TR antagonistic activity in the CV-1 cells, and AR antagonistic activity in the MDA-kb2 cells.

Study quality and assessment: The study is well-described and assessed to be of high quality. There is strong evidence for both an oestrogenic, anti-androgenic and anti-thyroid MoA of deltamethrin *in vitro*.

Birkhøj et al. (2004)

Summary: The study examined the *in vitro* anti-androgenic effect of 5 pesticides, including deltamethrin (no CAS no. reported, 99.0% purity), individually and in a mixture. Chinese Hamster Ovary (CHO) cells were transfected with androgen receptor (AR) and a reporter plasmid, and deltamethrin were added in 12 concentrations ranging from 0.025-50 μM with or without 0.1 nM of the AR agonist R1881 (3 biological replicates per concentration, read from figure). Luciferase activity and cytotoxicity was also assessed. Deltamethrin resulted in cytotoxicity at concentrations of 12.5 μM and above. A significant inhibition of the R1881-induced AR activation by deltamethrin was seen at sub-cytotoxic concentrations starting from 1.6 μM .

Study quality and assessment: The study is well-described although information on CAS no. and biological replicates are not stated in the material and methods. Overall, the study is assessed to be of high quality. The study provides moderate evidence for an anti-androgenic MoA in a single *in vitro* assay.

Andersen et al. (2002)

Summary: In this study 24 pesticides, including deltamethrin (CAS no. 52918-63-5, purity not reported), were tested for their (anti)oestrogenic and (anti)androgenic as well as aromatase inhibitory potential *in vitro*. First, deltamethrin was tested in concentrations from 10^{-8} - $5 \cdot 10^{-5}$ M in MCF-7 cells for cell proliferation and estrogen receptor (ER) transactivation activity in both the agonistic and antagonistic (using co-treatment with ER agonist 17β -estradiol). Cytotoxicity in the MCF-7 cells was assessed. Then deltamethrin was tested in Chinese hamster ovary (CHO) cells transfected with the human androgen receptor (AR) at 10^{-8} - $5 \cdot 10^{-5}$ M in agonistic and antagonistic mode (using co-treatment with AR agonist R1881). Cytotoxicity in the CHO cells was assessed. Finally, deltamethrin was tested in a single concentration of 50 μ M for effects on aromatase activity (CYP19) in human placental microsomes. At least two independent experiments were run with each concentration in triplicate or quadruplicate.

Deltamethrin resulted in weak effects on MCF-7 cell proliferation at concentrations below cytotoxicity (>25 μ M) but did not cause oestrogenic or anti-oestrogenic effects in the ER transactivation assay in MCF-7 cells. Also, no androgenic or anti-androgenic effects of deltamethrin were seen in the CHO cells, and no inhibition of CYP9 was observed in the human placental microsomes.

Study quality and assessment: The study is well-described and only information on the purity of deltamethrin is missing, and it is therefore assessed to be of high quality. The evidence for an oestrogenic MoA of deltamethrin is moderate due to the effects on cell proliferation but missing effects on ER transcription activity in MCF-7 cells.

4.10.3.3 *In vivo* effects with regard to an endocrine mode of action

Ben Slima et al. (2017)

Summary: The main purpose with this study was to investigate the adverse effects of deltamethrin (CAS no. 52918-63-5, 98% purity) on male mice reproductive performance. Adult male mice were administered 0 or 5 mg/kg deltamethrin (n=24 males/group) by oral gavage daily for 35 days, and then co-housed with untreated, fertile female mice. Male mating (number of females inseminated) and fertility (number of pregnant females) indices were recorded. Semen from electroejaculation were analysed for density, motility, viability and abnormal morphology, and blood samples were analysed for serum testosterone and inhibin B levels. Then the male mice were killed and reproductive organs were removed and weighed. The left testis was used for histopathological examination.

No significant signs of general toxicity were observed in any of the males, and no differences in body or reproductive organ weights were seen between the 2 groups. The deltamethrin-treated males had decreased mating and fertility indices and gave rise to fewer litters. Their semen had a reduced sperm count, motility and viability, while the number of abnormal sperm was increased. The serum testosterone and inhibin B levels in the treated males were both decreased, and histopathology was found in the testes.

Study quality and assessment: The description of the study is unclear, and the results in the text does not always correspond to the results reported in the tables with regard to statistical significance. Therefore, the study is assessed to be of medium quality. The evidence for male reproductive toxicity is strong with reduced fertility and effects on sperm parameters as well as histopathological changes in the testes, and decreased testosterone and inhibin B serum levels.

Ben Halima et al. (2014)

Summary: In this study, the effects of deltamethrin (CAS no. 52918-63-5, purity not reported) on testes and sperm parameters were studied as a part of a larger study on the anti-oxidative effect of oat oil. Adult male mice were exposed to 0 or 5 mg/kg/day deltamethrin (n=8 male/group) by oral gavage for 35 days. After 24h from the last treatment the mice were weighed and euthanized and testes and epididymides were removed and weighed. Testes were undergoing histological examinations and biochemical analyses, and the epididymides were used for sperm parameter studies. Sperm count, motility and viability were assessed and the number of morphologically abnormal sperms counted. Testicular lipid peroxidation (MDA formation), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxide (GPx) activity was estimated as was glutathione and protein levels.

It was found that deltamethrin caused significant decreases in sperm motility and density, and an increase in the number of abnormal sperm. In addition, significant increases in testicular lipid peroxidation production and GPx and SOD activities were seen, while CAT activity and GSH levels were decreased insignificantly. This suggests that deltamethrin causes oxidative stress in the testes leading to altered sperm parameters.

Study quality and assessment: The study is well-described but only investigates a single dose of deltamethrin. Overall the study is assessed to be of high quality. The study provides strong evidence for adverse male reproductive effects in mice with adverse effects on sperm parameters, but these effects are more likely driven by an oxidative stress MoA than an ED MoA.

Sekeroglu et al. (2014)

Summary: The purpose of the present study was to assess the effects of acute and subacute deltamethrin exposure (CAS no. and purity not reported) on thyroid stimulating hormone (TSH) and free triiodothyronine (FT3) and thyroxine (FT4) in rats. Adult male rats were given either 0 or 15 mg/kg (37.5% of LD₅₀) deltamethrin for in a single dose by oral gavage, or 0 or 3 mg/kg (7.5% of LD₅₀) deltamethrin per day by oral gavage for 30 days (n=6 males/dose group). At 24 h after last treatment the animals were euthanized and blood was sampled and analysed for TSH, FT3 and FT4 serum levels. Only insignificant increases in TSH after 24 h and decreases after 30 days was measured. Similarly, insignificant decreases in FT3 and FT4 serum levels were measured after 24 h exposure and increases after 30 days of exposure.

Study quality and assessment: Besides not reporting the CAS no. or purity of deltamethrin, the study does not include a positive control to confirm the use of the model for the given purpose. Also, the study description is not always clear, and due to the mentioned shortcomings it is assessed to be of low quality. The study only provides weak evidence for thyroid hormone alterations with insignificant increases in TSH, FT3 and FT4.

Ben Slima et al. (2012)

Summary: The purpose of the present study was to examine the reproductive effects of deltamethrin (CAS no. and purity not reported) on male offspring exposed *in utero*. Adult female mice were exposed to 0 or 5 mg/kg deltamethrin (n=5 females/group) by oral gavage from gestation day 3 to 21. Clinical signs of maternal toxicity, body weights and weights of ovaries at the time of necropsy were recorded. Four adult male offspring from each group were killed at postnatal day 60-65 and testes and epididymides were removed and weighed. Sperm from the right caudal epididymides were analysed for sperm count, motility, viability and number of abnormal sperm. Four testes per group were undergoing histological examination. No signs of maternal intoxication were observed but the deltamethrin-treated females had significantly lower body and ovary weights. In the male offspring, the weights of the testes were significantly reduced in the *in utero* exposed animals. These males also had significantly lower sperm count, motility and viability, and higher percentage of abnormal sperm, as well as histopathological changes in their testes.

Study quality and assessment: The description of the study is adequate in details except the missing information on the CAS no. and purity of deltamethrin. Overall the study is assessed to be of medium quality, and it provides strong evidence for adverse reproductive effects in male offspring exposed *in utero*.

Abdallah et al. (2010b)

Summary: The present study aimed at investigating the effects of deltamethrin (CAS no. and purity not reported) on sperm measures and the reproductive system in male mice. Adult male mice were exposed to 0 or 5 mg/kg deltamethrin for 21 days (n= 10/group, read from the tables), and after the treatment period the mice were weighed, killed and testes and epididymis were removed and weighed. Sperm motility, count, viability and morphology were assessed. No effects on body weight or absolute testes and epididymis weights were found after deltamethrin treatment. Sperm count, motility and viability were significantly decreased and the number of abnormal spermatozoa was increased in the treated group.

Study quality and assessment: The study does not report the CAS no. or purity of deltamethrin, and it is also not clear from the material and methods section how many animals were used in each group. Due to these shortcomings the study is assessed to be of medium quality. It provides strong evidence for adverse male reproductive effects in mice with adverse effects on sperm parameters.

Issam et al. (2009)

Summary: The aim of the present study was to evaluate the effects of deltamethrin (CAS no. and purity not reported) on male gonads and sex hormones in response to different doses and treatment periods. Adult male rats were randomly assigned to 3 treatment groups and 3 corresponding control groups (n=6/group): a 30 days s.c. exposure to 0.003 mg/kg deltamethrin, or vehicle; a 45 days s.c. exposure with 30 days of 0.003 mg/kg followed by 15 days of 0.03 mg/kg, or 45 days vehicle s.c. exposure; a 60 days s.c. exposure with 30 days of 0.003 mg/kg then 15 days of 0.03 mg/kg followed by 15 days of 0.3 mg/kg, or 60 days of vehicle s.c. exposure. After treatment, blood was samples for measurement of plasma malondialdehyde (MDA) (an oxidative stress marker), follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone levels, and testes were removed and undergoing histopathological evaluation. FSH levels increased in the 30-day exposed group but decreased in the 45- and 60-day groups. LH and testosterone levels increased after 30 and 45 days of

treatment but decreased after 60 days of deltamethrin treatment. MDA was elevated in all treatment groups. The histological examination showed histopathological changes with pyknotic cells and regression of the interstitial tissue in all treatment groups with arrested spermatogenesis in the 60 days treatment group.

Study quality and assessment: The study does not give information on CAS no. and purity of the tested compound and is not always clear in the description of the study design and results. Due to these shortcomings, the study is assessed to be of medium quality. The study provides moderate evidence of ED MoAs with altered hormone levels and effects on the gonads. These effects on the gonads might however be a result of the increased oxidative stress response observed.

Kilian et al. (2007)

Summary: In the present study a modified one-generation study was performed to assess the endocrine disrupting effect of deltamethrin (no CAS no. reported, 99.5% purity) alone or in mixtures on the reproductive parameters in male offspring exposed *in utero*. Adult female and male rats were paired and allocated into dosing groups (n= 6 pairs/group). After mating the pregnant females were exposed to either 0 or 1 mg/kg deltamethrin (n=6 /group) by oral gavage from gestation day 7 and until postnatal day 23, where the pups were weaned. Twelve male offspring from each group were selected and given 0 or 1 mg/kg deltamethrin by oral gavage for 10 weeks. At week 14 the male offspring were terminated and samples taken. All animals were assessed for general toxicity clinical signs. At termination of the male offspring, blood was sampled for liver functional analysis (n=6) and chemical analysis (n=6). Anogenital distance (AGD) was measured and testes, epididymides, seminal vesicles and liver were weighed, and the right testis was used for histological examination of spermatogenesis. Sperm count was determined.

No clinical signs of toxicity were observed in any of the animals. A significantly shorter AGD was found in the 1 mg/kg deltamethrin group, who also had a significant decrease in seminal vesicles weight compared to control group. On histology, decreases in seminiferous tubule diameter and epithelium thickness were found in the exposed group compared with control. No significant effects on liver function enzymes, testes and epididymides weights, spermatogenesis or sperm count were found.

Study quality and assessment: The study is well-described, but lack information such as CAS nos. and especially the method used for selecting the 12 males per group from the 6 litters per group. Also, it seems that the pup and not the litter was used as the statistical unit, which is incorrect. Thus the study is assessed to be of medium quality. The study provides moderate evidence for adverse effects on male offspring reproductive organs with a shortening of AGD and effects on seminal vesicles weight and histopathological changes in the testes.

Johri et al. (2006)

Summary: The present study examined the effects of prenatal exposure to deltamethrin (no CAS no. or purity reported) on rat offspring locomotor activity, as well as enzyme levels and activity in their liver and brain. Briefly, 54 pregnant female rats were exposed to 0, 0.25, 0.5 or 1 mg/kg deltamethrin by oral gavage on gestation day (GD) 5-21. The litters were culled to 8 pups with an equal distribution of sexes. Male offspring spontaneous locomotor activity was monitored at 3, 6 or 9 weeks and the animals were then sacrificed and liver and brain removed. Microsomes and RNA was isolated from

the livers and brains, and the activity of 3 CYP P450 monooxygenases, *N*-nitrosodimethylamine demethylase (NMDA-*d*), 7-pentoxoresorufin-*O*-dealkylase (PROD) and 7-ethoxyresorufin-*O*-deethylase (EROD), in the microsomes were measured as well the mRNA and protein levels of CYP1A1/1A2, CYP2B1/2B2 and CYP2E1.

Prenatal deltamethrin exposure resulted in altered spontaneous locomotor activity with most parameters affected at 3 weeks of age and some persisting into both 6 and 9 weeks of age. Increases in NMDA-*d*, PROD and EROD activities showed dose-dependent increases in both brain and liver at 3 weeks with some of the activity increases lasting into the 6th and 9th weeks. Similarly dose-dependent increases in CYP protein and mRNA levels were mainly seen at 3 weeks with some effects lasting into the 6th and 9th weeks of age especially at the highest exposure doses.

Study quality and assessment: The study is in general well-described and thorough but the exact number of animals in each group is not clearly stated and the figures are not optimally formatted. The study is therefore assessed to be of medium quality. Moderate evidence for neurodevelopmental toxicity, more specifically altered spontaneous motor activity, in the prenatally exposed animals exist but whether these effects are persistent after week 9 is not known. Also no links between these effects and an ED MoA is made here but deltamethrin seems to alter the expression and activity of multiple enzymes and may through this mechanism affect normal hormone turnover.

Birkhøj et al. (2004)

Summary: In the present study, deltamethrin (CAS no. and purity not reported) and 4 other pesticides were tested alone and in mixture in the Hershberger assay for anti-androgenic effects. Castrated male rats were treated with testosterone propionate (0.5 mg/kg sc) and 0 or 2.5 mg/kg BW deltamethrin po (n= 6 males/group) for 7 consecutive days. The positive control group were treated with the known anti-androgen Flutamide (20 mg/kg/day sc). After the exposure period, blood samples were taken for hormone analysis (testosterone, thyroxin (T4), prolactin and luteinizing hormone (LH) levels), and the animals were euthanized and the ventral prostate, seminal vesicles, levator ani/bulbocavernosus muscle (LABC), bulbourethral glands, liver, thyroid gland, paired adrenal glands and paired kidneys were removed and weighed. The ventral prostate was used for gene expression analysis. No effects on body weight, organ weights or hormone levels were observed after deltamethrin treatment. A reduction in gene expression of ODC (ornithine decarboxylase) relative to the positive control was the only affected gene measured in the ventral prostate gene expression analysis.

Study quality and assessment: The study is well-described and only lack information on CAS no. and purity, and the number of animals per dose group is not clearly stated in the material and methods section but can be read from tables and figures. It is assessed to be of high quality. The study provides only very weak evidence for an anti-androgenic MoA *in vivo* due to the effects on gene expression in the ventral prostate but no effects on organ weights or hormone levels were found in the exposed animals.

Andrade et al. (2002a)

Summary: The present study, investigated the effect of *in utero* exposure to deltamethrin (no CAS no. reported, 98.8% purity) on male offspring reproductive performance. Female rats were mated and administered to 0 (n=10), 1, 2 or 4 mg deltamethrin/kg (n=12/dose group) daily by oral gavage from gestation day (GD) day 1 to lactation day 21 (weaning). During this period maternal toxicity and

reproductive performance parameters were assessed. The male offspring (n=16/dose group) were assessed for testis descent and preputial separation but ano-genital distance was not measured in this study. Animals were kept until they were 150-180 days old, where they were killed and testes, epididymis, ventral prostates and seminal vesicles were removed and weighed, and blood samples were taken to measure testosterone levels. Sperm number and morphology were assessed. Before killing, 10 out of the 16 males/group were used at postnatal day 120 in a mating study with unexposed females where sexual behaviour and the reproductive performance were recorded. The testes from the 6 males/group not included in the mating study were used in a histological examination. No clinical signs of maternal toxicity or effects on maternal reproductive performance were seen. Testis descent and preputial separation, sperm number and morphology, serum testosterone levels, sexual behaviour and reproductive performance in the male offspring were not effected in the treated groups. Only significant effects were observed in the highest 4 mg/kg dose group, where the testis weight and seminiferous tubules diameters were reduced.

Study quality and assessment: The study is well-designed and -described. The CAS no. is not reported and the number of animals for the different examinations is not always clear from the material and methods section but can be found in figures and tables. In general, the study is of medium quality. The study provides weak evidence for male reproductive toxicity after *in utero* and lactational exposure to deltamethrin with effect on testes weight and histology.

Andrade et al. (2002b)

Summary: In the present study, deltamethrin (no CAS no. reported, 98.8% purity) was tested for (anti)estrogenicity and (anti)androgenicity in the 2 *in vivo* assays, Uterotrophic and Hershberger assay. In the Uterotrophic assay, immature (~21 days old) female rats were exposed to 0, 2 or 4 mg deltamethrin/kg daily by oral gavage for 3 days. To test for anti-estrogenicity, the rats were treated with hexahydrobenzoiate estradiol (0.4 mg/kg/day po), which was also used alone as a positive control, and then co-administered 4 mg/kg deltamethrin for 3 days. There were 7-10 females/dose group, and after the 3 days of exposure all rats were killed and their uterus removed and weighted. In the Hershberger assay, adult (~7 weeks old), castrated male rats were given deltamethrin daily for 7 days. In the anti-androgen mode, the rats were treated with testosterone cypionate (0.25 mg/kg/day sc) and co-administered 0, 2 or 4 mg deltamethrin/kg po. Flutamide (10 mg/kg/day sc) was used as the positive control for anti-androgenicity. In the androgen mode, the rats were given 4 mg deltamethrin/kg po. There were either 8 or 9 males/dose group. After the 7 days of exposure all rats were killed and their prostate and seminal vesicles were removed and weighted.

Neither oestrogenic nor anti-oestrogenic effects of deltamethrin were found in the Uterotrophic assay. Similarly, no effects were found in the Hershberger assay in both the androgenic or anti-androgenic mode.

Study quality and assessment: Overall, the study is well described and -performed and only a few details are missing such as CAS no., and the study is assessed to be of high quality. No evidence for ED MoAs of deltamethrin were found in the study.

Aziz et al. (2001)

Summary: The present study investigated the effects of low level *in utero* deltamethrin (no CAS no. or purity reported) exposure during gestation day (GD) 14-20 on neurobehavior, neurochemistry and

immunohistochemical parameters postnatally in rats. Female rats were mated and then randomly divided into 2 groups and administered either 1 mg/kg deltamethrin or only vehicle from GD 14 to GD 20 by oral gavage (n=10 females/group). After birth, litters were culled to 8 pups/litter with an equal distribution of males and females. The pups were examined daily, and a total of 10 offspring per group were evaluated for learning behaviour in a Y-maze. The offspring were sacrificed after either 6 or 12 weeks of age, and brains were removed and undergoing different analysis for acetylcholinesterase (AChE) and protein estimations.

Deltamethrin reduced the surface righting reflex, number of cholinergic receptors in the brain and learning performance, while AChE activity and GAP-43, a neuron specific protein related to neuron maturity, were increased.

Study quality and assessment: The study is assessed to be of medium quality based on the description with missing information on deltamethrin purity and sometimes less clear descriptions on the selection of animals for the different tests and analysis. Overall, the evidence for developmental neurotoxicity is strong with effects on learning, however these effects are not linked to an ED MoA in the present study but are rather associated with effects on the cholinergic system.

Lazarini et al. (2001)

Summary: In the present study the effects of prenatal deltamethrin (no CAS no. or purity reported) exposure on rat pup physical, reflex and behavioural development was investigated. Seventeen female rats were mated and randomly divided into 2 groups and given 0 (n= 8 females) or 0.08 mg/kg (n=9 females) deltamethrin by oral gavage from gestation day (GD) 6 to GD 15. After parturition the litters were culled to 8 offspring of equal sex distribution. An open-field test (locomotion frequency, rearing frequency and immobility time) and reflexes (surface righting reflex, negative geotaxis and palmer grasp) in one male and one female from each litter were assessed at weaning on parturition day (PND) 21. At PND 60 a swimming test followed by an open-field test was made on one animal per gender per litter to assess latency to start floating, duration of floating, locomotion frequency, rearing frequency and immobility time. At PND 140 the offspring were sacrificed, and brains were removed and undergoing neurochemical evaluation.

Deltamethrin treatment did not affect maternal or offspring weight, and no differences in reflexes or the open field test were seen at weaning. In adulthood at PND 60 a decreased latency to float as well as decreased locomotion and increased immobility in the open-field test were observed in exposed male offspring only. A general sexual dimorphic response in the open-field test was observed with males showing relative lower locomotion and rearing compared to females. In the neurochemical evaluation the only difference was a higher level of DOPAC (dopamine metabolite) and noradrenaline in the exposed male offspring.

Study quality and assessment: The study is well-described and assessed to be of medium quality due to shortcomings such as missing information on deltamethrin identity and the fact that only a single dose was tested. Overall, the study provide moderate evidence for developmental neurotoxicity of deltamethrin in males based on the effects in the swimming and open-field tests, however no links to ED MoAs are made.

Madsen et al. (1996)

Summary: In the present study, deltamethrin (CAS no. 52918-63-5, 99% purity) was tested for its effect on the immune system in a 28-days study in male rats. Deltamethrin was given by gavage daily to 4-week old male rats in doses of 0, 1, 5 or 10 mg/kg (n=16 males/dose group) for 28 days. On treatment day 23, 6 of the 16 rats were immunised. All animals were killed on day 28, and blood was sampled and organs removed and weighed. The spleens of the immunised animals were used for immunological tests assessing effects on sheep red blood cells (SRBC) plaque forming cells (PFC), mitogens and natural killer (NK) cell activity. Detection of lymphocyte subpopulations were made, and haematology of the blood from the non-immunised animals were performed. Bone marrow from the right femur was extracted to count the number of nucleated cells. Volume of thymus and mesenteric lymph nodes were quantified.

No effects on clinical appearance, haematological parameters, bone marrow cell number and volume of thymus and lymph nodes were seen in the exposed groups. Decreases in body weight gain, terminal weights, and thymus and adrenals weight were found in the high dose group. Deltamethrin caused an increase in the number of SRBC-PFCs and enhanced the activity of the NK cells at the 5 and 10 mg/kg doses. These results suggest that deltamethrin stimulates the immune system by increasing the number of antibody forming cells in the spleen and enhancing NK cell activity.

Study quality and assessment: The study is well-performed and -described although the methods could have included more detailed information. Overall the study is assessed to be of high quality. The study does not provide any evidence for adverse effects related to ED MoAs.

Abd El-Aziz et al. (1994)

Summary: The present study investigated the effect of deltamethrin (CAS no. and purity not reported) on male rat fertility. Adult male rats were exposed to 0, 1 or 2 mg/kg BW deltamethrin daily by oral gavage for 65 consecutive days (n=15/dose group). Blood samples for testosterone level measures were taken before and at 14, 28, 42 and 65 days of exposure as well as 21 days after exposure had stopped. From each dose group, 5 animals were killed after the 65 days exposure period, 5 animals were kept for 21 days, and the remaining 5 rats were kept for 60 days after treatment had stopped. The weight of the testes, epididymis, seminal vesicles and prostate as well as sperm parameters were recorded for the animals killed at day 65 and 65 + 21 days. Before this the 5 animals kept for 0 and 60 days post-treatment, respectively, were mated with non-treated females (n= 8/male/dose group), and the conception rates were noted.

Treatment with deltamethrin significantly decreased the weights of testes, epididymis, seminal vesicles and prostate both just after the 65 days treatment and 21 days after treatment had stopped. The same was seen in both groups with the sperm parameters with decreases in sperm density and motility and increase in abnormal morphology. Decreased testosterone serum levels were seen in all treatment groups at all the tested time points compared to the levels before treatment start. Male fertility was decreased in the two treatment group compared to control at day 65 of exposure as well as on day 60 post-treatment.

Study quality and assessment: The study is well-described although a number of important details are missing such as CAS no., purity, housing conditions etc. Overall the study is assessed to be of medium quality. The study provides strong evidence for adverse effects on male reproduction with effects on the gonads, sperm and testosterone levels. ED MoAs for these effects were not explicitly studied but the effects on the testosterone levels could provide a moderate evidence for ED MoA(s).

Husain et al. (1994)

Summary: The present study examined the mechanisms of deltamethrin (no CAS no. or purity reported) on neurodevelopment in young rats. A total of 100 young male rats were randomly divided into 2 groups and received either 0 or 7 mg/kg deltamethrin by oral gavage from postnatal day (PND) 22-37 (n=50/dose group). On PND 38, 5 animals from each group were killed and brains removed to estimate polyamine levels. Similarly, 5 other animals were killed at PND 38 and their brains were used to assess the 3 enzymes, monoamine oxidase (MAO), acetylcholinesterase (AChE) and Na⁺-K⁻ ATPase. Another set of 6 pups per group were sacrificed at PND 38 to estimate neurotransmitter receptor binding. Finally, the remaining 34 rats/group were used for behavioural testing 24 hrs after the last treatment. Ten rats were tested for spontaneous locomotor activity and then reused in a test for aggressive behaviour using a total of 12 pairs (i.e. 24 animals/group). Another set of 10 rats/group were used to test learning or the conditional avoidance response.

No general toxicity such as weight reductions were seen in the treated group. The wet weight of hippocampus was significantly reduced and the activities of MAO and AChE were elevated. A significant increase in the spontaneous locomotor activity and impairment of learning were seen. Changes in polyamine levels and neurotransmitter receptor binding were also found in the treated animals.

Study quality and assessment: The study is assessed to be of medium quality based on the many animals used but a sometimes unclear explanation of the study design and missing information on the purity of deltamethrin as well as the limitations of testing only a single dose. Moderate evidence for developmental neurotoxicity with effects on behaviour is provided however no conclusions with regard to links to ED MoA can be made since these mechanisms were not investigated here.

Abdel-Khalik et al. (1993)

Summary: In the present study, deltamethrin (CAS no. and purity not reported) was given orally at doses of 0, 1, 2.5 and 5 mg/kg to pregnant female rats (n=20/dose group) from gestation day (GD) 6-15. At GD 19 the pregnant rats were killed and number of implantations, live foetuses, weight and length of foetuses were recorded. One third of the foetuses were subjected to visceral examination and the remaining 2/3 were subjected to skeletal examinations. Deltamethrin caused dose-dependent early embryonic death and growth retardations. Likewise, a dose-related increase in foetuses with hypoplasia of the lungs and dilations of the renal pelvis were also observed in the foetuses exposed *in utero* to deltamethrin. The placentas from the exposed female rats had increased weight.

Study quality and assessment: The description of the study could be more detailed, and relevant information such as CAS no., purity, vehicle type and housing conditions are missing. The study is due to these missing details in the description assessed to be of medium quality. The evidence for adverse developmental defects in the foetuses is moderate, while evidence for ED MoA related to these effects have not been studied.

Husain et al. (1992)

Summary: This study investigated *in utero* effects of deltamethrin (no CAS no. or purity reported) on early rat brain development. Pregnant female rats were randomly divided into a control group and a group receiving 7 mg/kg deltamethrin daily by oral gavage from gestation day (GD) 5-21. The offspring were evaluated for behavioural teratogenicity (n=10 pups/group). Included in the test were pinna detachment, incisor eruption, surface righting, air righting, grip strength, auditory startle, ear opening, eye opening and growth as well as motor activity (assessed at both pre-weaning (PND 21) and post-weaning (PND 42)). At PND 22, 5 pups from each group were killed and brains removed to estimate polyamine levels in the different brain regions.

No general toxicity was seen in the maternal animals but deltamethrin caused an increase in the number of foetal resorptions and death of neonates. In addition significant delays in the onset of fur development, incisor eruption, eye and ear opening, and reductions in surface righting reflex and grip strength were seen in animals after deltamethrin exposure. Spontaneous motor activity was also significantly reduced at both PND 21 and 42 compared to the control animals. The levels of polyamines in the different brain areas were also significantly reduced following deltamethrin exposure.

Study quality and assessment: The study does not specify the exact number of pregnant females and litters per group or the distribution of male and female pups in the different tests and if this differed between the treated group and the control group. That in addition to missing information on the purity of deltamethrin and the fact that only a single dose was tested results in the study being assessed as of low quality. The study provides moderate evidence for a developmental neurotoxic (DNT) effect of deltamethrin due to the observed alterations in behaviour. The study does not investigate effects on hormone levels or ED MoAs and therefore no conclusions regarding ED links to the DNT effects can be made.

Eriksson and Fredriksson (1991)

Summary: The purpose of this study was to investigate the effects of postnatal exposure to deltamethrin (no CAS no. or purity reported) on young and adult mice behaviour and brain expression of muscarinic cholinergic receptors (mAChRs). Ten-day old male mice were administered 0 or 0.7 mg/kg deltamethrin (n=12/group, from 3 different litters) by oral gavage from postnatal day (PND) 10-17. Behavioural tests were made at PND 17 and again at 4 months of age, hereafter they were killed and brains removed to measure mAChR density.

No clinical signs of pyrethroid poisoning were seen. No significant changes in behaviour were seen at PND 17, but at 4 months of age the deltamethrin-treated mice showed a significant increase in locomotion and total activity when compared to control animals. A tendency to a decrease in the density of mAChRs was seen in the cortex but not in the other brain areas of the deltamethrin-treated animals.

Study quality and assessment: The study description is rather short and important information is absent from the description and figures such as clearly stating the number of animals per group in each test/analysis. Overall the study is assessed to be of low quality. There is moderate evidence for developmental neurotoxicity of deltamethrin in mice due to the altered adult behaviour but no conclusions on links to ED MoAs can be made.

Draft Assessment Report (DAR) (1998)

Summary: In the draft assessment report for deltamethrin several repeated dose studies and an old two-generation study (1992) are included. Hardly any effects on weight of reproductive organs were found in the repeated dose studies and no data on testis histology is reported. It is assumed that histology was performed, but that no treatment-related findings were found on histological assessment of reproductive organs.

Study quality and assessment: The quality of the studies cannot be assessed based on the summaries available in the draft assessment report. The DAR is from 1998 and the studies are therefore performed according to the OECD TG before 1998 which means that some ED relevant endpoints are not included (e.g. AGD, nipple retention and sperm quality). The studies included in the draft assessment report indicate that there are no adverse effects on reproductive or developmental parameters with regards to the endpoints that can be assumed to be evaluated at the time the study was performed.

4.10.3.4 Summary of the plausible link between adverse effects and endocrine mode of action

Available studies on ED MoAs of deltamethrin (Table 1) provide inconsistent results, for example are 2 *in vitro* studies indicating weak oestrogenic activity while no oestrogenic or anti-oestrogenic activity was found in another *in vitro* assay and in the Uterotrophic *in vivo* assay. A number of studies indicate anti-androgenic effects of deltamethrin in *in vitro* assays (AR antagonism) and *in vivo* with decreased testosterone and shortening of ano-genital distance in adult male offspring (Table 1 and 2). However, no anti-androgenic effects were seen in 2 independent Hershberger *in vivo* assays using castrated males. Multiple studies have shown altered testes histopathology, adversely altered sperm parameters, and decreased testosterone serum levels in both rat and mouse after exposure during adulthood or *in utero* and reduced weights of testes and accessory sex organs. These male reproductive toxicity effects have been attributed to oxidative stress induced by deltamethrin but a link to an anti-androgenic MoA, i.e. effect on steroidogenesis leading to low testosterone, is also plausible.

Various studies on developmental neurotoxicity (DNT) of deltamethrin have been conducted in both rats and mice, and overall these studies indicate that deltamethrin is a developmental neurotoxicant with effects on behaviour and the weight of some brain regions after pre-or postnatal exposure. These effects have primarily been attributed to alterations in the cholinergic system, enzyme activities and polyamine levels in the brain and to our knowledge no links to ED MoAs have been studied. A single study in rats showed non-significant effects on TSH, free T3 and T4 in serum, and in an *in vitro* study weak TR antagonism of deltamethrin was found. More studies are needed to evaluate a plausible link between these potential effects on the thyroid system and developmental neurotoxicity of deltamethrin.

The total evidence for an AR antagonistic MoA is weak due to conflicting *in vivo* and *in vitro* data. Based on the published literature, the evidence for adverse effects on male reproductive organs is strong, but when taking the DAR into account the evidence is assessed to be moderate due to the lack of reported effects on testis weights and histopathology of reproductive organs. The evidence for a plausible link between this adverse effect and an anti-androgenic MoA is moderate. The evidence for DNT of deltamethrin is moderate due to the low to medium quality of the related studies that often does not report the purity of the tested compound or has an unclear study design description. The evidence for a plausible link between DNT and a thyroid MoA is weak mainly due to very few available studies for this MoA.

In conclusion, deltamethrin does not meet the WHO definition of an endocrine disruptor, but fulfil the WHO definition of a potential endocrine disruptor. Also deltamethrin fulfil the proposed Danish criteria for being a suspected ED.

Additional literature not directly used in the evaluation

OEHHA report 2012: This report reviews results on developmental, female and male reproductive toxicity of deltamethrin from animal studies. It reports effects on all three endpoints with most evidence for male reproductive toxicity and developmental neurotoxicity.

S.C. Johnson & Son (2012): This report from the industry with the title ‘Deltamethrin Has Not Been Clearly Shown to Cause Reproductive Toxicity’ is a response to the OEHHA report. It highlights the in general missing quality assessment of the studies included in the OEHHA report, and concludes that deltamethrin has not been clearly shown through scientifically valid testing to cause developmental toxicity or male and/or female reproductive toxicity. Public available references in the report has been included in the above evaluation if assessed relevant.

EFSA Scientific Opinion (2008): The studies included in the Opinion were assessed to have several limitations and could not provide any clear evidence for a developmental neurotoxic effect of deltamethrin. The conclusion from the experts was that deltamethrin has been adequately tested for developmental neurotoxicity and that the available data do not indicate that deltamethrin is a developmental neurotoxic agent. Publicly available references in the Opinion has been included in the above evaluation if assessed relevant.

Shafer et al. (2005): This is a review of developmental neurotoxicity of pyrethroids including deltamethrin and describes results and strengths and limitations of multiple studies of which the relevant references have been included in the above evaluation. It also refers to results from an unpublished study by Muhammed and Ray but due to the unpublished nature of this data it will not be included here.

Table 1. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of deltamethrin.

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Christen et al. (2014)	Weak AR antagonism in MDA-kb2 cells.		Low	Moderate
Abdallah et al. (2010a)	Effects on spermatozoa viability, motility and morphology <i>in vitro</i> that are attributed to oxidative stress induced by deltamethrin and not an ED MoA.		High	Weak
Du et al. (2010)	Weak ER agonism and TR antagonism in CV-1 cells, as well as AR antagonism in MDA-kb2 cells.		High	Strong
Issam et al. (2009)		Altered FSH, LH and testosterone plasma levels in adult male rats exposed s.c. to different levels and periods of deltamethrin.	Medium	Moderate
Birkhøj et al. (2004)	Anti-androgenic at sub-cytotoxic concentrations in AR transfected CHO cells	No anti-androgenic MoA in the rat Hershberger assay (castrated males) except reduced expression of a single gene in the ventral prostate	High	Weak-Moderate
Andersen et al. (2002)	Weak, but significant effects of deltamethrin on cell proliferation but no ER transactivating effects in MCF-7 cells.		High	Moderate
Andrade et al. (2002b)		No (anti)oestrogenic or (anti)androgenic MoAs in the rat oral Uterotrophic or the Hershberger assay (castrated males), respectively	Medium	None

androgen receptor (AR), thyroid hormone receptor (TR), estrogen receptor (ER), follicule-stimulating hormone (FSH), luteinizing hormone (LH), Chinese Hamster Ovary (CHO)

Table 2. Overview of potential endocrine-related adverse effects of deltamethrin.

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Ben Slima et al. (2017)	Mouse, 24 males/group	Decreased fertility, and decreases in sperm count, viability and motility, and increase in the number of abnormal sperm. Decreased testosterone and inhibin B serum levels and histopathology in the testes	Medium	Strong
Ben Halima et al. (2014)	Mouse, 8 males/group	Decreases in sperm count and motility, and increase in the number of abnormal sperm. Altered oxidative stress markers in the testes.	High	Strong
Sekeroglu et al. (2014)	Rat, 6 males/group	Non-significant effects on TSH, FT3 or FT4 serum levels were found after acute and subacute exposure to deltamethrin.	Low	Weak
Ben Slima et al. (2012)	Mouse, 5 females/group and 4 male offspring/group	Effects in the <i>in utero</i> exposed male offspring: reduced testes weights, decreased sperm count, vitality and motility, and increased number of abnormal sperm, histopathological changes in the testes.	Medium	Strong
Abdallah et al. (2010b)	Mouse, 10 males/group	Decreases in sperm count, motility and viability and increase in the number of abnormal spermatozoa.	Medium	Strong
Issam et al. (2009)	Rat, 6 males/group	Altered testicular histopathology in adult male rats exposed s.c. to different levels and periods of deltamethrin.	Medium	Moderate
Kilian et al. (2007)	Rat, 6-12 animals/group	<i>In vivo</i> study using a modified one-generation study: shortening of AGD and effects on seminal vesicles weight and histopathological changes in the testes of adult male offspring.	Medium	Moderate
Johri et al. (2006)	Rat, 13-14 females/dose group w. 8 offspring each	Altered spontaneous motor activity as well as altered enzyme activity and levels in liver and brain with most pronounced effects after the 3rd and declining through the 6 th and 9 th weeks of age in prenatally exposed offspring.	Medium	Moderate
Andrade et al. (2002a)	Rat, n=16 male offspring/ group	Effects on male rat offspring testes weight and histology in the highest treatment group (4 mg/kg)	High	Weak
Aziz et al. (2001)	Rat, 10 female/group w. 8 offspring each	Decreased learning and memory capacity in offspring exposed <i>in utero</i> (GD14-20) as well as effects on the cholinergic system and maturation parameter (GAP-43) in the brain.	Medium	Strong
Lazarini et al. (2001)	Rat, 8-9 females/group w. 8 offspring each	Decreased latency to float in swimming test, and decreased locomotion and increased immobility in the open-field test at PN60 as well as a higher level of DOPAC (dopamine metabolite) and noradrenaline in the at PN 140 were observed in exposed male offspring only.	Medium	Moderate
Abd El-Aziz et al (1994)	Rat, 15/males/dose group	Potential irreversible effects on male fertility with decreased weights of the accessory sex organs, adversely affected sperm parameters and decreased testosterone serum levels.	Medium	Strong

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Husain et al. (1994)	Rat, 50 young males/group, 10-12 per behavioural test	Increased spontaneous locomotor activity and impaired learning as well as altered levels of polyamines, enzyme activities and neurotransmitter receptor binding in the brains were seen in postnatally exposed male rats.	Medium	Moderate
Abdel-Khalik et al. (1993)	Rat, 20/females/dose group	Early embryonic death and growth retardation of <i>in utero</i> exposed foetuses. Increase in cases with visceral malformations were also seen	Medium	Moderate
Husain et al. (1992)	Rat, 10 pups/behavioural test group	Behavioural teratogenicity parameters such as eye and ear opening, fur development and incisor eruption were delayed and motor activity was reduced in the <i>in utero</i> exposed offspring	Low	Moderate
Eriksson and Fredriksson (1991)	Mouse, 12 offspring/group, from 3 different litters	Changes in adult behaviour of postnatally deltamethrin-treated male mice indicative of developmental neurotoxicity.	Low	Moderate

Thyroid stimulating hormone (TSH), free triiodothyronine (FT3), free thyroxine (FT4), Anogenital distance (AGD), gestation day (GD), parturition day (PND)

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Prochloraz, CAS no. 67747-09-5

Synonyms: N-propyl-N-[2-(2,4,6-trichlorophenoxy)ethyl]imidazole-1-carboxamide.

Prochloraz (Figure 1) is a broad-spectrum imidazole fungicide. Prochloraz is widely used in gardening and agriculture. It is used on wheat, barley, mushrooms, cherries, turf on golf courses, and in flower production, for instance, in Ecuador, where roses are treated with prochloraz prior to export to the USA. Its fungicidal activity is due to inhibition of 14 alpha-demethylase (CYP 51), an enzyme required for the synthesis of fungal cell walls. Prochloraz is an agricultural imidazole fungicide that inhibits a CYP enzyme involved in ergosterol synthesis, but has also been reported to inhibit other CYP enzymes, and to act as a potent aromatase inhibitor. This EU approval expires 31st December 2021. At the EU Member State level it has been approved in 25 EU countries (except Malta and Denmark) (EU, 2016).

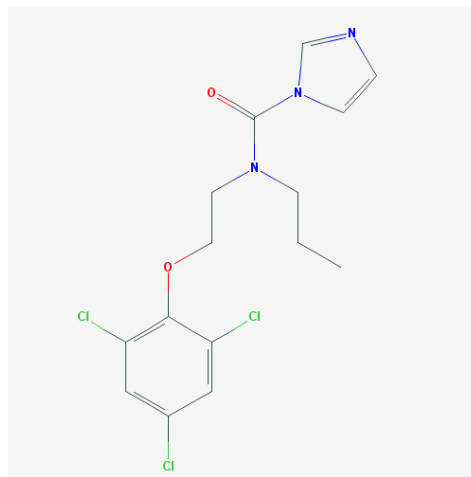


Figure 1. 2D structure from PubChem

4. Human health hazard assessment

4.10.3 Endocrine disruption

4.10.3.1 General approach – human health

4.10.3.2 *In vitro* information indicative of endocrine activity

Kjærstad et al. (2010)

Summary: In this study widely used conazole antifungals were tested for endocrine disruptive effects using a panel of *in vitro* assays. They all showed endocrine disrupting potential and ability to act via several different mechanisms. Overall the imidazoles (conazole, ketoconazole, miconazole, prochloraz) were more potent than the triazoles (epoxiconazole, propiconazole, tebuconazole). The critical mechanism seems to be disturbance of steroid biosynthesis. In the H295R cell assay, the conazoles including prochloraz decreased dose-dependently the formation of estradiol and testosterone, and increased the concentration of progesterone. Effects at 0.01 μM (lowest concentration tested). Maximum effects (at 3.0 μM) were less than 10% of the solvent control. This was indicating inhibition of enzymes involved in the conversion of progesterone to testosterone. Prochloraz was most potent. In the MCF-7 cell proliferation assay, the conazoles showed anti-estrogenic effect, including aromatase inhibition, since they inhibited the response induced by both 17 β -estradiol (incl. prochloraz) and testosterone (incl. prochloraz).

Study quality and assessment: The study is well-described. In this study prochloraz was assessed in a battery of *in vitro* tests. Purity (99.5%) and CAS no. is given and cytotoxicity was evaluated by using the Cytotox 96 Non-Radioactive Cytotoxicity assay. Overall the quality of the study is assessed to be high, and the study provides strong evidence for an anti-androgenic, anti-estrogenic effect and also aromatase inhibiting MoA of prochloraz.

Hecker et al. 2006

Summary: In this study an *in vitro* screening assay based on measuring alterations in hormone production was developed using the H295R human adrenocortical carcinoma cell line. The objective of the H295R Steroidogenesis Assay (later OECD TG 456) is to detect substances that affect production of estradiol (E2) and testosterone (T) and P(progesterone/pregnenolone). Prochloraz is included in this Test Guideline as a positive control as it is a strong inhibitor of steroid hormone secretion. The aim of the current study was to develop and standardize an *in vitro* Tier 1 screening assay using the H295R cell line to prioritize chemicals that act to alter hormone production. CAS and purity was not given in this study. Model chemicals with different modes of action on steroidogenic systems were tested in this study. Exposure to forskolin resulted in dose-dependent increases in all three hormones with the greatest relative increase being observed for E2. This differed from cells exposed to prochloraz (or ketoconazole) where P concentrations increased while T and E2 concentrations decreased in a dose-dependent manner at prochloraz concentrations greater than 0.03 and 0.003 μM , respectively. Based on these results, the H295R *in vitro* system has potential for high throughput screening to not only characterizes the effects of chemicals on endocrine systems but also to prioritize chemicals for additional testing.

Study quality and assessment: The study is well-described and prochloraz was included as positive control in this study (as later in the final OECD TG 456). More information on CAS-number would have been preferred and cytotoxic chemical concentrations were not included in the hormone concentration measurements. Overall the quality of the study is still assessed to be high, and the study provides strong evidence for an anti-estrogenic and anti-androgenic MoA of prochloraz.

Overall, a large battery of published *in vitro* screening studies indicates that prochloraz is able to induce endocrine imbalance by affecting the androgen and oestrogen receptor, to inhibit the aromatase activity and to interfere with steroidogenesis probably at the level of P450c17. A review of the endocrine-related data available for prochloraz has been conducted in GD 181 by OECD which led to the conclusion that “The combined dataset provides sufficient evidence of endocrine activity” (OECD 2012). Also the review by Melching-Kollmuss et al. (2017) gives an overview of *in vitro* assays on prochloraz.

4.10.3.3 *In vivo* effects with regard to an endocrine mode of action

Melching-Kollmuss et al. (2017)

Summary: This paper provides a good review of regulatory and peer reviewed studies of prochloraz. Prochloraz was administered per gavage in oil from gestation day (GD) 6 to postnatal day (PND) 83 to pregnant and lactating Wistar rats and their respective offspring, at doses of 0.01 mg/kg bw/day (acceptable daily intake of prochloraz), 5 mg/kg bw/day (expected no-observed-effect-level (NOEL)) and 30 mg/kg bw/day. At 30 mg/kg bw/day maternal and offspring effects (decreased viability, lower number of live offspring) were seen including a delayed entry into male puberty (plus one day) accompanied by lower male offspring body weights, increased ano-genital distance/index in females and (transiently) retained nipples in males at PND 12 (not seen at PND 20). The only finding at the expected NOEL was increased incidences of transiently retained nipples in males which the authors do not consider an adverse finding. No effects were seen in the low-dose group.

Study quality and assessment: The study is well-described and evaluates prochloraz at very low levels (ADI, NOEL, 6 times NOEL). The study is of moderate quality as it has some limitations. It is reported that 20 mated females per dose group are used. Unfortunately, it is unclear how many litters are evaluated for each endpoint. Additionally, it is not clear whether they make statistics (e.g. ano-genital distance (AGD)) on individual pups and take litter effect into account (as described) or if they are just looking at individual pups which is not nearly as sensitive. They find retained nipples at day 12-13. The authors do not believe that a significant incidence of preserved nipples is an adverse effect. However, it is possible to set a NOAEL on this finding according to OECD guidance documents. Overall the study provides strong evidence for adverse effects such as increased nipple retention (even at low doses and thereby an anti-androgenic MoA of prochloraz).

Blystone et al. (2007)

Summary: Prochloraz was administered by gavage to pregnant rats at doses of 0, 7.8, 15.6, 31.3, 62.5, and 125 mg/kg/day from gestational day 14 to 18. On gestational day 18, hormone production from ex vivo fetal testes was examined and prochloraz levels in amniotic fluid and maternal serum were measured. Fetal P and hydroxy-P production levels were increased significantly at every prochloraz dose, whereas T levels were significantly decreased only at the two high doses. These results suggested that prochloraz inhibits the conversion of P to T through the inhibition of CYP17. Prochloraz had no effect on testicular CYP17 gene expression (mRNA levels) but CYP17 hydroxylase activity was significantly inhibited when tested in vitro ($K_i = 865$ nM). Amniotic fluid prochloraz concentrations ranged from 78 to 1512 ppb and T production was reduced when prochloraz reached approximately 500 ppb, which compared favourably with the determined CYP17 hydroxylase K_i (326 ppb).

Study quality and assessment: The study is well-described and results demonstrate that prochloraz lowers testicular T synthesis by inhibiting CYP17 activity which likely contributes to the induced malformations in androgen-dependent tissues of male offspring. Overall the quality of the study is assessed to be high, and the study provides strong evidence for decreased testicular T synthesis and thereby an anti-androgenic MoA of prochloraz.

Laier et al. (2006)

Summary: Prochloraz was administered orally to pregnant rats at doses of 50 and 150 mg/kg/day, from gestational day 7 to postnatal day (PND) 16. Male and female offspring were examined, a subset of foetuses were examined after Caesarean section of dams at gestational day 21.

Prochloraz caused mild dysgenesis of the male external genitalia, reduced ano-genital distance (AGD) and retention of nipples in male pups. In female pups AGD was increased AGD. In male foetuses, testicular and plasma levels of testosterone (T) were decreased and levels of progesterone (P) increased. Immunohistochemistry of fetal testes showed increased expression of 17 α -hydroxylase/17,20-lyase (P450c17) and a reduction in 17 β -hydroxysteroid dehydrogenase (type 10) expression. Increased expression of P450c17 mRNA was observed in fetal male adrenals, and the androgen-regulated genes ornithine decarboxylase, prostatic binding protein C3 as well as insulin-like growth factor I mRNA were reduced in ventral prostates at PND 16. These results indicate that reduced activity of P450c17 may be a primary cause of the disrupted fetal steroidogenesis and that altered androgen metabolism may also play a role

Study quality and assessment: The study is well-described and evaluates prochloraz at high doses. The study is of moderate quality as it uses small group sizes 5-8 litters per group. This study find decreased AGD (6-12%) in the males (from 50 and 150 mg/kg group respectively). This decrease in male AGD has not been seen in several other studies (Christiansen et al. 2009, Noriega et al 2005; Vinggaard et al. 2005; Melching-Kollmuss et al. 2017). The effects on NR and/or increase in female AGD have also been seen in several studies (Christiansen et al. 2009; Vinggaard et al 2005; Melching-Kollmuss et al 2017). Overall the study provides strong evidence for adverse effects such as dysgenesis and increased nipple retention and thereby an anti-androgenic MoA of prochloraz.

Noriega et al. (2005)

Summary: Prochloraz was administered by gavage to pregnant rats at doses of 31.25, 62.5, 125, and 250 mg/kg/day from gestational day 14 to 18. Prochloraz delayed delivery in a dose-dependent manner and resulted in pup mortalities at the two highest doses. In male offspring, anogenital distance (AGD) adjusted for body weight was not affected, but in females adjusted AGD was increased at 250 mg/kg. In females VO (vaginal opening) was unaffected. Nipple retention (NR) was observed in males at 13 days of age at frequencies of 31%, 43%, 41%, and 71% in the lowest-dose to highest-dose groups, respectively. Weights of sexual accessory tissues showed dose dependent reductions. Hypospadias and vaginal pouches were noted in all males treated with 250 mg/kg, whereas those defects were observed in 12.5% and 6.25%, respectively, of males treated with 125 mg/kg. Treatment did not affect age of PPS in animals without penile malformations. Despite severe malformations in males, no malformations were noted in females.

Study quality and assessment: The study is well-described and evaluates prochloraz at high doses. The study is of moderate quality as it uses very high doses (general toxic doses). This study find decreased absolute AGD at 125 and 250 mg/kg, but no effect on AGD when body weight was included as covariate (which is normally done). The effects on increased NR seen at 125 and 250 mg/kg, but not at the lower doses 31 and 63 mg/kg have also been seen in several other studies (Christiansen et al 2009; Vinggaard et al 2005; Melching-Kollmuss et al 2017). Overall the study provides strong evidence for adverse effects such as increased nipple retention and thereby an anti-androgenic MoA of prochloraz.

Vinggaard et al. (2005)

Summary: Prochloraz was administered orally to pregnant rats at a dose of 30 mg/kg/day, from gestational day 7 to postnatal day (PND) 16. Male and female offspring were examined, a subset of foetuses were examined after Caesarean section of dams at gestational day 21. Gestational length was increased by prochloraz. Plasma and testicular T levels in gestational day 21 male foetuses were reduced. Chemical analysis of the rat breast milk showed that prochloraz was transferred to the milk. In males nipple retention was increased, and the bulbourethral gland weight was decreased, whereas other reproductive organs were unaffected. CYP1A activities in livers were induced by prochloraz. Behavioural studies showed that the activity level and sweet preference of adult males were significantly increased.

Study quality and assessment: The study is well-described and evaluates prochloraz at lower doses and in many litters per group (N=16). The study is of high quality and find increased number of retained nipples and also behavioural effects. Overall these results provide strong evidence for

adverse effects and that prochloraz feminizes the male offspring after perinatal exposure. Moreover this study indicates that these effects are due, at least in part, to diminished fetal steroidogenesis.

Prochloraz has been tested in a full set of regulatory toxicological studies including two multi-generation reproductive toxicity studies, which was performed according or comparable to the US EPA OPPTS 870-3380 or OECD TG 416 (Two generation test) These TG 416 studies was made before the update in 2001, so none of them were to current standards (OECD, 2012b)

These regulatory toxicological studies are summarized below and in the DAR of Prochloraz and in EFSA conclusion (EFSA, 2007; EFSA, 2011).

Prochloraz was evaluated in two key two-generation toxicity studies from 1993 and 1982 where overall reproductive performance was impaired following prochloraz administration to rats (Cozens et al. (1982) as summarized in DAR and Reader et al. (1993) as summarized in EFSA conclusion). Effects on reduction in body weight and body weight gain, increased liver weight and deaths were associated with dystocia and extended gestation length. Developmental toxicity was observed as reduced mean litter size, increased total litter loss, reduced live birth index, impaired growth and adverse effects on organ weights. In the 1993 study the agreed parental and reproductive NOAEL was 50 ppm (2.26 mg/kg bw/d), and the offspring NOAEL is 150 ppm (6.58 mg/kg bw/d). In the study from 1983 the agreed parental NOAEL is 150 ppm (13 mg/kg bw/d), the reproductive NOAEL is 37.5 ppm (3.1 mg/kg bw/d), and the offspring NOAEL is 150 ppm (13 mg/kg bw/d) (EFSA conclusion 2011).

In the developmental toxicity studies, there was no evidence of teratogenicity, and the relevant maternal and developmental NOAELs are 25 mg/kg bw/d for the rat and 40 mg/kg bw/d for the rabbit.

Prochloraz has been tested in these regulatory toxicological studies as summarised above. These guidelines, however, precede OECD harmonization lack specific parameters to sensitive to endocrine disruption and to identify anti-androgenicity (e.g., sperm parameter, onset of puberty, AGD and nipple retention). The effect on dystocia was the only endocrine endpoint found in these studies.

As reported in published papers (some of them mentioned above), rats displayed typical signs of anti-androgenicity after treatment with prochloraz, like retained nipples, reduced testosterone, increased progesterone levels, reduced male reproductive organ weights and delayed entries into male puberty (Blystone et al. 2007; Laier et al. 2006; Vinggaard et al. 2002). A NOAEL identified for the most sensitive endpoint retained nipples was 5 mg/kg bw/day in rats was determined by Christiansen et al. (2009). A summary of the findings seen in both regulatory studies and in *in vivo* studies published in peer-reviewed journals is provided in GD 181 (OECD 2012b) and the review by Melching-Kollmuss et al. (2017).

4.10.3.4 Summary of the plausible link between adverse effects and endocrine mode of action

Prochloraz is recognized as an endocrine disrupter as there is strong evidence from *in vitro* assays that the substance acts by multiple mechanisms of action in non-target species including inhibition of enzymes of steroidogenesis (CYP 19, CYP 17 and 5 α -reductase) and AR antagonism. Moreover, *in vivo* studies have shown adverse effect on male reproductive development as development of several androgen-dependent tissues has been affected as summarized below.

Prochloraz has been shown to react through several endocrine disrupting mechanisms (Vinggaard et al., 2005; Vinggaard et al. 2006). Moreover *in vivo* prochloraz can affect the development of several androgen-dependent tissues (Vinggaard et al., 2002; Vinggaard et al. 2005, Laier et al. 2006, Taxvig et al. 2008). Common features for the azole fungicides are that they increase gestational length and affect steroid hormone levels in fetuses and/or dams. In the majority of studies, male offspring, exposed *in utero* to prochloraz often showed no statistically significant changes in anogenital distance (AGD) with doses from 25-150 mg/kg but find significant nipple retention (NR) (Vinggaard et al. 2005; Christiansen et al. 2009; Noriega et al. 2005 and Melching-Kollmuss et al. 2017). One study has found both a decrease in male AGD at 50 and 150 mg/kg and also increased NR (Laier et al. 2006). Several studies find an increase in female AGD after *in utero* exposure to prochloraz (Laier et al. 2006; Melching-Kollmuss et al. 2017).

In addition to its anti- androgenic MoA, interference with testosterone synthesis and steroidogenic MoA, prochloraz has also been shown to affect thyroid hormone levels and cause effects on the sexually dimorphic development of the brain (Vinggaard et al. 2005).

The results of several *in vitro* assays, which have been published over the last 15 years, point out that prochloraz has endocrine mode(s) of action. These *in vitro* screening studies were carried out in various test systems such as steroid hormone synthesis assay (e.g., OECD TG 456 (2011)). And in the final TG 456 prochloraz is used as a positive control as it is a strong inhibitor of steroid hormone secretion (OECD, 2011). Prochloraz was found to be able to interact with estrogen and/or androgen receptors, with aromatase and with the steroid hormones (Andersen et al. 2002; Birkhøj et al. 2004; Grünfeld and Bonefeld-Jorgensen 2004; Hecker et al. 2006; Kojima et al. 2004; Laier et al. 2006; Sanderson et al. 2002; Trösken et al. 2004; Vinggaard et al. 2000, 2002, 2005). More recent studies suggest that prochloraz interferes with steroidogenesis in *in vitro* systems by inhibition of P450c17 (17 α -hydroxylase and 17,20-lyase) (Nielsen et al. 2012). Cortisol and corticosterone levels were shown to decrease after exposure of H295R cells to prochloraz (Winther et al. 2013; Ohlsson et al. 2010). In some of these studies also gene expression of relevant steroidogenesis genes were investigated: H295R cells exposed to 0.03 μ M prochloraz showed decreased expressions of some of the genes involved in steroidogenesis were seen (Ohlsson et al. (2009); Ohlsson et al. 2010).

Overall, this battery of *in vitro* screening studies shows that prochloraz is able to induce hormonal imbalance by affecting both the androgen and estrogen receptor, to inhibit the aromatase activity and to interfere with steroidogenesis probably at the level of P450c17.

In OECD GD 181 (OECD, 2012b) prochloraz was one of the cases used for the OECD GD 150 (OECD, 2012a). In GD 181 a review of the endocrine-related data available for prochloraz was collected (on both *in vitro*, mammals and fish) and this to evaluate whether the conclusions and next

steps recommended in the guidance document for identification of ED was sensible and helpful when assessed in light of comprehensive datasets

In GD 181 it says: *“The combined dataset indicates that the ER and AR antagonism and S disruption shown in vitro also occur in vivo in mammals and fish. The antagonist response in the Hershberger assay provides confirmation that this mechanism may act in vivo, whilst the positive results in male PP assay (Pubertal Development and Thyroid Function Assay in Peripubertal Male Rats) and fish assays suggest that A, S or E modalities may be responsible for the effects seen on endocrine endpoints. The combined dataset provides sufficient evidence of endocrine activity”*. Moreover in relation to the generation studies: *“Although the 2-generation study was negative for endocrine effects, the combined dataset provides sufficient evidence of concern for endocrine activity in mammals. NOAELs for reproduction and development could be derived from the combined dataset, thus avoiding further testing. The combined dataset indicates that the ER and AR antagonism and S disruption shown in vitro also occur in vivo in mammalian species. These effects also give cause for concern in wildlife species although the physiological consequences of the effects are likely to be different.”*

The total evidence for adverse effects of prochloraz is strong (Table 2), the evidence for an anti-androgenic MoA (incl. anti-estrogenic MoA) of prochloraz and inhibition of enzymes in steroidogenesis is strong (Table 1) and the evidence for a plausible link between the MoA and adverse effects is also strong.

In conclusion, prochloraz meet the WHO definition of an endocrine disruptor.

Table 1. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of prochloraz

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
OECD, 2012b, Review	The combined dataset provides sufficient evidence of endocrine activity.	The combined dataset indicates that the ER (estrogen receptor) and AR (androgen receptor) antagonism and S (steroidogenic) disruption shown <i>in vitro</i> also occur <i>in vivo</i> in mammals and fish. The combined dataset provides sufficient evidence of endocrine activity.	High	Strong

Table 2. Overview of potential endocrine-related adverse effects of prochloraz

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
OECD, 2012b Review	Rats	Malformed genitalia in males, altered AGD (females), caused nipple retention in males, decreased serum Testosterone, reduced sexual accessory tissue weight males, sweet preference increased males.	High	Strong

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Prochloraz,

5. Environmental hazard assessment

5.6.2 Endocrine Disruption

5.6.2.1 General approach – environment

The peer reviewed literature was investigated by use of Web of Science including all databases. Search terms included prochloraz + endocrine, prochloraz + fish, prochloraz + amphibian, prochloraz + vitellogenin. A google search including the search terms prochloraz + endocrine, prochloraz + fish, prochloraz + amphibians and prochloraz + vitellogenin were also performed and revealed the OECD case study with prochloraz (OECD GD 181, 2012)

Studies solely investigating effects of prochloraz in invertebrates were not taken into account due to lack of endocrine specific endpoints. Studies using mixtures of chemicals where the effects could not be directly related to prochloraz were also not taken into account. For studies where one of the authors was also an author of this document, the study quality was evaluated by another person.

Four studies included in Table 3 are not evaluated in the present document but were copied from the summary table of OECD GD 181 where they have been evaluated. The studies are Ankley et al., 2005, Biever et al., 2007, Jensen & Ankley, 2006 and OECD, 2006 (ring test results).

Due to the substantial amount of literature on prochloraz and endocrine disruption, only a selection of key studies in relation to identification of endocrine disruption according to the WHO/ICPS definition was evaluated. A search for fish and amphibian studies including endocrine biomarkers and endpoints but showing no endocrine effects of prochloraz was also conducted to investigate the Weight of Evidence of ED-effects of prochloraz in vertebrate wildlife. Searching prochloraz + endocrin* + vitellogenin as well as prochloraz + sex ratio and prochloraz + zebrafish and prochloraz + fathead minnow and prochloraz + Japanese medaka revealed approximately 30 studies in Web of Science related to fish and ED. None of these studies were “negative” on prochloraz ED effects.

5.6.2.2 *In vitro* information indicative of endocrine activity.

Covered by the toxicological evaluation of prochloraz.

5.6.2.3 *In vivo* effects with regard to an endocrine mode of action

Flynn et al. (2017)

Summary: The authors used a Japanese medaka (*Oryzias latipes*) multigeneration assay (modification of OECD TG 240 with much less replication and lower power of endpoints) to investigate effect of prochloraz (5.3, 9.2, 17.5, 25.0 and 41.1 µg/l mean measured) on several endpoints including ED relevant endpoints. The exposure was 29 weeks and spanned 2 full generations. The test was terminated after reproduction in the F2. Six replicates of breeding pairs (one male and one female) per concentration was used where the final OECD TG 240 used 24 pairs for controls and 12 pairs for exposures. Prochloraz did not affect the tissues and organs of male fish, but caused endocrine related pathologies in the ovaries of female fish (decreased yolk and follicular hyperplasia/hypertrophy). The LOEC for fecundity was 41µg/L in F0 and 25 µg/L in F2. The phenotypic sex ratio in prochloraz exposed fish was not discussed, possibly because of low power due to only 12 fish per exposure in F1 and F2. Vtg-gene expression was decreased in females. The authors argue that this could be plausibly

linked to aromatase inhibition, causing circulating E2 to decrease, which in turn would lower the expression of hepatic vitellogenin.

Study quality and assessment: The study is very well described and detailed but due to the test design of only 6 breeding pairs per test concentration the power of the endpoints was low. For example, the power to detect a 30% fecundity decrease was 0.38, which is very far from the desired 0.8. is strong. Based on this the study is assessed to be of moderate quality. Despite low power, the results do though support prochloraz to inhibit aromatase activity by decreasing Vtg-gene expression in females and causing ED related pathologies in the ovaries of female fish. This link is moderate-strong.

Bauman et al. (2015)

Summary: The authors investigated the persistence of effects of prochloraz exposure during sexual development of zebrafish. Fish were exposed (10, 30, 100, and 300 µg/l prochloraz) from fertilization to 60 days post hatch and transferred to clean water for 40 days thereafter. Two replicates of 100 fertilized eggs were used per concentration. Subsampling of fish was performed at day 30 and 60 post hatch and final sampling at 100 days post hatch. No chemical analysis was performed but a reference to Baumann (2012) was given because the same exposure system was used. Baumann et al (2012) is evaluated other place in this document. Survival was not described so it is difficult to assess whether prochloraz affected this. The number of sampled fish are though equal to the number of initial fish so it is expected that mortality was extremely low. Histological investigations of the gonads revealed persistent effects on sexual differentiation. The sex ratio was skewed towards males and significantly more intersex individuals were found and this decrease in phenotypic females was dose dependent with no females at 300 µg/l prochloraz. A 40-day depuration period showed that the masculinization of the fish was irreversible.

Study quality and assessment: The study is very well described and detailed and the results are dose-dependent and expected. It is not explained how survival could be close to 100% and prochloraz was not quantified so the overall quality is assessed to be medium-high. The data on the change in phenotypic sex ratio is population relevant and adverse. The link to aromatase inhibition which is expected for prochloraz in fish is strong.

Bauman et al. (2013)

Summary: Five zebrafish (*Danio rerio*) FSDTs with EDCs with different modes of action including prochloraz (CAS no. 64447-09-5) were conducted, and the experimental setups followed the OECD TG 234. Each tank contained 40 eggs (two replicates), and the prochloraz exposure started at 1 hpf and ended 60 dpf and with the following nominal concentrations: 10, 30, 100, and 300 µg/L. Chemical determination of the actual exposure concentrations was performed by LC-MS (< 20% deviation from the nominal concentration over the entire test period).

No significant effects on hatchability or mortality were observed after exposure to prochloraz, but the body size was reduced in the highest concentrations compared with the control. The results document that not only sex ratio and VTG production of the prochloraz exposed fish were massively affected, but also gonad maturation. The sex ratio was shifted towards males and intersex in the groups exposed to the two highest exposure concentrations; only male and intersex individuals were observed at 300 µg/L and the number of females was significantly reduced at 100 µg/L ($\leq 10\%$ females). The VTG response in both males and females was non-monotonic, and the only significant effect was an

increase in male VTG at 30 µg/L. At this concentration, a non-significant increase in VTG was also observed in females, whereas both male and female VTG levels tended to decrease at higher exposure concentrations, albeit not significantly. The maturation index followed the same pattern in both males and females but statistical significant differences were not observed when compared with the control.

Study quality and assessment: The experimental setup follows the OECD TG 234 and the experimental design and laboratory procedures are very well described. However, the authors state that prochloraz did not influence the mortality, but information about the exact mortality in the groups is not mentioned, and n is not provided in any of the figures or figure legends, which complicates evaluation of the data quality. Therefore, the study quality is assessed as medium-high. Clearly, it is demonstrated that prochloraz exposure during sexual development of zebrafish has an adverse effect since the sex ratio is shifted towards males at the two highest exposure concentrations. The only significant increase in VTG is observed in males in the intermediate exposure (30 µg/L). The exact mechanism is unclear but a clear endocrine MoA is demonstrated and the link to an endocrine mediated MoA is assessed as strong.

Holbech et al. (2012)

Summary: Zebrafish or fathead minnow (0-60 dph or 0-120 dph) were exposed to prochloraz (CAS no 67747-09-5) in different laboratories in order to investigate the inter-laboratory robustness of the endocrine parameters in the OECD FSDT TG 234 (whole body VTG and sex ratio) and to compare the sensitivity of the two test species. The experimental setup followed the OECD TG 234 and included two or four replicates with a minimum of 40 eggs per replicate in a flow-through system. The following nominal prochloraz concentrations were used: 32, 100, and 320 µg/l or 38, 75, 150, 300 and 600 µg/L. The exposure concentrations were determined by LC-MS or HPLC-UV. Generally, no significant effects of prochloraz on hatchability and growth was observed, but mortality was affected in a few exposure groups in some of the labs. Prochloraz induced a monotonic concentration-response reduction in VTG levels of female fathead minnows with effect at the lowest (29 µg/L at 60 dph) or intermediate concentration (106 µg/L at 120 dph). In zebrafish, a monotonic concentration-response reduction in female VTG levels was also observed with effects observed at 48, 99 or 183 µg/L, respectively. In two out of three labs a reduction in zebrafish males was also observed with effects at 44 or 135 µg/L. In both fathead minnow experiments the sex ratio was significantly skewed towards males in the highest exposure group (284 and 301 µg/L) but no effect at the intermediate concentration in any of the experiments (≈100 µg/L). The sex ratio of zebrafish was skewed towards males in a monotonic concentration-response relationship in one lab with effects at the lowest concentration (60 µg/L) and in the second lab no effect at the intermediate concentration (48 µg/L) but significantly more males and undifferentiated individuals at 320 µg/L. The third lab had significantly more males at the two intermediate concentrations (99 and 197 µg/L) but not at the highest concentration (434 µg/L), however most of the water samples from this lab were lost.

Study quality and assessment: The experimental setups are solid with regards to the number of animals, replicates, histological procedures, and chemical analysis of water samples, and the results are well described. However, based on the information in the M&M section and the figures it is unclear how many embryos each laboratory had in each replicate and the exact mortality in each experiment is not mentioned, also the number of control animals is much higher than the number in the exposure group, which is not explained. But n is generally high and it is stated the prochloraz generally did not affect the mortality. The experiment was run with the same setup and in different labs and with different strains of fish, and thereby a broad picture of the effects is obtained. The

study is assessed to be of high quality. The reduction in female VTG and number of females (adverse effect) clearly demonstrate an endocrine MoA. Prochloraz has multiple endocrine modes of action, including inhibition of aromatase activity, inhibition of androgen synthesis and AR antagonism, in fish and mammals, and based on these experiments it is not clear if the exact mechanism is anti-estrogenic, androgenic or inhibition of aromatase activity. However, the evidence for an ED MoA is assessed as strong.

Thorpe et al. (2011)

Summary: In the present study, fathead minnow (*pimephales promelas*) and zebrafish (*Danio rerio*) were exposed to prochloraz (32, 100 and 320 µg/l) from embryo until 95-125 days post hatch (DPH) and 60 DPH respectively. The exposure periods cover the sexual development of the two fish species. 2x25 embryos per replicate in 6 replicates for fathead minnow and 2x30 embryos per replicate in 4 replicates for zebrafish were used until 30 DPH where each replicate was adjusted to 30 fish by random removing excess fish to equalize fish density. Exposure to 320 µg/L inhibited somatic growth in both species. Larval survival in zebrafish was decreased from 32 µg/l where such effect was not seen in fathead minnow. Prochloraz exposure caused a decrease in the proportion of phenotypic females in both species with a zebrafish LOEC of 100 µg/l and a fathead minnow LOEC of 320 µg/L. A delay in the completion of sexual differentiation was also seen in both species.

Study quality and assessment: The study is very well described and detailed and replication is high. The quality is high. The data on the change in phenotypic sex ratio is population relevant and adverse. The link to aromatase inhibition is strong due to the decreased VTG concentration in female fathead minnow and zebrafish.

Brande-Lavridsen et al. (2008)

Summary: In this amphibian study, tadpoles of the Common frog (*Rana temporaria*) were exposed to prochloraz or EE2 from hatch through metamorphosis. In two different experiments, tadpoles were exposed to 15 and 150 µg/l prochloraz (nominal concentrations) and 11, 115 and 252 µg/l prochloraz respectively. Two replicates of 150 tadpoles were used per concentration. Exposure was semi-static with water exchange 3 times per week. Tadpoles were sampled at the end of metamorphic climax (Gosner stage 44–46). Testosterone and E2 was quantified in whole body homogenate by commercial ELISA-kits. Calcium levels was measured in whole body homogenate as a surrogate for vitellogenin. Gonadal histology was performed to investigate effects on gonads and to document phenotypic sex. Survival rates ranged between 71.3 and 84.3%. The sex ratio was significantly skewed toward males from 115 µg/l prochloraz and above and testosterone concentrations decreased in the same groups. E2 increased in the 115 µg/l group but not at the highest exposure concentration of 252 µg/l. Calcium was not affected by prochloraz.

Study quality and assessment: The study is well described. The use of calcium as surrogate for vitellogenin is not recommendable so these results are not taken into account. The study is assessed to be of moderate-high quality. The results on the phenotypic sex ratio is population relevant and adverse and highly linked to an endocrine mechanism. The reduced T concentrations could be caused by different mechanisms but is not directly linked to aromatase inhibition.

Kinnberg et al. (2007)

Summary: The objective of this study was to assess the effects of prochloraz on the sexual development of zebrafish (*Danio rerio*) exposed to prochloraz (Information about purity and CAS no. not provided) for 60 days from 24 h post fertilization (0, 16, 64 or 202 µg/l). The experimental setup followed the OECD TG 234 and included two replicates per exposure with 80 eggs per replicate in a flow-through system. The exposure concentrations were determined by LC-MS. The sex ratio was significantly skewed towards males in the highest exposure group and this group also had a significantly higher number of intersex individuals. Male VTG concentrations were significantly increased at the low and intermediate prochloraz concentration and at the highest concentration both male and female VTG concentrations were significantly decreased.

By histological examination of the gonad development it was shown that females in all exposure had gonads with less developed oocytes compared with the control, and when the males of all prochloraz treatments were pooled 82% of the males had gonads in the most developed stage (stage III: abundant spermatozoa) compared with 66% in the control group. By inhibition of the aromatase activity 17β -estradiol synthesis is reduced and a decrease in female VTG and female individuals would be expected. The observed decrease in male and female VTG levels at the highest concentration is consistent with aromatase inhibition, and the enhanced sperm production in males and increased number of males could also be related to an increased androgen concentration or decreased E2/T ratio. The increase in male VTG concentration at the low and intermediate concentration may be due to the ability of prochloraz to target multiple steroidogenic enzymes, thus leading to effects elsewhere in the steroidogenic pathway.

Study quality and assessment: The experimental setup is solid with regards to the number of animals, replicates, histological procedures, and chemical analysis of water samples, and the results are well described. The mortality is approximately 50% but not different between the exposure groups and the control. The study is assessed to be of high quality. The results on the phenotypic sex ratio is population relevant and adverse. The link to aromatase inhibition is strong due to the decreased VTG concentration in female zebrafish but other endocrine MoAs cannot be ruled out.

Additional information used:

OECD Case study with prochloraz OECD GD 181

([http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono\(2012\)34&doclanguage=en](http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2012)34&doclanguage=en)).

5.6.2.4 Summary of the plausible link between adverse effects and endocrine mode of action

The evidence from the referred studies strongly support that prochloraz is an endocrine disrupter according to the IPCS/WHO definition (summarized in Table 3). It was not possible to find any study with fish that was investigating phenotypic sex ratio without finding an effect of prochloraz exposure.

5.6.2.5 Environmental relevance

Adverse effects (Table 4) in fish and amphibians are seen around and below 100 µg/l. Barret (1995) calculated the maximal predicted environmental concentration (PEC) to 195 µg/l. The PEC was based on an overspray of water 30 cm deep at a maximum recommended application rate of 0.585 kg active ingredient ha⁻¹. An EFSA risk assessment of prochloraz did though conclude a low risk of prochloraz to aquatic organisms (EFSA, 2011).

Table 3 Overview of *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of prochloraz in fish and amphibians

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Flynn et al. (2017)		Japanese medaka multi-generation test. Dosing 5-40 µg/l prochloraz. Decreased fecundity. Decreased Vtg-gene expression. ED related pathologies in the ovaries of female fish (decreased yolk and follicular hyperplasia/hypertrophy).	medium	Moderate-strong
Baumann et al. (2015)		Zebrafish exposed from 0-60 dpf. Depuration period from 60-100 dpf. Skewed phenotypic sex ratio. NOEC 30 µg/l. LOEC 100 µg/l (no females at 300 µg/l).	Medium-high	Strong
Baumann et al. (2013)		Zebrafish exposed from 0-60 dpf. The sex ratio was shifted towards males and intersex in the groups exposed to the two highest exposure concentrations; only male and intersex individuals were observed at 300 µg/L and the number of females was significantly reduced at 100 µg/L	High	Strong
Holbech et al. (2012)		Zebrafish exposed from 0-60 dpf. Fathead minnow exposed from 0-60/120 dpf. Dose-range 32-600 µg/l. Five studies were conducted as a part of the OECD TG 234 validation. Effects on sex ratio (decreased proportion of females) and Vtg protein levels were seen in all experiments.	High	Strong

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Thorpe et al. (2011)		Zebrafish exposed from 0-60 dph. Fathead minnow exposed from 0-95 or 125 dph. Dosing: 32, 100 and 320 µg/l. Phenotypic sex ratio skewed with less females. LOEC zebrafish, 100 µg/l. LOEC fathead minnow, 320 µg/l. Delayed completion of sexual differentiation in both species.	High	Strong
Brande-Lavridsen et al. (2008)		Common frog <i>Rana temporalis</i> tadpoles exposed from hatch through metamorphosis. The sex ratio was significantly skewed toward males from 115 µg/l prochloraz and above and T decreased in the same groups. E2 increased in the 115 µg/l group but not at the highest exposure concentration of 252 µg/l.	Moderate-high	Strong
Ankley et al. (2005)		From OECD GD 181: OECD TG 229: Fish Short Term Reproduction Assay (FSTRA): Dosing range = 30 -300 µg/L Fecundity ↓ >3-fold in fathead minnow (LOEC = 100 µg/L; NOEC = 30 µg/L). ♀ VTG ↓ approx. 3-fold in (LOEC = 100 µg/L; NOEC = 30 µg/L). Significant reductions were also observed in ♂ testosterone, 11ketotestosterone and brain aromatase activity, and ♀ estradiol.	High	Strong
Biever et al. (2007)		From OECD GD 181: OECD TG 229: Fish Short Term Reproduction Assay (FSTRA): Dosing range = 20 -300 µg/L Fecundity ↓ approx. 5-fold in fathead minnow (geomean LOEC = 300 µg/L; geomean NOEC = 100 µg/L).	High	strong

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Kinnberg et al. (2007)		Zebrafish exposed from 0-60 dpf. Dose-range 16-202 µg/l (measured). Effects on sex ratio (decreased proportion of females) and Vtg protein levels were seen. NOEC sex ratio 64 µg/l, LOEC 202 µg/l	High	strong
Jensen & Ankley (2006)		From OECD GD 181: Three individual studies following OECD TG 229: Fish Short Term Reproduction Assay (FSTRA): Dosing range = 20 -300 µg/L Fecundity↓ in fathead minnow (geomean LOEC = 58 µg/L; geomean NOEC = 16 µg/L). Secondary sexual characteristics (tubercle score) ↓ in fathead minnow (geomean LOEC = 144 µg/L; geomean NOEC = 34 µg/L). ♀VTG↓ in fathead minnow (LOEC = 20 µg/L; NOEC = <20 µg/L)	High	Strong
OECD (2006)		From OECD GD 181: Ring test of OECD TG 230 with fathead minnow, Japanese medaka and zebrafish: Dosing range = 20 - 300 µg/L ♀VTG↓ by up to 10-fold in medaka (geomean LOEC = 116 µg/L; geomean NOEC = 38 µg/L), fatheads (geomean LOEC = 208 µg/L; geomean NOEC = 58 µg/L), and zebrafish (geomean LOEC = 182 µg/L; geomean NOEC = 49 µg/L)2 Fecundity was reduced in medaka (4/4 labs) and zebrafish (1/2 labs).	High	Strong

Table 4. Overview of potential endocrine-related adverse effects of prochloraz in fish and amphibians.

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Baumann et al (2015)	Zebrafish	Skewed phenotypic sex ratio. No females at 300 µg/l (nominal)	Medium-high	high
Baumann et al (2013)	Zebrafish	Skewed phenotypic sex ratio.	Medium-high	high
Holbech et al (2012)	Zebrafish	Skewed phenotypic sex ratio.	high	high
Holbech et al (2012)	Fathead minnow	Skewed phenotypic sex ratio.	high	high
Thorpe et al (2011)	Zebrafish	Skewed phenotypic sex ratio.	high	high
Thorpe et al (2011)	Fathead minnow	Skewed phenotypic sex ratio.	high	high
Brande-Lavridsen et al (2008)	Rana temporalis	Skewed phenotypic sex ratio. No females at 115 µg/l and above (measured)	high	high
Kinnberg et al (2007)	zebrafish	Skewed phenotypic sex ratio.	high	high

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Triclocarban (TCC), CAS no. 101-20-2

Synonyms: Tcc

Substance type: TCC (Figure 1); 3,4,40-trichlorocarbanilide; 3-(4-Chlorophenyl)-1-(3,4-dichlorophenyl)urea).

TCC is an antimicrobial agent used widely in various personal hygiene products including soaps, toothpaste and shampoo and is used in 100-1000 tonnes per annum. TCC is remarkably stable and resistant to biological and chemical treatments, so there is also a potential for exposure by ingestion of contaminated water and/or agricultural products exposed to TCC-containing sludge (Duleba et al 2011). Several studies have reported that serum concentrations of triclocarban in humans are in the nM range (from Wu et al. 2016).

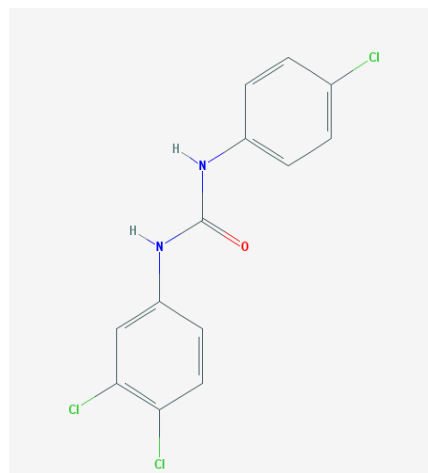


Figure 1. 2D structure from PubChem

4. Human health hazard assessment

4.10.3 Endocrine disruption

4.10.3.1 General approach – human health

4.10.3.2 *In vitro* information indicative of endocrine activity

Wu et al. (2016)

This study examined the effects of TCC on sodium/iodide symporter (NIS)-mediated iodide uptake and the expression of genes involved in thyroid hormone (TH) synthesis in rat thyroid follicular FRTL-5 cells (at concentrations from 0.01 to 1000 μM), and on the activity of thyroid peroxidase (TPO) using rat thyroid microsomes. TCC inhibited NIS-mediated iodide uptake in a concentration-dependent manner at ranges below effects on cell viability. A decrease in the iodide uptake was also observed in the absence of sodium iodide (NaI) at non-cytotoxic concentrations. Kinetic studies showed that TCC is a non-competitive inhibitor of NIS. FRTL-5 cells were also used to measure the transcriptional expression of three genes involved in TH synthesis, *Slc5a5*, *Tpo*, and *Tgo*, as well as three thyroid transcription factor genes, *Pax8*, *Foxe1*, and *Nkx2-1*. No significant changes in the expression of any genes were observed for TCC. Furthermore, TCC inhibited the activity of TPO in a concentration-dependent manner, however with a low potency, i.e. IC_{50} of $>300 \mu\text{M}$. The observations that TCC inhibited NIS-mediated iodide uptake, but did not influence the transcriptional expression levels of *Slc5a5*, the mRNA that codes for NIS, suggest that TCC may affect NIS at the post-translational level. When FRTL-5 cells were incubated with TCC in the absence of NaI, inhibition of iodide uptake was observed at as low as $0.3 \mu\text{M}$. This concentration was much lower than the IC_{50} value for iodide uptake ($17.2 \mu\text{M}$) when FRTL-5 cells were exposed to the TCC and NaI simultaneously, and this indicates that inhibition of iodide uptake by TCC is mediated primarily through regulating NIS at the post-translational level. Although TCC inhibited the TPO activity in a concentration-dependent manner, TPO seemed not a primary target of TCC, because the IC_{50} value for TPO activity inhibition ($>300 \mu\text{M}$) was higher than those for iodide uptake. In summary, TCC acted as non-competitive inhibitors of NIS and inhibited NIS-mediated iodide uptake in FRTL-5 cells. Compared to iodide uptake, TPO seemed not to be a primary target of TCC.

Study quality and assessment: TCC inhibited NIS-mediated iodide uptake in a concentration-dependent manner has been shown at ranges below effects on cell viability. Kinetic studies showed that TCC is a non-competitive inhibitor of NIS. TCC inhibited the TPO activity in a concentration-dependent manner, but compared to iodide uptake, TPO seemed not to be a primary target of TCC. Overall, the quality of the study is evaluated as high and the *in vitro* evidence for NIS inhibition is evaluated as strong.

Kolšek et al. (2015)

The effect of TCC on glucocorticoid receptor (GR) antagonistic activity was investigated using the MDA-kb2 cell line stably transfected with MMTV luciferase reporter gene. TCC at concentrations of 2 μ M was shown to enhance hydrocortisone (HC) (500nM) induced luciferase activity to ~156% compared to control (DMSO 100%). The same effect was seen when investigating TCC androgen receptor (AR) antagonistic activity. 2 μ M TCC enhanced dihydrotestosterone (DHT) induced signal to ~166% when co-administrated with 0.5 nM DHT.

Study quality and assessment: The study is well-written although some details on methods are described in the results section. The chosen assays appear to be well performed, e.g. the tested concentrations of TCC were below cytotoxicity and each experiment was at least performed in duplicates. However, a study has shown that TCC stabilizes the luciferase enzyme, which may have confounded the read-outs from the AR reporter gene assay. The study is thus assessed to be of medium quality. This study provides weak evidence that TCC acts as a modulator of the GR and AR activity.

Huang et al. (2014)

TCC exerted estrogenic activity by inducing luciferase activity in an ER reporter gene assay, promoting the proliferation of human mammary carcinoma MCF-7 cells, up-regulating the expression of estrogen-inducible pS2 gene and down-regulating ER α expression at both mRNA and protein levels in the MCF-7 cells. Furthermore, TCC could alter the expression of multiple microRNAs (mir-22, mir-206 and mir-193b) in the MCF-7 cells.

Study quality and assessment: The study is well-described, all assays were performed in triplicates and included both positive and vehicle controls. However, more information on CAS-number would have been preferred. Cytotoxicity was not evaluated but most of the results including increased proliferation, increased luciferase activity, increased protein levels (of pS2) and upregulation of gene expression (of pS2) and microRNA expression levels indicate that cytotoxicity is not present at the concentration tested. Similar TCC concentrations were used in all assays and cytotoxicity is not considered an issue in this study. A study has shown that TCC stabilizes the luciferase enzyme which may have confounded the read-out on luciferase activity and the results from the ER α reporter gene assay has limited scientific value. The study is assessed to be of medium quality. The study provides moderate evidence of an estrogenic mode of action *in vitro*.

Tarnow et al. (2013)

In a MDA-kb2 reporter cell line transfected with a stable MMTV.luciferase.neo reporter gene construct, $1\mu\text{M}^1$ TCC was shown to induce androgen-mediated amplification of luciferase-activity by ~40%. $1\mu\text{M}$ of TCC was also shown to induce estrogen-mediated amplification of luciferase-activity by ~50% in HeLa9903 cells stably transfected with human estrogen receptor (ER) α and an estrogen responsive element (ERE) driven luciferase reporter gene. TCC did not affect the transcription of genes known to be regulated by androgen receptor (AR): specifically androgen-regulated gene protein (SARG), N-myc downstream regulated 1 (NDRG1) and sorbitol dehydrogenase (SORD). Also, no effect of TCC was seen on estrogen responsive genes in human mammary carcinoma MCF-7 cells co-exposed to 17 β -estradiol, Bisphenol A, butylparaben or genistein. The induced estrogen mediated enhancement of luciferase-activity was further investigated using an assay (E-screen), where estrogen-dependent cell proliferation is used as the endpoint. These findings show that treatment with E₂ resulted in a dose-dependent increase in MCF-7 cell number, however addition of $1\mu\text{M}$ TCC was not able to further enhance estrogen-dependent cell proliferation, as was seen with the ~50% enhanced luciferase activity in HeLa9903 cells. Due to the conflicting results, the effect of TCC on firefly luciferase enzyme heat stability was examined using thermal shift. The thermal shift assay showed a stabilizing effect of TCC (above $5\mu\text{M}$) on the luciferase enzyme, which was specifically enhanced in the presence of adenosine triphosphate (ATP) as enzymatic co-factor. The authors argue that in cellular assays the effective concentration is likely to be lower due to more physiological buffered conditions. Thus, these results point to the effects of TCC on ER and AR as being false positive due to a stabilizing effect of TCC on the luciferase enzyme. TCC showed a co-stimulatory effect on transcription of CYP1A1 and CYP1B1, which are classical target genes of the transcription factor aryl hydrocarbon receptor (AhR). The authors argue that TCC interfere with AhR, which are connected to ER α via complex regulatory crosstalk, and interference due to AhR-ER crosstalk may lead to adverse outcomes (reviewed in Tarnow et al. 2013).

Study quality and assessment: The study is well described and the authors appear to be thorough in the methods used. Each assay was performed in triplicates, they include positive and negative controls, and they verified the presence and absence of relevant receptors (e.g. AR and ER) in the cell lines. As discussed above, TCC stabilizes the luciferase enzyme and this may have confounded the read-out on luciferase activity and the results from the AR reporter assay and the ER reporter gene assay have limited scientific value. Moreover, they did not test for cytotoxicity. Finally, more information on CAS number and purity of the substances would have been preferred. The study is assessed to be of medium quality. The study provides weak evidence of an endocrine disrupting mode of action of TCC.

Duleba et al. (2011)

Summary: Luciferase reporter plasmids containing probasin (probasin-luc) or three repeats of the androgen response element ligated to the luciferase reporter (ARE-luc) was transfected into LNCaP and C4-2B cells (human prostate cancer cell lines). The transfected cells were exposed to testosterone (T) (1nmol/L), dihydrotestosterone (DHT) (1nmol/L), TCC ($1\mu\text{mol/L}$) or a combinational treatment of TCC + T or TCC + DHT. Furthermore, the signal transduction independency via androgen receptor (AR) was tested using the AR inhibitor, bicalutamide. T and DHT significantly increased luciferase activity in both LNCaP and C4-2B cells containing ARE-luc or probasin-luc. TCC alone had no effect on luciferase activity, however in combination with androgens, the luciferase activity was further

¹ corresponds to the maximal levels realistically expected in human blood – stated by the authors

increased by 221% (probasin promoter, DHT) and 175% (ARE promoter, T) in LNCaP cells compared to androgen treatment alone. The same enhancement of luciferase activity was seen in C4-2B cells where TCC + androgen co-treatment further enhanced luciferase activity by 25.9% (probasin promoter, DHT) and 38.5% (ARE promoter, T) compared to androgen treatment alone. Furthermore, the AR binding inhibitor, bicalutamide, significantly suppressed the enhanced effect of TCC and androgens down to levels comparable to when TCC was administered alone.

Study quality and assessment: The study is well-described and is assessed to be of high quality. However, a study has shown that TCC stabilizes the luciferase enzyme which may have confounded the effect. This study provides moderate evidence for amplification of androgen activity.

Hinther et al. (2011)

Summary: This study assessed the effects of TCC (10, 100 or 1000 nM), alone or in combination with triiodothyronine (T3) (10 nM), on thyroid hormone (TH) signaling and cellular stress using the cultured frog tadpole tail fin biopsy (C-fin) assay and the TH-responsive rat pituitary GH3 cell line. mRNA abundance of TH receptor β (TR β), Rana larval keratin type I (RLKI), both indicating a TH response was measured in the C-fin assay. The TH-responsive gene transcripts encoding growth hormone (Gh), deiodinase I (Dio1), and prolactin (Prl) were measured in the GH3 cells. In the C-fin assay, TCC alone significantly decreased RLKI transcript levels ($p = 0.021$) at the highest concentration (1000 nM) but did not affect TR β transcript levels. In the presence of T3, TCC did not affect TR β or RLKI steady-state transcript levels. In the GH3 cells, TCC (1000 nM), alone or in the presence of T3, significantly reduced the levels of TH-responsive gene transcripts: Gh, Dio1 and Prl.

Study quality and assessment: This study has been criticized by Fort et al. (2011) and DeLeo et al. (2011) for inadequate/wrong citations and the applied method, respectively. DeLeo et al. (2011) further noted that since TCC induced down-regulation of RLKI, but did not alter TR β , TCC should not necessarily be considered a disruptor of thyroid axis without further evidence. Overall, the quality of the study is evaluated as low and the evidence of TH MoA is evaluated as weak.

Christen et al. (2010)

Summary: MDA-kb2 cells were stably transfected with murine mammalian tumor virus (MMTV)-luciferase. TCC was analyzed in concentration that was previously shown not to be cytotoxic. In the analysis of anti-androgenic activity the MDA-kb2 cells were co-exposed to dihydrotestosterone (DHT) and TCC (+ a solvent control (negative control) and DHT (positive control)). Luciferase activity was measured after 24 h of incubation. TCC showed cytotoxicity at concentrations higher than 5 μ M. TCC showed no androgenic activity in itself, but potentiated the DHT response (0.5nM) up to 130% at 0.01-5 μ M TCC. Addition of 10 μ M flutamide inhibited the induction of this response, thereby excluding that the potentiation is due to/originates from co-activation of the glucocorticoid receptor (GR) that can also stimulate the expression of luciferase by TCC.

Study quality and assessment: The study is well-described and is assessed to be of high quality. However, a study has shown that TCC stabilizes the luciferase enzyme which may have confounded the effect. This study provides moderate evidence for amplification of androgen activity.

Chen et al. (2008)

Summary: The androgen and/or anti-androgenic activity of TCC was investigated using human embryonic kidney 293 (2933Y) cells, which were transfected with PCDNA6-human androgen receptor (AR) and MMTV-Luc.neo plasmid containing a luciferase reporting gene. No cell cytotoxicity was detected for TCC when administered alone or in combination with 0.125 nM testosterone (T). When administered alone TCC (1.0 μ M) showed no effect on transcriptional luciferase activity, however in combination with T (0.125nM), TCC amplified the T-induced transcriptional activity by 45%. This amplifying effect was both time and dose dependent. Flutamide, a competitive inhibitor of androgen binding to the AR, suppressed this amplification effect of 1.0 μ M TCC at a flutamide concentration of 10 μ M.

TCC was tested in a competitive binding assay at multiple concentrations up to 200 μ M, but showed no competitive binding to the AR. Also, TCC did not activate cyclic adenosine monophosphate (cAMP)/ protein kinase A (PKA)-mediated luciferase activity or enhanced the signal transduction induced by human chorionic gonadotropin (hCG).

The effect of TCC on AR protein expression was investigated by western blot analysis. MDA-kb2 and 2933Y cells were treated for 48h with either 0.1 nM T, 1.0 μ M TCC or a combination of T (0.1 nM) and TCC (1.0 μ M). Subsequently, western blot analysis was conducted on cell lysates. Results show that treatment with T as well as T+TCC increased AR immunoreactive (AR-ir) protein in both cell lines compared to vehicle control. In the MDA-kb2 cells TCC + T resulted in more AR-ir protein compared to treatment with T alone. However, in 2933Y cells no statistical difference on AR-ir protein was observed between the T and T+TCC treatment. The authors state that this difference could be due to the inherent differences between the exogenous AR in the 2933Y cells and the endogenous AR MDA-kb2 cells.

Study quality and assessment: The study is well-described and is assessed to be of high quality. However, a study has shown that TCC stabilizes the luciferase enzyme which may have confounded the results. This study provides moderate evidence for amplification of androgen activity in two different in vitro assays.

Ahn et al. (2008)

Summary: 1 μ M TCC was shown to significantly enhance estradiol (E_2) induced luciferase activity in recombinant human ovarian cells [BG1Luc4 E_2 , ER- α -positive] containing estrogen receptor (ER)-responsive firefly luciferase reporter gene plasmid, pGudLuc7ERE, when E_2 was co-administrated at concentrations of 1-10 nM. Furthermore, TCC (1 μ M) enhanced testosterone dependent induction of AR-mediated luciferase gene activity in recombinant human cells [T47D-androg-responsive-element (ARE)] containing AR-responsive firefly luciferase reporter gene plasmid, pGudLuc7ARE, but only at testosterone concentrations of 10 μ M (the highest concentrations). TCC showed weak ER activity at concentrations of 1-10 μ M.

Study quality and assessment: The study is well-described and is assessed to be of high quality. However, a study has shown that TCC stabilizes the luciferase enzyme which may have confounded the effect. This study provides moderate evidence for weak ER activity.

4.10.3.3 *In vivo* effects with regard to an endocrine mode of action

Kennedy et al. (2015)

Summary: This study (from the same group as Chen et al. 2008 and Duleba et al. 2011) investigated how exposure to TCC during early life affects the trajectory of fetal and/or neonatal survival and development. Sprague Dawley rats were provided control, 0.2% weight/weight (w/w), or 0.5% w/w TCC-supplemented chow through a series of 3 experiments that limited TCC exposure to critical growth periods: 1) gestation, 2) gestation and lactation, or 3) lactation only (cross-fostering). In the first experiment, the level of TCC was measured in serum and amniotic fluid of gestation day (GD) 19 in dams fed from GD5-19 with either TCC-supplemented (n=5 in each group) or standard chow (n=4). They also assessed circulating hormone levels (E₂, progesterone, T, triiodothyronine (T3), thyroxine (T4) and thyroid-stimulating hormone (TSH)), number of implantation sites, systemic (liver, kidney and adrenal) organ weights, and sex organ (ovary) weights and histology. The only observed effect from TCC exposure was a decreased T3 level in the 0.5% TCC group. In the second experiment, the effect of *in utero* and lactational exposure to TCC on neonate survival was first examined by feeding dams either standard or TCC-supplemented chow from GD5 until weaning at postnatal day (PND) 21 (n=5 in each group). While pups born and raised by control rats survived beyond weaning, pups born and raised by TCC-treated dams did not survive beyond PND8. Histological examinations of mammary glands collected from the TCC-treated dams showed evidence of increased lobule separation by interstitial mature fat, increase in epithelial vacuolation with fat and thinning of epithelial height. To further investigate if the effect of TCC exposure on mammary tissue and reduced lactation was involved in the reduced neonatal survival a new study was performed. Here daily estimates of the size of milk band on pups were used as an indicator for the amount of milk consumed by pups. After PND3 a decrease in milk band size was observed in the pups born/raised by dams receiving 0.5% TCC compared to control pups born/raised by control dams. The concentration level of TCC in dam milk and serum from dams and pups was measured during lactation. Interestingly, the level of TCC in the milk was almost four times higher than serum levels of TCC in the dams. Finally, in the third experiment, the effect of TCC exposure during lactation on the survival of F1 female pups was investigated in a cross-fostering study. The results from this study show decreased survival and body weight as well as distended abdomen and diarrhea of pups raised by TCC-treated dams compared to pups raised by control dams, regardless of their *in utero* exposure. No effect of TCC was seen on anogenital distance (AGD), vaginal opening (VO) date, or first date of estrus after VO in any of the exposure scenarios.

Study quality and assessment: The quality of the study is evaluated as low due to the low number of litters per group (N=3-5). Thus, the lack of effect of TCC on anogenital distance, vaginal opening (VO) date, or first date of estrus after VO as well as the observed decrease of T3 level do not provide evidence for absence or presence of adverse endocrine effects of TCC.

Duleba et al. (2011)

Summary: This study was an extension of the study by Chen et al. 2008. In this study the same group of researchers examined whether TCC induces effects on intact peripubertal male rats. Twenty-four intact male SD rats aged 48-52 days were randomly assigned into two groups with 12 rats in each group: a control group receiving normal standard diet, and a TCC group receiving 0.25% (2500 ppm, 2500 mg/kg diet) TCC in the diet for 10 days. Rats receiving TCC in the diet had a significantly higher absolute and relative weight of all accessory sex organs except from the testes compared to

control rats. The relative weight of the seminal vesicle was increased by ~38%, ventral prostate by ~25%. Levator ani-bulbocavernosus (LABC) muscle by ~126% and glands penis by ~29%. The absolute and relative liver weight was also significantly increased by ~14% and ~8%, respectively, and post-treatment body weight (BW) was non-significantly increased by ~5% when compared to the control group. This increased organ weight seen in TCC treated animals was also applicable for the seminal vesicle, LABC muscle and glands penis when looking at dry weight. The kidney also had a small but significant increase in dry weight even though this significant increase was not seen in the other weight measures. Furthermore, the TCC group also had significantly higher protein and DNA content (mg/organ) of the ventral prostate, LABC muscle and glands penis compared to the control group. When looking at water content of the organs only the LABC muscle had a small but significant increase in % water. No effects on serum T and luteinizing hormone (LH) levels were measured, and no visible abnormalities or histological differences of accessory sex organs were observed between treated and control rats.

This paper is included in the REACH registration dossier for Triclocarban

Study quality and assessment: The study is well-described and used a sufficient number of males per group. The study included only two dose groups, which is a limitation with regards to assessment of dose-response relationship. The study is therefore evaluated to be of medium quality. The study provides strong evidence for endocrine activity, i.e. androgen activity, in intact peripubertal male rats. The marked effect on male reproductive organ weights, in the absence of significant effects on body weight, is suggestive of either cell hypertrophy or hyperplasia. Ventral prostate, LABC and glands penis had significantly increased protein and DNA content indicating that exposure to TCC resulted in increased number of cells per organ, i.e. hyperplasia. The lack of histological differences between accessory sex glands from treated and control rats suggest that the increased growth associated with TCC was proportional in the epithelium and surrounding parenchyma of each organ. Overall, this study is evaluated to provide moderate evidence for adverse reproductive toxicity effects of TCC.

Chen et al. (2008)

Summary: The potential amplification effect of TCC on androgen ligands was investigated *in vivo* using an established and widely used rat model (corresponding to the Hershberger assay) to investigate androgenic/anti-androgenic effects of substances on accessory sex tissue. Castrated 48-52 days old male Sprague Dawley (SD) rats (n=12) were treated for 10 days with sc. injections of testosterone propionate (TP) (0.2 mg/kg bw), TCC (0.25% in the diet, corresponding to 2500 ppm or 2500 mg/kg in the diet), and a combination of TP injections and TCC-supplemented diet. The control group received vehicle (sesame oil) and normal diet. There were no effects of the treatments on body weights. TP treatment alone significantly increased the weight of all accessory sex organs, except from glands penis, compared with the control group and with the group receiving TCC alone. Rats receiving only TCC showed no effect on weight of the seminal vesicles, Cowper's gland, Levator ani-bulbocavernosus (LABC) muscle and glands penis, however a slightly but significantly increased liver weight (~17%) was observed, as well as an increased ventral prostate weight (~47%), when compared to the control group. The co-treatment of TCC and TP showed an additional increase in the weight of all accessory sex organs (~78% for the seminal vesicles, ~67% for the ventral prostate, ~35% for the gland penis, ~65% for the Cowper's gland and ~13% for the LABC muscle) when compared to the effect of TP treatment alone.

Study quality and assessment: The study is well-described, used an established rat model (corresponding to the Hershberger assay) and is evaluated to be of high quality. This study provides strong evidence for endocrine disruptive activity, i.e. amplification of androgen activity, in castrated rats.

REACH registration dossier

Summary: Two potentially relevant studies are mentioned. In the first study, Sprague-Dawley rats (10 rats/sex/group) were dosed with 25% aqueous solution of TCC at 0, 500 or 1000 mg/kg bw by intubation 5 days per week for a thirty day period. Food consumption and weight gain were recorded weekly and observations were made for outward symptoms of toxicity such as reduced activity and non-grooming. At the end of the 30 day period, representative animals from each group were sacrificed. The viscera of the 1000 mg/kg bw and control groups were examined microscopically and saved for possible future examination. Macroscopic examination was made of mounted tissue from liver, kidneys, gonads, adrenals, brain, heart, and lungs. No effects of 1000 mg/kg bw was found on food consumption, growth data, and at the macroscopic tissue examination.

Study quality and assessment: The quality of the study is evaluated as high with regards to the number of doses and animals per dose group. However, the endpoints included are not likely to be sensitive to endocrine disruption and especially the performance of only macroscopic examination of the gonads is of very limited value. Overall, the quality of the study is evaluated as low due to the limited endpoints included. The study does not provide evidence for absence or presence of adverse endocrine effects of TCC.

The second study included in the robust study summary refers Duleba et al. 2011 (see above under the heading Duleba et al 2011).

4.10.3.4 Summary of the plausible link between adverse effects and endocrine mode of action

A vast amount of *in vitro* studies indicate that when administered alone TCC show no estrogenic (Huang et al. 2014, Ahn et al. 2008, Tarnow et al. 2015) or androgenic (Christen et al. 2010, Duleba et al. 2011, Ahn et al. 2008, Tarnow et al. 2015, Chen et al. 2008) activity in itself, when using luciferase activity as the indirect read out. When co-administered together with androgens (T or DHT) or estrogens (E₂), TCC induced both androgen- and estrogen-mediated amplification of luciferase activity (Christen et al. 2010, Duleba et al. 2011, Ahn et al. 2008, Tarnow et al. 2015, Chen et al. 2008). The androgen receptor (AR) inhibitors, flutamide and bicalutamide, significantly suppressed the enhanced effect of co-administered TCC and androgens to the levels comparable to control or when TCC was administered alone (Chen et al. 2008, Christen et al. 2010, Duleba et al. 2011). The amplifying effect of TCC is supported by *in vitro* results on AR protein expression, where co-exposure to TCC + T resulted in a higher expression of AR-ir protein compared to treatment with T alone (Chen et al. 2008). The TCC amplified estrogen-mediated activity from the luciferase activity assay could not be reproduced using the E-screen assay. In contrast another study found that TCC has estrogenic properties in concentrations ranging from 1x10⁻⁹-1x10⁻⁶M, and that co-treatment with an ER antagonist inhibited the effect of TCC. Furthermore, mRNA analysis using qPCR showed that two estrogen-responsive genes were upregulated (pS2) and downregulated (ER α), respectively, when exposed to TCC, which was confirmed when measuring the protein levels of pS2 and ER α . Studies on micro-RNAs (mir-22, mir-206 and mir-193b), which have recently been identified as potent regulators of ER α in the MCF-7 cells, showed that TCC (and E₂) upregulates the expression of these. A study has shown that TCC stabilizes the luciferase enzyme. This stabilization may explain why estrogenic effects of TCC were seen in the luciferase assay, whereas no effect of TCC was observed in the E-screen or in levels of estrogenic responsive genes. The same confounding effect may explain why TCC with androgens amplified AR activity in the luciferase assay, and is supported by results showing that TCC does not affect transcription of AR regulated genes. TCC showed co-stimulatory effect on transcription of CYP1A1 and CYP1B1, which are classical target genes of the regulon² of the aryl hydrocarbon receptor (AhR). This may indicate that TCC interferes with AhR and thereby indirectly affects ER α activity due to AhR-ER crosstalk.

In conclusion, TCC show no estrogenic or androgenic activity in itself in the above mentioned *in vitro* studies (table 1). When co-administered together with androgens (T or DHT) or estrogens (E₂), TCC have induced amplification of both androgen- and estrogen-mediated activity in many studies. However, a confounding effect due to TCC-induced stabilization of the luciferase enzyme may partly explain the activity in luciferase assay. Thus, the *in vitro* evidence for estrogenic and androgenic MoA of TCC is evaluated as moderate.

In vivo studies have shown effects of TCC corresponding to the enhancing effects of androgen and estrogen induced activity seen *in vitro* (Duleba et al. 2011, Chen et al. 2008) (table 2). Investigations in castrated males and intact peripubertal rats show that in presence of androgens, either exogenous or endogenous, TCC further increases the weight of all accessory sex organs compared to the effect of androgen treatment alone (Chen et al. 2008) or control rats (Duleba et al. 2011). Thus, there is both data showing *in vivo* androgenic MoA of TCC as well as effects typically induced by androgens.

² A regulon is a group of genes that are regulated as a unit, generally controlled by the same regulatory gene that expresses a protein acting as a repressor or activator.

The *in vivo* evidence for endocrine MoA is evaluated as strong and moderate evidence for adverse reproductive toxicity effects of TCC.

There is a strong biologically plausible link between the adverse effects in peripubertal male rats and the enhancing effects of androgen and estrogen induced activity seen *in vitro* and especially the androgen MoA seen *in vivo*.

There is *in vitro* evidence for thyroid disturbing MoA of TCC, especially inhibition of NIS. Only one *in vivo* study of low quality has investigated relevant endpoints for thyroid effects and found decreased triiodothyronine (T3) in the absence of effects on thyroxine (T4) or TSH. This does not provide *in vivo* evidence for thyroid effects of TCC. As the *in vivo* evidence is evaluated as absent/weak, TCC is not evaluated to fulfil the WHO-definition for being considered as an ED with a thyroid MoA. However, TCC is evaluated as suspected ED with a thyroid MoA based on the *in vitro* data.

There are two studies on MoA related to effects on the thyroid axis. Indications of thyroid disruption have been reported by Hinthner *et al.*, 2011, but the study is evaluated to have low quality and the results appear unclear, i.e. provide only weak evidence. TCC inhibited NIS-mediated iodide uptake in a concentration-dependent manner has been shown at ranges below effects on cell viability (Wu et al. 2016). The, the *in vitro* evidence for TH MoA manifested as NIS inhibition is considered strong.

In conclusion, triclocarban meets the WHO-definition of an endocrine disruptor with an androgenic MoA.

Table 1. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of triclocarban

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Wu et al. 2016	TCC acted as non-competitive inhibitors of NIS and inhibited NIS-mediated iodide uptake in FRTL-5 cells. Compared to iodide uptake, TPO seemed not to be a primary target of TCC.		High	Strong
Kolšek et al 2015	TCC was shown to enhance hydrocortisone (HC) (500nM) induced luciferase activity to ~156% compared to control (DMSO 100%). The same effect was seen when investigating TCC androgen receptor (AR) antagonistic activity. 2 µM TCC enhanced dihydrotestosterone (DHT) induced signal to ~166% when co-administrated with 0.5 nM DHT.		Medium	Weak
Huang et al.2014	TCC exerted estrogenic activity by inducing luciferase activity in an ER reporter gene assay, promoting the proliferation of human mammary carcinoma MCF-7 cells, up-regulating the expression of estrogen-inducible pS2 gene and down-regulating ERα expression at both mRNA and protein levels in the MCF-7 cells		Medium	Moderate
Tarnow et al. 2013	TCC was shown to induce androgen-mediated amplification of luciferase-activity by ~40%. no effect of TCC was seen on estrogen responsive genes in human mammary carcinoma MCF-7 cells co-exposed to 17β-estradiol, Bisphenol A, butylparaben or genistein. The induced estrogen mediated enhancement of luciferase-activity was further investigated using an assay (E-screen), where estrogen-dependent		Medium	Weak

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
	cell proliferation is used as the endpoint. These findings show that treatment with E ₂ resulted in a dose-dependent increase in MCF-7 cell number, however addition of 1μM TCC was not able to further enhance estrogen-dependent cell proliferation.			
Duleba et al.2011	TCC alone had no effect on luciferase activity, however in combination with androgens, the luciferase activity was further increased by 221% (probasin promotor, DHT) and 175% (ARE promoter, T) in LNCaP cells compared to androgen treatment alone. The same enhancement of luciferase activity was seen in C4-2B cells where TCC + androgen co-treatment further enhanced luciferase activity by 25.9% (probasin promotor, DHT) and 38.5% (ARE promoter, T) compared to androgen treatment alone. Furthermore, the AR binding inhibitor, bicalutamide, significantly suppressed the enhanced effect of TCC and androgens down to levels comparable to when TCC was administrated alone.		High	Moderate
Hinther et al.2011	In the C-fin assay, TCC alone significantly decreased RLKI transcript levels (p =0.021) at the highest concentration (1000 nM) but did not affects TRβ transcript levels. In the presence of T3, TCC did not affect TRβ or RLKI steady-state transcript levels. In the GH3 cells, TCC (1000 nM), alone or in the presence of T3, significantly reduced the levels of TH-responsive gene transcripts: Gh, Dio1 and Plr.		Low	Weak

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Christen et al. 2010	TCC showed no androgenic activity in itself, but potentiated the DHT response (0.5nM) up to 130% at 0.01-5 µM TCC. Addition of 10µM flutamide inhibited the induction of this response, thereby excluding that the potentiation is due to/originates from co-activation of the glucocorticoid receptor (GR) that can also stimulate the expression of luciferase by TCC.		High	Moderate
Chen et al. 2008	Treatment with T as well as T+TCC increased AR immunoreactive (AR-ir) protein in both cell lines compared to vehicle control. In the MDA-kb2 cells TCC + T resulted in more AR-ir protein compared to treatment with T alone. However, in 2933Y cells no statistical difference on AR-ir protein was observed between the T and T+TCC treatment. The authors state that this difference could be due to the inherent differences between the exogenous AR in the 2933Y cells and the endogenous AR MDA-kb2 cells.		High	Strong
Ahn et al. 2008	TCC showed weak ER activity at concentrations of 1-10µM.		High	Moderate

Thyroid peroxidase (TPO), dihydrotestosterone (DHT), androgen receptor (AR), Rana larval keratin type I (RLKI), TH receptor β (TRβ), triiodothyronine (T3), testosterone (T), estrogen receptor (ER)

Table 2. Overview of potential endocrine-related adverse effects of triclocarban.

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Kennedy et al. 2015	Rats n = 3-5	The lack of effect of TCC on anogenital distance, vaginal opening (VO) date, or first date of estrus after VO as well as the observed decrease of T3 level do not provide evidence for absence or presence of adverse endocrine effects of TCC.	Low	None
Duleba et al. 2011	Rats N=12/group	The marked effect on male reproductive organ weights, in the absence of significant effects on body weight, is suggestive of either cell hypertrophy or hyperplasia. Ventral prostate, LABC and glans penis had significantly increased protein and DNA content indicating that exposure to TCC resulted in increased number of cells per organ, i.e. hyperplasia. The lack of histological differences between accessory sex glands from treated and control rats suggest that the increased growth associated with TCC was proportional in the epithelium and surrounding parenchyma of each organ.	Medium	Moderate

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Octamethylcyclotetrasiloxane (D4), CAS no. 556-67-2

Synonyms: Cyclic dimethylsiloxane tetramer, OMCTS.

Octamethylcyclotetrasiloxane (C₈H₂₄O₄Si₄) is a cyclic siloxane/ silicone with low molecular weight (Figure 1). D4 is used in the synthesis of larger silicone polymers and organosilicon as well as in consumer products such as cosmetics and personal care products, washing and cleaning products, polishes and wax blends. D4 is used in 100000-1000000 tonnes per annum. D4 is classified in ECHA as a substance suspected of damaging fertility or the unborn child (Repr. 2) and may cause long lasting harmful effects in aquatic life (Aquatic Chronic 4)).

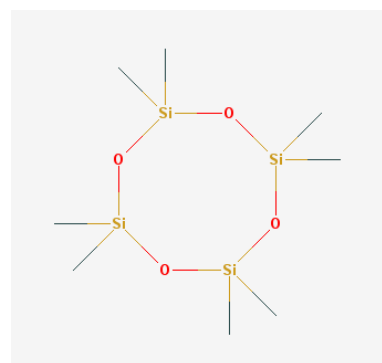


Figure 1. 2D structure from PubChem

4. Human health hazard assessment

4.10.3 Endocrine disruption

4.10.3.1 General approach – human health

4.10.3.2 *In vitro* information indicative of endocrine activity

Lee et al. (2015)

Summary: The oestrogenic activity of D4 was investigated *in vivo* and *in vitro* in the present study. The GH3 cell line (from rat pituitary) was exposed to D4 (1×10^{-5} M) or to 17 β -estradiol (E2) (1×10^{-9} M) for 1 day. Gene and protein expression levels of CaBP-9K, ER α , PR and CYP2B1 were measured in the cells. Simultaneous exposure to ICI 182-780 (ICI) was used to investigate if the effects on gene expression level were regulated by the ER mediated pathway.

CaBP-9K gene expression was increased in GH3 cells exposed to E2 or D4. When cells were exposed to ICI 182-780 in combination with D4 or E2, the gene expression level of CaBP-9K was not affected. Protein expression of CaBP-9K was slightly increased by E2 and D4 but not when ICI was administered simultaneously. Similarly, PR gene- and protein expression levels were increased by D4 and E2, an effect that was blocked by ICI. Conversely, gene- and protein expressions of ER α were downregulated by E2 and D4 and ICI blocked the effect.

Study quality and assessment: The study is well described and thorough and it is assessed to be of high quality although no CAS-number or purity of the chemical is given. The study provides strong evidence of an estrogenic mode of action of D4.

Quinn et al. (2007a)

Summary: The purpose of the study was to determine the estrogenic, progestagenic and androgenic activity of two cyclic siloxanes *in vitro* and *in vivo*. In the *in vitro* part of the study receptor binding experiments and luciferase reporter gene assays were performed for oestrogen receptor (ER) α , ER β , progesterone receptor (PR) α or PR β . Androgenic activity was investigated in an *in vivo* assay (see below). Weak binding of D4 to ER α (compared to diethylstilbestrol) was observed. D4 did not appear to bind to ER β , PR α or PR β . The ER α reporter gene assay showed activity at 10 μ M of D4, whereas no activation of the PR β reporter gene assay was observed for any of the tested doses of D4 (0.1-10 μ M).

This paper is included in the REACH registration dossier for D4 on toxicity to reproduction.

Study quality and assessment: The *in vitro* studies are well described, the tests are performed in triplicates and although it is not stated whether cytotoxicity is evaluated, the findings do not reflect results from cytotoxicity. The study is assessed to be of high quality. The study provides strong evidence of an estrogenic mode of action of D4 through binding and activation of ER α .

He et al. (2003)

Summary: The objective of this study was to characterise the estrogenic mode of action of D4 *in vivo* and to investigate the estrogenic mechanism of D4 *in vitro*. An oestrogen receptor (ER) binding assay using human ER α and ER β receptors was performed. The siloxane D4 bound competitively to ER α but not to ER β .

Study quality and assessment: The study is well-described and appears to be well-performed. The study is assessed to be of high quality. The study provides strong evidence of an estrogenic mode of action and the *in vivo* data support this conclusion.

4.10.3.3 *In vivo* effects with regard to an endocrine mode of action

Jean and Plotzke (2017)

Summary: This study presents data of a chronic toxicity and oncogenicity study of D4. In the oncogenicity part of the study, male and female rats (60/sex/group) were exposed to D4 (0, 10, 30, 150 or 700 ppm) for 6 hours per day for 24 months in whole-body inhalation chambers. Organ weights were recorded and histologic examination of organs was performed.

Uterine (absolute and relative to body weight) and testis weights (relative to body weight) were increased in animals in the 700 ppm group. An increased incidence of uterine cystic endometrial hyperplasia (from 19 % in controls to 50 % in treated females) was found in females exposed to 700 ppm D4. Although few high-dose females had cervical squamous epithelial hyperplasia and/or ovarian atrophy, the incidence of these changes (5 % and 6.7 %, respectively) was statistically significantly increased compared to controls (0 % and 1.7 %, respectively). The severity of the changes in uterus and ovaries were increased by the exposure, but no information on the statistical significance for these is given. Similarly, a modest but significant increase in the incidence of testicular interstitial cell hyperplasia was observed after 24 months of exposure in the two highest exposure groups (150 and 700 ppm).

Study quality and assessment: The study is well-described and assessed to be of high quality although it would have been preferred if the CAS-number of the substance had been given. The study provides moderate evidence of reproductive adverse effects.

Jean et al. (2017)

Summary: This study describes the effects of chronic exposure to 700 ppm of D4 on markers of reproductive senescence in female rats (49-50 weeks of age). The animals (50 per group) were exposed through whole-body inhalation from 11 to 24 months of age. Estrous cycle was monitored throughout the study and blood samples were collected monthly (for measurement of prolactin, progesterone, estradiol and corticosterone). At necropsy, blood samples were collected (FSH, estradiol and estradiol metabolites), organ weights were recorded (adrenal glands, pituitary glands, uterus, ovaries with oviducts and other non-endocrine related organs) and histopathology (of ovaries, uterus and vagina) was performed. Histological assessment of reproductive organs was also used to evaluate the estrous cycle and the types of corpora lutea and to record the number of atretic and healthy antral follicles and primordial follicles.

Increased number or percent of estrogen-predominant days (proestrous and estrous) in the estrous cycle was found in D4 treated animals compared to controls. Progesterone levels were elevated in exposed rats 2-10 weeks after start of treatment and estradiol was reduced over the total study period after exposure start. As a consequence, lower estradiol:progesterone ratios compared to controls were found in D4 treated females. Corticosterone concentrations was increased in exposed animals during the almost the entire study period. Statistical analysis of histological changes was not performed, but a marked decrease was observed in the incidence of antral-size atretic follicles and an increase in severity was observed for vaginal mucification.

Study quality and assessment: The study is well described and thorough. Although information on CAS-number and feed had been preferred, the study is assessed to be of high quality. The study provides strong evidence of an endocrine mode of action of D4 and moderate evidence for reproductive adverse effects.

Lee et al. (2015)

Summary: The oestrogenic activity of D4 was investigated *in vivo* and *in vitro* in the present study. A Uterotrophic assay in immature female rats (18 days old) dosed s.c. for 4 days (5/group) with ethinyl estradiol (EE) (3 µg/kg) or D4 (500 or 1000 mg/kg) was performed. 4 more groups of rats were co-treated with the ER α inhibitor ICI 30 min before EE or D4 administration. Animals were killed 1 day after the last dosage. Livers were weighed and gene- and expression of CYP2B1/2 was measured. Gene and protein expression was measured in uteri from the Uterotrophic assay for evaluation of the expression levels of the estrogenic biomarkers CaBP-9K, ER α and PR.

Relative liver weights were increased by D4 but not by EE. Gene expression of CYP2B1/2 was increased markedly compared to controls in the D4 dose-group in a dose-dependent manner and ICI did not change the effect significantly compared to D4. In contrast, CYP2B1/2 gene expression was decreased by EE and co-administration of ICI and EE decreased the gene expression additionally compared to EE alone. In the Uterotrophic assay, the positive control EE increased the relative uterus weight markedly. No effects on uterus weight were seen with administration of D4. Gene-and protein

expression of CaBP-9K, ER α and PR was measured in rat immature uterus. The gene expression levels of CaBP-9K were increased by EE and D4 and the effect was blocked by ICI. Protein expression of CaBP-9K was similarly induced by EE and D4 and the effect was inhibited by ICI. Both protein and gene expression levels were more elevated after EE exposure than with D4. Expression of PR mRNA was suppressed by EE and by the high dose of D4. Co-treatment of ICI further decreased PR gene expression levels in controls, in EE treated animals and in the low-dose D4 group. In the high-dose D4 exposure group ICI treatment increased PR levels compared to D4 alone. For ER α , gene expression was reduced by EE and D4, and simultaneous exposure to ICI reduced the expression level further for EE exposure and increased expression in the high D4-group compared to D4 alone.

Study quality and assessment: The study is generally well described and the Uterotrophic assay followed the OECD guideline for this test. However in the *in vivo* part of the result section there seem to be a mistake in referring to E2 instead of EE and there is conflicting description of data compared to the depicted data in a figure (PR gene expression levels in uterus). Moreover, no CAS-number or purity of the chemical is given. The study is assessed to be of medium quality. Data on the estrogenic mode of action of D4 are somewhat conflicting as some effects of D4 are similar to EE and the ER α inhibitor ICI block some of the effects of D4, but some effects are not comparable between EE and D4. Also, D4 induced no effect on uterus weight. The study provides weak evidence of an estrogenic mode of action of D4.

Meeks et al. (2007)

Summary: The aim of this study was to investigate which phases of the female reproductive cycle are affected by D4. A study design allowing investigation of the complete female reproductive cycle from pre-mating and throughout gestation was performed. Several different cohorts were performed in female rats exposed by whole-body vapour inhalation for 6 hours per day. In the first experiment, female rats were exposed to D4 (0, 70, 300, 500 or 700 ppm) from 28 days prior mating to GD19 (n=24/ group) or for a shorter period covering the ovarian phase, the fertilisation phase or the implantation phase. In these experiments, females were exposed to 700 ppm of D4 from 31 days prior to mating to 3 days before mating (n=26 and 50 pregnant dams in controls and exposed groups, respectively), from 3 prior to mating until GD3 (n=28 and 19 pregnant dams in controls and exposed groups, respectively) or from GD2-GD5 (n=23 pregnant females/ group), respectively. In the second experiment with focus on the fertilisation and implantation phases, females were exposed during 1 day only on days 1, 2, 3 or 4 prior to mating or daily either for 3 days prior to mating or from 3 days prior to mating until GD3 (n=11-40 pregnant dams). Necropsies were performed on GD20 in the first experiment and on GD8 in the second experiment. The body weight gain, number of corpora lutea (CLs) in ovaries, number of foetuses, early resorptions and implantation sites and weight of uterus, ovaries, thyroid gland, adrenal glands and brain were assessed in all the studies.

Exposure concentrations were found to be very close to the targeted exposure levels, e.g. in the 700 ppm group, mean exposure concentrations were found to range between 689 and 702 ppm in the different studies. Overall, the paper shows effects of D4 on female fertility. In the first experiment, effects on the investigated parameters were seen in the study with exposure to D4 during the fertilisation phase and in the study with exposure during the whole mating to gestation period. In the second study, decreased fertility was seen in females exposed at a time-point close to mating.

In the females exposed from before mating and throughout gestation reduced body weight gain during gestation and increased weight of adrenal glands was seen in the highest exposure group. The number of CLs was reduced by D4 in the three highest exposure-groups, but only statistically significantly in

the 300 and 500 ppm groups and increased pre-implantation loss was seen in the two highest dose-groups of 500 and 700 ppm. The number of viable foetuses was lower compared to controls as a result of lower numbers of CLs and the higher level of pre-implantation losses. The gravid uterine weight was reduced in the highest exposure group due to a lower number of viable foetuses. In the fertilisation phase, pregnant females exposed to 700 ppm of D4 had a lower body weight gain, a lower number of CLs and an increased percentage of pre-implantation loss. The number of implantation sites, the number of viable foetuses and the uterine weight were reduced. No effects were found in females exposed during the ovarian or the implantation phases. In the second study, a reduced number of pregnant dams were found among the confirmed mated females exposed to D4 1 day prior to mating. A decreased number of CLs, decreased uterine weight and an increased number of small implantation sites were seen in females exposed from 3 days before mating until GD 3.

This paper is included in the REACH registration dossier for D4 on toxicity to reproduction.

Study quality and assessment: The study is well-described and although the paper includes many sub-studies with different exposure periods it is clear how it was done, what the results are and the link between the different results is well explained. The study is assessed to be of high quality even though no CAS-number of D4 is given. The study provides strong evidence of adverse effects on female fertility around ovulation and fertilisation.

Quinn et al. (2007a)

Summary: The purpose of the study was to determine the estrogenic, progestagenic and the androgenic activity of two cyclic siloxanes *in vitro* and *in vivo*. In the *in vivo* part of the study the rat Uterotrophic and Hershberger assays for investigation of the estrogenic and androgenic activity, respectively, were performed. In both assays exposure to D4 (700 ppm) was through whole-body inhalation, 16 hours/day. In the Uterotrophic assay, exposure in Wistar and Fisher 344 rats (n=6 controls and 10 treated for each strain) lasted 3 days and in the Hershberger assay exposure of Fisher 344 rats (n=10/group) lasted 10 days.

In the Uterotrophic assay uterine (wet and blotted) weight was increased, uterus was fluid filled and had increased luminal and epithelial cell height in both strains tested. The anti-estrogenic effect was investigated, however the results are unclear. The D4 but not the ER antagonist ICI 182,789 suppressed the ethinyl estradiol induced increase in uterine weight, indicating a weak anti-estrogenic activity of D4. The Hershberger assay showed no androgenic or anti-androgenic activity of D4.

Study quality and assessment: The study is well described but the CAS-number of the substance and housing conditions are not described. The study is assessed to be of high quality. The study provides strong evidence of an estrogenic mode of action of D4 and weak evidence of an anti-estrogenic mode of action.

Quinn et al. (2007b)

Summary: The objective of the study was to investigate the effects of D4 on ovulation and on reproductive hormones, including luteinizing hormone. Two studies in female rats (13 weeks old) were performed with whole-body vapour inhalation exposure (generally 6 hours per day) to D4 at 700 or 900 ppm for 3 days from the day of diestrus until proestrus. In the first study, called phase I (n=24, 22 and 27 for controls, 700 and 900 ppm, respectively), rats were euthanized on the day of proestrus. Blood was collected for hormone analysis (Follicle stimulating hormone (FSH), 17 β -

estradiol, estrone and progesterone). In the second study, called phase II (n=138 in total at study start), blood was collected at 2, 4, 6, 8 and 10 p.m. on the day of proestrus and the animals were euthanized on the day of oestrus. The serial blood samples from phase II were analysed for luteinizing hormone (LH) and prolactin levels. On the day of necropsy body weight and weight of uterus, ovaries and brain were assessed and the number of ova in the oviducts was counted in both studies/ phases. Step sections of ovaries from phase II were evaluated histologically for assessment of large follicles, new corpora lutea and atretic antral follicles for classification of ovulators and non-ovulators. Terminal blood samples were used for hormone analysis (FSH, estradiol, estrone and progesterone).

Body weight was reduced in both treatment groups in phase I and in the 900 ppm group in phase II. The absolute ovary and relative ovary and uterus weights were reduced in the highest exposure group in phase II as well as the ovary relative to brain weight. More non-ovulating females were seen in the treatment groups (n=26, 12 and 10 ovulating females in controls, 700 ppm and 900 ppm groups, respectively compared to n=9, 19, 23 non-ovulating females in the controls, 700 ppm, 900 ppm groups, respectively). In general, plasma LH levels and the peak levels of LH were lower compared to controls, but the changes in LH peaks were related to the ovulatory status, i.e. lower mean levels of LH were related to a higher number of non-ovulators in the treatment groups. However, in ovulating females, prolactin levels were reduced at 2 p.m. in the 900 ppm group. In phase I and II, estrone hormone levels were increased in both treatment groups and in phase II, 17 β -estradiol levels were elevated in both exposure groups, both in ovulating and in non-ovulating rats. The ratio between estrone and 17 β -estradiol was reduced in the 900 ppm group in non-ovulating females. FSH was decreased in both treatment groups. In phase I, progesterone was increased in the highest exposure group on the day of proestrus. Histological assessment of phase II ovaries showed a tendency to increased number of large antral follicles, which correlated well with the increased estradiol levels. The number of ova found in the oviducts was reduced in the treated groups compared to controls, but not in the ovulating females. Overall, there were no signs of follicular toxicity, but D4 appeared to disrupt the LH surge and decreased the portion of ovulating females.

This paper is included in the REACH registration dossier for D4 on toxicity to reproduction.

Study quality and assessment: The study is well described and the interpretation of data and relations between the different observed effects are elucidated. The number of animals used per group in the phase II study is not stated, but the number of ovulators and non-ovulators in the different groups are listed in a figure. The study is assessed to be of high quality although no CAS-number of D4 is given. The study provides strong evidence of an endocrine mode of action of D4 and strong evidence of adverse effects on female reproduction. However, disruption of the LH surge that probably lead to the observed effects may be a rodent species specific mode of action and thus the effects on ovulation may not be relevant to humans.

Siddiqui et al. (2007)

Summary: The aim of the study was to evaluate the effects of D4 on reproductive function in F0 and F1 animals and on survival, growth and development of the offspring. A two-generation toxicity study with whole-body vapour inhalation of 0, 70, 300, 500 or 700 ppm of D4 for 6 hours per day was performed in rats. Male and female rats (F₀, 165 per sex, 44-45 days old) were exposed from 70 days before mating and until weaning of the pups (F₁, n=23-27 litters per group). The F₁ offspring were exposed in utero and through lactation and directly exposed from weaning throughout adulthood with at least 70 days of exposure prior to first mating. F₁ offspring were mated twice delivering two litters (F_{2a} and F_{2b}, n= 17-29 and n=12-26 litters per group, respectively) and male offspring were mated a

third time with untreated females while males were still exposed. Exposure was interrupted for 5 days at the time of delivery from GD20 until PND4 for all pregnant female rats.

General toxicity (food consumption, body weight gain and weight and histology of liver, kidney and lung) was assessed. Reproductive (e.g. mating and fertility index, number of implantation sites, pups born per litter etc.) and developmental parameters (e.g. sperm parameters, AGD and sexual maturation) were assessed.

Food consumption in F₀ males was reduced compared to controls in the 700 ppm group during weeks 1-2 which was consistent with a reduced weight gain observed in these males at that time-point. Increased organ weights were seen in livers (absolute and relative weights in F₀ and F₁ males and females), kidneys (absolute and relative weights in F₀ males and F₁ females and relative weights in F₁ males) and pituitary glands (absolute weights in F₁ females and relative weights in F₀ males and F₁ females). Histologically, changes were seen in livers, kidneys and lungs. Hepatocytic hypertrophy was increased in F₁ females in 500 and 700 ppm groups and in F₁ males in the 700 ppm group. In the F₁ males in the 700 ppm group, increased bile duct hyperplasia and bile pigment were observed. Tubular mineralisation was seen in the kidneys of F₀ and F₁ males in the 700 ppm group.

Reproductive parameters were also affected. Female body weight gain was reduced during GD14-20 in the 700 ppm group (F₀ and F₁), a change considered to be related to a decreased number of foetuses in the uterus, as a reduced number of pups were born in this group. The percentage of mated rats with successful delivery of litters was reduced and the gestation length was increased in F₁ offspring in the 700 ppm treatment group. The oestrous cycle length was increased in F₁ females in the highest exposure-group. Most (8 out of 9) females with extended diestrus did not show evidence of mating and this led to decreased mating indices (number of successful matings out of number of animals used for mating) during the second mating in F₁ animals. Histologically, the number of corpora lutea was decreased in F₁ females and half of the females (15 out of 30) in the highest exposure group were anovulatory or had disturbed oestrous cycles. In males, no effects were seen on sperm parameters. The number of litters was reduced in the 700 ppm group for the first mating in the F₁ generation and in the two highest dose-groups for the second mating. The number of live pups per litter was reduced in the 500 and 700 ppm groups in the F₀ and F₁ generations and the number of implantation sites was reduced in the 700 ppm group in F₀ females. Pup survival on PND 0 was reduced in F_{2b} pups from the 700 ppm group.

This paper is included in the REACH registration dossier for D4 on toxicity to reproduction. Reduction of fertility indices of F_{1b} males and females (second mating) in the 500 and 700 ppm groups is reported as statistically significant compared to control levels in the REACH dossier but not in the published paper.

Study quality and assessment: The study was conducted according to EPA OPPTS Health Effects Test guideline (which is equivalent or similar to the OECD guideline 416). It looks like there is a mistake in table 1 showing data on reproductive and developmental parameters from F₀, F_{1a}, F_{1b} and F_{1c} animals. The table covers more than one page and it is probably data from F_{1b} and F_{1c} that are shown on page 2 and not data from F₀ and F_{1a} as stated in the first line of the table on page 2. Apart from this detail, the study is well-described and thorough and the study is assessed to be of high quality. The study provides strong evidence of adverse effects on female reproduction of rats exposed as adults and especially *in utero* and throughout lactation. However, the F₁ females showed more signs of toxicity (increased organ weights and histological changes in livers indicating metabolising activity) compared to F₀ females and this may explain why more marked effects on reproductive parameters such as

gestation length and oestrous cycle length were seen in the F1 females. Thus, the study provides moderate evidence of adverse effects on female reproduction that could be explained by an endocrine disrupting mode of action.

He et al. (2003)

Summary: The objective of this study was to characterise the estrogenic mode of action of D4 *in vivo* and *in vitro*. Several *in vivo* studies were performed and other siloxanes were also tested in the Uterotrophic assay. Female mice (6-7 weeks old, n=6/group) were dosed orally with 1000 mg/kg D4 for 7 days and serum oestradiol was measured. Subsequently intact, sham operated and adrenalectomised (ADX) mice were dosed similarly as the previous study and serum oestradiol and corticosterone were measured. The siloxane D4 was tested in an Uterotrophic assay in ovariectomised female mice dosed orally to 1000 mg/kg/day D4 for 3 days (n=5/group). A uterine peroxidase assay was performed on uteri from the ovariectomised mice. A dose-response study was additionally performed for the Uterotrophic assay using 0, 1, 10, 50, 100, 250, 500 and 1000 mg/kg D4 for a 3-days exposure period (n=5/group) in ovariectomised mice. To investigate if the effects on uterine weight were mediated through ER, two more studies were performed. The Uterotrophic assay was repeated in ovariectomised mice with pre-treatment with the ER antagonist ICI 182,780. Finally, ER α knockout mice (α ERKO) and wild-type controls were ovariectomised (n=5) and used in an Uterotrophic assay with oral dosing of 1000mg/kg D4 for 3 days.

Serum oestradiol was reduced by approximately 50 % by D4 exposure. In the ADX study serum oestradiol was reduced in intact, sham operated and ADX mice. Serum corticosterone was increased in intact and sham operated mice, whereas corticosterone was reduced in ADX mice. This suggested that the D4 induced decrease in oestradiol was independent of corticosterone levels and an association between stress-like elevated corticosterone levels and suppression of the hypothalamic-pituitary-gonadal axis could be excluded. D4 induced increased uterine weight in the Uterotrophic assay and increased uterine peroxidase activity in ovariectomised rats. The dose-response study in the Uterotrophic assay showed effects at doses of 250mg/kg D4 and above. Pre-treatment with ICI 182,780 blocked the D4-induced increase in uterine weight, indicating that the effects on uterus weight are ER-mediated. Additionally, the D4 induced increase in uterus weight was absent in exposed α ERKO mice, suggesting that the effects on uterus weight were more specifically mediated through ER α .

Study quality and assessment: Information of the CAS-number and purity of the compound would have been preferred, but otherwise the study is well-described although it is a complex study describing several smaller studies. The study is assessed to be of high quality. The study provides strong evidence of an estrogenic mode of action of D4.

Burns-Nass et al. (2002)

Summary: The objective of the study was to investigate the subchronic toxicity of D4 in a 3 months inhalation study. Male and female rats (approximately 10 weeks old) were exposed to 0, 35, 122, 488 or 898 ppm for 6h/day, 5 days a week for 3 months (20/sex/group) to D4 by nose-only inhalation. A recovery study was performed with 10 rats per group exposed to 0 or 898 ppm as described previously for 3 months and with a following recovery period of 1 month. Blood was collected for hematologic and clinical chemistry, urine analysis was performed and organs were weighed, including liver,

spleen, heart, lung, thymus, ovaries, testes, kidneys, adrenals and brain. Histopathological assessment of a full set of organs was conducted.

Changes in endocrine related endpoints were found. Testes weight was increased in the 488 ppm group but not at the highest dose, and the change was considered not to be exposure related. Ovary weight was decreased (by 38% compared to controls) in the highest exposure group.

Histopathological evaluation of the ovaries showed an increased incidence of hypoactivity seen as a lack of active corpora lutea in the 898 ppm dose-group after a 3 months exposure period. Follicular development appeared normal. The morphological changes indicated a decreased ovulatory activity of the ovaries and the reduced ovary weight was most likely due to the lower number of corpora lutea. After the 1 month recovery period, the ovaries showed normal follicular development. Increased incidence and thickness of mucification of the vaginal mucosa was seen in the uterus in females exposed to 898ppm for 3 months.

Study quality and assessment: The study is well-described and is assessed to be of high quality. The changes observed in female reproductive organs may be related to an endocrine mode of action of D4. The study provides strong evidence of adverse effects on ovaries which can be explained by an endocrine mode of action of D4.

McKim et al. (2001)

Summary: The objective was to investigate the estrogenic and anti-estrogenic activity of the cyclic D4 and the linear siloxane hexamethyldisiloxane in an Uterotrophic assay. Immature female rats (12 pups per group) from two different strains were used in the study. Sprague-Dawley rats (18 days old) and Fisher-344 rats (21 days old) were dosed by oral gavage with 0, 10, 50, 100, 250 or 1000 mg/kg/day of D4 for 4 days. For evaluation of anti-estrogenic properties of D4, EE was co-administered with either D4 (500 mg/kg/day) or ICI 182-780 (positive control). The uterine weight was measured and the epithelial cell height in uterus was measured histologically (n=6 per group). Relative potency of the different chemicals tested (EE, diethylstilbestrol dipropionate and Coumestrol) was evaluated based on the effects on uterine weight.

Female body weight was decreased in the highest D4 dose-group on day 21 (SD rats) or on days 23, 24 and 25 (Fisher rats). Compared to the other compounds tested, D4 was the least potent in increasing uterine weight (absolute and relative). Higher doses of D4 were necessary to get the same increase in uterine weight compared with the weak estrogenic compound Coumestrol. Uterus weight was significantly increased by 250, 500 and 1000 mg/kg/day of D4. Co-administration of D4 with EE attenuated the effect of EE on uterine weight suggesting an anti-estrogenic effect of D4. The compound D4 was less effective in inhibiting the effect of EE on uterine weight compared to ICI. Uterine epithelial cell height was increased by EE and D4 in a dose-dependent manner. In conclusion, D4 appeared to have weak estrogenic and anti-estrogenic properties.

Study quality and assessment: The study has some limitations as the number of litters is not mentioned, which means it is unclear if litter mates were used. Moreover, more details on housing conditions would have been preferred as well as the CAS-number of D4. The study is assessed to be of medium quality. The study provides strong evidence of a weak estrogenic mode of action of D4.

Hayden and Barlow (1972)

Summary: The purpose of the study was to investigate the effects of a series of organosiloxanes in immature female rats and to establish structure-activity relationships. However D4 does not appear to have been investigated in this study

REACH registration dossier

Toxicity to reproduction:

Key Experimental results and Supporting Experimental results

Summary: The applicant reported data from 7 additional study reports. Data from two of these studies are published in Meeks et al. (2007) and Siddiqui et al. (2007) and these papers have already been included here. The other 5 studies are one-generation reproductive toxicity studies in male and/or female rats with inhalation exposure. The studies do not add significant value or new information on the endocrine mode of action or endocrine-related adverse effects of D4, except for a reversible increase in the weight of the thyroid gland in adult males. In general, the studies show decreased weight gain and food consumption in adult rats and adverse effects on reproduction (e.g. reduced mean litter size, reduced pup viability and reduced number of implantation sites).

Study quality and assessment: The quality of the unpublished studies cannot be assessed based on the summaries available. The studies are performed under GLP with 20-22 animals per dose-group, but not all of them follow test guidelines. The studies provide strong evidence of adverse effects on reproduction based on reduced litter size, pup viability and number of implantation sites.

Developmental toxicity/ teratogenicity:

001 Key Experimental results

Summary: The first key study in the REACH registration dossier with data on developmental toxicity is published as an abstract in *The Toxicologist* in 1994 (York and Schardein 1994). This publication was not possible to retrieve from PubMed or by a Google search (24/11-2017). In this study pregnant rats (n=30 per group) were exposed through whole-body inhalation to 100, 300 or 700 ppm D4 for 6 hours per day for 4 weeks (GD6-15). A caesarean section was performed GD20 for assessment of developmental parameters. No effects were found on embryo lethality, litter size, sex distribution and foetal body weight. Mean maternal food consumption was reduced as well as the body weight gain in the highest exposure group.

Study quality and assessment:

The study is performed under GLP with 30 females per dose-group and the study design was equivalent or similar to OECD Guideline 414 (Prenatal Developmental Toxicity Study) with some (unspecified) missing details. Overall the study seems to be of good quality, but as only a summary is available and details on data and methods are not given, the quality of the study cannot be assessed. The study provides no evidence of relevance for evaluating adverse effects related to endocrine mode of action.

002 Key Experimental results:

Summary: The second key study in the REACH registration dossier for D4 with data on developmental toxicity was published in the same abstract in *The Toxicologist* in 1994 (York and Schardein 1994) as the data from the first key study on rats. The publication was not possible to retrieve from PubMed or by a Google search (24/11-2017). In this study 20 rabbits per group were exposed to D4 through whole-body inhalation of 0, 100, 300 or 500 ppm on GD6-18 or during different intervals in this gestational period. Decreased maternal food consumption was observed. No effects were found on mortality, malformations, developmental variations or body weight gain. Slight increase in postimplantation loss was seen but the levels were within historical control levels. No treatment-related effects were found on the number of viable foetuses per dam, mean foetal body weight, foetal malformations or developmental variations.

Study quality and assessment: The study is performed under GLP with 20 females per dose-group and the study design was equivalent or similar to OECD Guideline 414 (Prenatal Developmental Toxicity Study) with some (unspecified) missing details. Overall the study seems to be of good quality, but as only a summary is available and details on data and methods are not given, the quality of the study cannot be assessed. The study provides no evidence of relevance for evaluating adverse effects related to endocrine mode of action.

Supporting Experimental results

Summary: Several supporting experimental results are included in the REACH registration dossier of D4 on developmental toxicity and teratogenicity. Overall, the (3 GLP) studies showed decreased food consumption and reduced body weight gain in dams and 1 study showed increased abortions (from 500 mg/kg) and increased post implantation loss (1000 mg/kg), but the reduced food consumption was considered responsible for these findings on reproduction. One study in rabbits showed decreased weight of gravid uterus and a reduced number of live foetuses (1000 mg/kg).

Study quality and assessment: Three out of 5 studies were performed under GLP compliance with 6 female rats or rabbits/ group and the study design was equivalent or similar to OECD Guideline 414 (Prenatal Developmental Toxicity Study). Overall the studies seem to be of good quality, but as only summaries are available and details on data and methods are not given, the quality of the studies cannot be assessed. Two study summaries were retrieved from IUCLID and included in this part of the REACH registration dossier of D4 but very few information on those studies are available. They are not GLP compliant and no details on the doses or the number of animals used are given. In conclusion, the quality of the unpublished studies cannot be assessed based on the summaries available. The studies appears to provide strong evidence of maternal toxicity based on food consumption and body weight gain and weak evidence of reproductive adverse effects but no conclusion can be made on the relation to endocrine disruption.

Toxicity to reproduction: other studies

Summary: Fifteen additional studies on reproductive toxicity and *in vitro* studies on endocrine related activity are included in the REACH registration dossier for D4. Some of these refer to published papers already assessed here (Meeks et al. 2007, He et al. 2003, Quinn et al. 2007 and McKim et al 2001). No significant additional data on fertility and reproduction are given on these studies in the REACH registration dossier compared to the published papers. The only supplemental information given in the REACH registration dossier for the published studies is reduced food consumption in

females with reduced weight gain. The remaining studies included supporting data published in the papers assessed above. In these studies, D4 had an estrogenic effect in MCF-7 cells and in the Uterotrophic assay, D4 exposure led to fewer ovulating female rats, attenuated the LH surges, decreased serum prolactin levels and *in vitro* studies showed a lack of receptor binding and receptor activation of PR. Some data appear to support the hypothesis that D4 may be a dopamine D2-receptor agonist.

Study quality and assessment: Most of the studies are non-GLP studies and do not follow specific guidelines. The quality of the studies cannot be assessed based on the restricted information given in the summaries. The studies appear to provide strong evidence of an endocrine mode of action of D4 on female reproductive system, including strong evidence of an estrogenic mode of action and strong evidence for adverse effects, although the mechanism behind reduced ovulations may be species specific.

Repeated dose studies

Summary: Overall, repeated dose toxicity studies with exposure to D4 through inhalation showed effects on the female reproductive tract. Several of the studies found decreased ovary weight, ovary atrophy/ hypoactivity and vaginal mucification. Additionally, increased uterus weight and endometrial epithelial hyperplasia was seen in a study after 24 months of D4 exposure. Some studies found increased weight of adrenal glands in females and vacuolation of zona fasciculata of the adrenal glands was shown. Effects were also observed in the male reproductive tract, more specifically the testes. One study showed increased testes weight without histopathological changes after 24 months of exposure while another study found testes tubular atrophy after exposure to D4 for 13 weeks.

Study quality and assessment: The studies referred to above were all GLP compliant and followed a test guideline. In these studies 10 rats or more were used per group. Overall the studies seem to be of good quality, but as only summaries are available and details on data and methods are not given, the quality of the studies cannot be assessed. The studies appear to provide strong evidence of reproductive adverse effects.

Specific investigations: Other studies

Summary: *In vivo* studies investigating effects of D4 on thyroid glands and on serum prolactin levels are included. One of the studies showed increased thyroid gland weights, hyperplasia and increased proliferation in thyroid glands after 6 or 13 days of exposure. No such effects were seen after 5 days of exposure. This suggests a proliferative effect of D4 on cells in the thyroid glands after continuous exposure. Other studies showed time-dependent effects on serum prolactin levels. No changes in prolactin levels were seen in any of the studies immediately after exposure. However, 18 hours after D4 exposure decreased prolactin levels were observed in one study whereas another study showed increased prolactin levels 4 and 8 hours after D4 exposure.

Study quality and assessment: The studies are not performed under GLP compliance and do not follow any guidelines. The quality of the studies cannot be assessed based on the restricted information given in the summaries. The studies provide weak evidence of an endocrine mode of action of D4 based on effects on serum prolactin levels *in vivo* and hyperplasia in thyroid glands.

4.10.3.4 Summary of the plausible link between adverse effects and endocrine mode of action

A large number of studies have found adverse effects on female reproductive organs and reproductive function in rodents (Table 2). Most studies were performed in adult rodents and only one study in the published literature investigated developmental effects (Siddiqui et al. 2007). The reproductive effects reported include reduced fertility, disturbed oestrous cycles, reduced ovulations, increased uterus weights with endometrial hyperplasia, vaginal mucification, reduced ovary weight and atrophy of ovaries. These effects could be related to an endocrine disrupting mode of action involving female reproductive hormones such as oestrogens, LH or FSH or a combination of these (OECD GD 106). No additional effects were found in offspring in the developmental study, but the effects appeared to be more marked than in adult females (Siddiqui et al. 2007). This could be due to more general toxicity in the offspring as evidenced by increased organ weights of liver, kidney and pituitary glands and histologically observed hypertrophy of hepatocytes, indicative of increased metabolising activity of the liver. The evidence of effects on reproductive function related to endocrine disruption observed in the developmental study is thus weak. Reproductive effects observed in adult animals in the other studies are more convincingly related to an endocrine disrupting mode of action and the studies provide strong evidence of adverse effects on female reproduction.

One published paper and a study registered in the REACH registration dossier reported work that has been done to identify whether D4 could lead to changes in plasma LH levels and if the reduced ovulations and fertility observed *in vivo* could be related to such changes (Quinn et al. 2007b). Lower plasma LH peaks in non-ovulating females compared to controls was reported and it is very likely that the link between low LH peak levels and reduced ovulations can explain some of the effects observed on lower fertility in females (e.g. reduced litter size) and disturbed oestrous cycles (Siddiqui et al. 2007; Meeks et al. 2007; Jean et al. 2017). However, disruption of the LH surge may be a species specific mode of action and thus the effects on ovulation may not be relevant to humans.

In concordance with the observed adverse effects in females (e.g. uterus and vaginal changes), several *in vivo* and *in vitro* studies showed an estrogenic mode of action of D4, although some studies show conflicting data on the estrogenic effects *in vivo* (Table 1). Overall, *in vitro* studies showed binding to and activation of ER α and estrogenic effects at gene- and protein expression level in GH3 cells. Compared to the positive controls ethinyl estradiol and 17 β -estradiol, D4 showed similar effects *in vitro* (i.e. gene- and protein expression of the estrogenic biomarker CaBP-9K and of ER α in pituitary GH3 cells) and *in vivo* (i.e. gene- and protein expression of the estrogenic biomarker CaBP-9K and of ER α in the uterus and increase in uterine epithelial cell height) and the effects were blocked by the anti-estrogenic (ER α inhibitor) substance ICI 182-780 both for the effects observed after exposure to D4 and the positive control (Lee et al. 20015; McKim et al. 2003). Other *in vitro* studies showed binding of D4 to ER α (but not ER β) and activation of the ER α reporter gene assay (Quinn et al. 2007a; He et al. 2003) and this was supported by estrogenic effects of D4 in MCF-7 cells reported in the REACH registration dossiers.

Some conflicting data on *in vivo* estrogenic effects in the Uterotrophic assay have been reported. One study did not find effects of D4 on uterus weight in the assay (Lee et al. 2015), whereas other studies did (Quinn et al. 2007a; He et al. 2003; McKim et al. 2001). This could be explained by the administration route as D4 was dosed s.c. in the study by Lee et al. (2015) whereas first-pass metabolism was possible in two of the other studies, where exposure was conducted through oral gavage (HE et al. 2003; MicKim et al. 2001). Moreover, the studies that found increased uterus

weight in the Uterotrophic assay had a larger number of animals per group compared to the study performed by Lee et al. (2015). Supporting studies submitted by the applicant in the REACH registration dossier also showed an estrogenic effect of D4 in the Uterotrophic assay. The anti-estrogenic compound, ICI 182-780, was shown to have an inhibiting effect on the increase in uterine weight after D4 exposure in the Uterotrophic assay, indicating an ER-mediated effects of D4 on the uterus (He et al. 2003). Conflicting data on estrogenic effects of D4 were also seen at the hormonal level in rat studies. Some studies showed reduced serum estradiol levels (Jean et al. 2017; He et al. 2003), whereas another study showed increased estrone and 17 β -estradiol levels in plasma (Quinn et al. 2007b). The lack of effect in the study by Jean et al. (2015) could be explained by the differences in dosing period. In the study by Jean et al (20017), exposure was conducted in aging females. In contrast, females in the Quinn et al. (2007b) study were in the reproductive stage (13 weeks old), and the data on hormone levels are evaluated to be more reliable in the study performed by Quinn and co-workers (2007b). In summary, the weight of evidence of the available *in vivo* and *in vitro* studies point to an estrogenic mode of action of D4. Altogether, the published data provide strong evidence of estrogenic mode of action of D4 and strong evidence for adverse effects linked to anti-estrogenic mode of action of D4.

In addition to the effects on female reproductive function and female reproductive organs, some studies reported adverse effects on male reproductive organs. Increased testis weight and histological changes of testis (i.e. testicular interstitial cell hyperplasia and testis tubular atrophy) were described in the REACH registration dossiers and in the published literature (Burns-Nass et al. 2002; Jean and Plotzke, 2017). However, studies on the androgenic or anti-androgenic mode of action of D4 are sparse. A Hershberger assay was performed in a single study but no signs of androgenic or anti-androgenic activity of D4 were found (Quinn et al. 2007b). There is moderate evidence of adverse effects on male reproductive organs, but the mode of action behind the testicular effects is unclear. However, it could be speculated, that the estrogenic potential of D4 could play a role in the testis. Thus, there is weak evidence for a plausible link between the estrogenic mode of action of D4 and adverse effects on male reproductive organs.

In addition to the above mentioned effects on reproductive organs, one mechanistic *in vivo* study reported in the REACH registration dossiers showed increased thyroid gland weights, thyroid hyperplasia and increased proliferation in thyroid glands. Based on this study there appear to be moderate evidence of a thyroid disrupting potential of D4, and more studies on these endpoints are necessary to conclude on this kind of endocrine disrupting capacity of D4. Thyroid hormones play a role in the regulation of prolactin and the effects observed on plasma prolactin levels (Quinn et al. 2007b; REACH registration dossier) may be related to the thyroid gland effects reported in the REACH registration dossier.

Summary and conclusions

All in all there is strong evidence that D4 has adverse effects on the reproductive system as well as weak evidence of adverse effects on the thyroid gland. There is strong evidence for an estrogenic mode of action of D4, and strong evidence for adverse effects on female reproductive system that can be related to this estrogenic mode of action of D4 together with an endocrine mode of action through LH. However, changes in LH levels may be species specific. Changes in LH levels are probably responsible for some of the adverse effects observed, but D4 also had a strong estrogenic activity and it is unclear which adverse effects can be linked to this mode of action alone. The male reproductive effects are likely related to an endocrine mode of action as well, but the few data available on androgen-related mode of action did not confirm an anti-androgenic mode of action of D4. It is possible that the estrogenic mode of action of D4 could be responsible for the testicular effects

observed. The mode of action behind the effects observed on thyroid glands cannot be determined based on the available data.

In conclusion, D4 does meet the WHO definition of an endocrine disruptor with an estrogenic mode of action leading to adverse effects on the female reproductive system.

Additional literature not included in the evaluation

Reviews on D4 have been used to check for additional literature not found in our literature search (Dekant *et al.* 2017; Franzen *et al.* 2017). Both reviews referred to an abstract with *in vitro* data on ligand binding assays and reporter gene assay for progesterone receptors, but this abstract was not accessible (Jean *et al.* 2005).

Table 1. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of octamethylcyclotetrasiloxane (D4).

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Jean et al. 2017		Progesterone levels were elevated in exposed rats 2-10 weeks after start of treatment and estradiol was reduced over the total study period. As a consequence, lower estradiol:progesterone ratios compared to controls were found in D4 treated females. Corticosterone concentrations were increased in exposed animals during almost the entire study period.	High	Strong
Lee et al. 2015	CaBP-9K gene expression was increased in GH3 cells exposed to E2 or D4. When cells were exposed to ICI 182-780 in combination with D4 or E2, the gene expression level of CaBP-9K was not affected. Protein expression of CaBP-9K was slightly increased by E2 and D4 but not when ICI was administered simultaneously. Similarly, PR gene- and protein expression levels were increased by D4 and E2, an effect that was blocked by ICI. Conversely, gene- and protein expressions of ER α were downregulated by E2 and D4 and ICI blocked the effect.	In the Uterotrophic assay, no effects on uterus weight were seen with s.c. administration of D4. Gene- and protein expression levels of CaBP-9K, an estrogenic biomarker, were increased in the uterus by EE and D4 and co-administration of ICI inhibited the effect. PR gene expression in the uterus was decreased by EE and the high D4 dose. ER α gene expression was reduced in the uterus by EE and D4. Gene expression of CYP2B1/2 in the livers was increased markedly compared to controls in the D4 dose-group in a dose-dependent manner.	High	Strong (in vitro)- Weak (in vivo)
Quinn et al. 2007b		A decrease in plasma LH peak levels in female rats were related to the ovulatory status, i.e. lower mean levels of LH were related to a higher number of non-ovulators in the treatment groups. In ovulating females, prolactin levels were reduced. In the 900 ppm group. Plasma estrone and 17 β -estradiol hormone levels were increased in both	High	Strong

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
		treatment groups (700 and 900 ppm). The ratio between estrone and 17 β - estradiol was reduced in the 900 ppm group in non-ovulating females. FSH was decreased in both treatment groups. Progesterone was increased in the highest exposure group.		
Quinn et al. 2007a	Weak binding of D4 to ER α was observed. D4 did not bind to ER β , PR α or PR β . Activation of the ER α reporter gene assay was seen, whereas no activation of the PR β reporter gene assay was observed for D4.	In the Uterotrophic assay with inhalation exposure to D4, uterine weight was increased, uterus was fluid filled and had increased luminal and epithelial cell height in both strains tested. D4 but not ICI 182,789 showed weak anti-estrogenic activity. The Hershberger assay showed no androgenic or anti-androgenic activity of D4.	High	Strong
He et al. 2003	D4 bound competitively to ER α but not to ER β in an ER binding assay.	Serum oestradiol was reduced by approximately 50% by D4 exposure. In adrenalectomised (ADX) mice serum oestradiol was reduced with D4 exposure. D4 exposure induced increased serum corticosterone in intact and sham operated mice, whereas corticosterone was reduced in ADX mice. D4 induced increased uterine weight in the Uterotrophic assay and increased uterine peroxidase activity. Pre-treatment with ICI 182,780 blocked the D4-induced increase in uterine weight, indicating that the effects on uterus weight are ER-mediated. Additionally, the D4 induced increase in uterus weight was absent in exposed α ERKO mice.	High	Strong
McKim et al. 2001		Body weight was decreased in the highest D4 dose-group. Uterus weight was significantly increased by 250, 500 and 1000 mg/kg/day of D4. Co-administration of D4 with EE	Medium	Strong

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
		attenuated the effect of EE on uterine weight suggesting an anti-estrogenic effect of D4. D4 inhibited the effect of EE on uterine weight. Uterine epithelial cell height was increased by EE and D4 in a dose-dependent manner.		
REACH registration dossier, Toxicity to reproduction: other studies	An estrogenic effect of D4 was found in MCF-7 cells. A lack of receptor binding and receptor activation of PR was found for D4.	An estrogenic effect of D4 was found in the uterotrophic assay. Moreover, D4 attenuated the LH surges and decreased serum prolactin levels.	Not assessable	Appears to be strong
REACH registration dossier, Specific investigations		Studies showed time-dependent effects on serum prolactin levels. No changes in prolactin levels were seen in any of the studies immediately after exposure. However, 18 hours after D4 exposure decreased prolactin levels were observed in one study whereas another study showed increased prolactin levels 4 and 8 hours after D4 exposure.	Not assessable	Weak

Ethinyl estradiol (EE); 17 β -estradiol(E2), estrogen receptor (ER); progesterone receptor (PR), luteinizing hormone (LH).

Table 2. Overview of potential endocrine-related adverse effects of octamethylcyclotetrasiloxane (D4).

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Jean and Plotzke, 2017	Rats, n=60/sex/group	Uterine and testis weights were increased in animals in the 700 ppm group exposed through inhalation for 24 months. An increased incidence of uterine cystic endometrial hyperplasia was found in high-dose females. Small but statistically significantly increased incidence of cervical squamous epithelial hyperplasia and/ or ovarian atrophy was observed. Similarly, a modest but significant increase in the incidence of testicular interstitial cell hyperplasia was observed after 24 months of exposure to 150 and 700 ppm D4.	High	Moderate
Jean et al. 2017	Rats, n=50/group	Increased number or percent of estrogen-predominant days (proestrous and estrous) in the estrous cycle was found in D4 treated animals compared to controls. A marked decrease was observed in the incidence of antral-size atretic follicles and an increase in severity was observed for vaginal mucification.	High	Moderate
Lee et al. 2015	Rats, n=5/group	D4 but not ethinyl estradiol increased the relative liver weights in immature female rats after s.c. exposure for 4 days.	Medium	Weak
Meeks et al. 2007	Rats, n=24/group or 60/group	Decreased fertility was observed in female rats exposed at a time-point close to mating and fertilisation or when the exposure period included this time-point.	High	Strong
Siddiqui et al. 2007	Rats, n=23-27 litters/ group in the first generation	Reproductive parameters were adversely affected, e.g. reduced number of litters, reduced number of pups per litter and extended oestrous cycles.	High	Moderate
Quinn et al. 2007b	Rats, n=22-27 in phase I, n=31-35 in phase II	Body weight was reduced in both treatment groups (700 and 900 ppm) in phase I and in the highest exposure group in phase II. The absolute ovary and relative ovary and uterus weights were reduced in the highest exposure group in phase II as well as the ovary relative to brain weight. More non-ovulating females were seen in the treatment groups. Histological assessment of ovaries showed a tendency to increased number of large antral follicles, which correlated well with the increased estradiol levels. The number of ova found in the oviducts was reduced in	High	Strong

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
		the treated groups compared to controls, but not in the ovulating females.		
Burns-Nass et al. 2002	Rats, n=20/group	Ovary weight was decreased in the 898 ppm group and histopathological evaluation of the ovaries showed an increased incidence of hypoactivity in the 898 ppm dose-group after a 3 months exposure period. Increased incidence and thickness of mucification of the vaginal mucosa was seen in the uterus in females exposed to 898 ppm. Testes weight was increased in the 488 ppm group but not at the highest dose, and the change was considered not to be exposure related.	High	Strong
REACH registration dossier, toxicity to reproduction	Rats, n=20-22/group	The studies show decreased weight gain and food consumption in adult rats and adverse effects on reproduction (e.g. reduced mean litter size, reduced pup viability and reduced number of implantation sites).	Not assessable	Strong
REACH registration dossier, developmental toxicity	Rats, n=30 females/group	Maternal toxicity was observed as reduced food consumption and reduced body weight gain in the 700 ppm group.	Not assessable	None
REACH registration dossier, developmental toxicity	Rabbits, n=20/group	Decreased maternal food consumption was observed. Slight increase in postimplantation loss was seen but the levels were within historical control levels.	Not assessable	None
REACH registration dossier, Repeated dose: inhalation	Rats, n=10 or more/group	Several of the studies found decreased ovary weight, ovary atrophy/hypoactivity and vaginal mucification. Additionally, increased uterus weight and endometrial epithelial hyperplasia was seen in a study after 24 months of D4 exposure. Some studies found increased weight of adrenal glands in females and vacuolation of zona fasciculata of the adrenal glands was shown. One study showed increased testes weight without histopathological changes after 24 months of exposure while another study found testes tubular atrophy after exposure to D4 for 13 weeks	Not assessable	Strong

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
REACH registration dossier, Specific investigations	NA	Increased thyroid gland weights, hyperplasia and increased proliferation in thyroid glands after 6 or 13 days of exposure. No such effects were seen after 5 days of exposure, suggesting a proliferative effect of D4 on cells in the thyroid glands after continuous exposure.	Not assessable	Weak

NA: not applicable (e.g. if represents several studies)

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Tris(methylphenyl)phosphate (TMPP), CAS no. 1330-78-5

Synonyms: Tricresyl phosphate (TCP)

TMPP is a mixture of the 3 isomers ortho-, meta-, and para cresols (Figure 1) and is used in the manufacturing of e.g. plastics, organophosphate flame retardants and solvents. TMPP is registered under REACH with a tonnage band of 1 000 - 10 000 tonnes per annum and is classified as Rep. 2 or self-classification as Repr. 1B; H360: May damage fertility or the unborn child . It is used in a variety of products such as furniture, electronics, textiles etc., and has been detected in the environment and in humans. The ortho isomer of TMPP, known as tri-*ortho*-cresyl phosphate (TOCP), is the most toxic of the 3 isomers and have been identified to cause neurotoxicity in humans and susceptible animals.

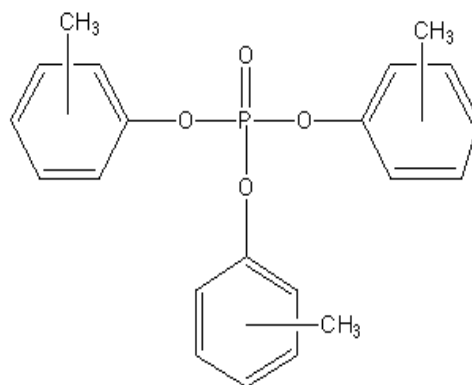


Figure 1. 2D structure from <https://www.huidziekten.nl/allergie/stoffen/tricresyl-phosphate.htm>

4. Human health hazard assessment

4.10.3 Endocrine disruption

4.10.3.1 General approach – human health

4.10.3.2 *In vitro* information indicative of endocrine activity

Reers et al. (2016)

Summary: In the present study, the effects of 6 organophosphate flame retardants (OPFRs), including TMPP (CAS no. 1330-78-5, purity not reported), on androgen receptor (AR), estrogen receptor (ER) and aryl hydrocarbon receptor (AhR) agonism and antagonism *in vitro* were studied. AR-mediated gene expression was studied by exposing metastatic prostate cancer LNCaP cells to chemicals in doses between 0.01-20 μ M and measuring mRNA and protein accumulation. Effects on ER α and AhR were studied in endometrial carcinoma cells (ECC-1 cells), at the same dose ranges. For both studies both the agonistic activity and the antagonistic activity (with positive controls: E2 for ER activity in ECC-1, TCDD (a dioxin) for AhR activity, and R1881 (synthetic androgen) for AR-activity in LNCaP cells). TMPP did not cause any effects on AR or ER α and AhR gene expression in the exposed LNCaP and ECC-1 cells, respectively, in both the agonistic and antagonistic mode.

Study quality and assessment: The material and methods section is not explicit with missing information on the measured mRNA and proteins in the two cell lines, purity of the tested chemicals, cytotoxicity information. The results from the first part of the study are also not explicitly presented. Overall, the quality of this study is assessed to be low. The evidence for lack of ER α , AR or AhR agonism or antagonism is weak due to the low quality of the study.

Schang et al. (2016)

Summary: In the present study, the effects of 7 OPFRs, including TMPP (CAS no. 1330-78-5, purity not reported), on MA-10 mouse Leydig tumor cells were compared to those of the brominated flame retardant BDE-47. First cell viability and cell count was assessed after exposing the MA-10 cells for 48h with 0, 1, 2, 5, 10, 20, 50 and 100 μ M TMPP. Next, cells were treated with 0, 10.1, 1 and 10 μ M TMPP for 48 h followed by measuring the superoxide production. Similarly the production of progesterone was assessed, both with cells only exposed to TMPP for 48 h, or TMPP and 2h post-treatment with dbcAMP (1 mM) or luteinizing hormone (LH) (100 ng/ml), and the progesterone production was normalized to cell number. Finally, the MA-10 cells were exposed to 10 μ M TMPP for 48 h with or without 2 h post treatment with dbcAMP or LH, and the quantification of the following steroidogenesis related genes: *Star*, *Tspo*, *Hsd3b*, *Lhcgr*, *Cyp11a1*, *Adcy3*. Each test was run in 5-7 independent experiments. TMPP significantly decreased cell viability at concentrations of 20 μ M or above and reduced the cell number at all tested concentrations except at 2 μ M. Superoxide and progesterone production was increased in the 10 μ M dose, but no significant effects were seen on the gene expression on steroidogenic genes.

Study quality and assessment: The study well-described and –designed and only information on the purity of TMPP is missing. Overall, the study is assessed to be of high quality. The study provides moderate evidence of a steroidogenic MoA due to increased progesterone production although the exact mechanisms underlying this effect were not identified.

Bradley et al. (2015)

Summary: The present study used chicken eggs to test TMPP (CAS no. 1330-78-5, \geq 89.0% purity) for effects on chicken embryo neurodevelopment. On day 0 of incubation, eggs were injected into air cell with 50 μ M TMPP in dimethyl sulfoxide (DMSO) in doses of 0 (n=15), 10, 100 or 1000 ng/g egg (n=20/dose group), or used as a non-injected control ((n=15). Incubation mortality, successful pipping and hatching were recorded. At day 7 through 9 post-hatching the chicks were tested in multiple behavioural tests such as open field, righting reflex, angled balance beam, gait analysis and wing flap reflex to assess their locomotor function, fear, desire for social reinstatement, motor reflexes, coordination, balance, and neuromuscular strength. At day 10 post-hatching, the chicks were euthanized and left cerebrum was used for cholinergic analyses, while cerebellum was used for histological evaluation of purkinje cell number.

The two control groups, non-incubated and DMSO incubated did not show any differences and were combined into a single control group. TMPP did not affect, survival or hatching, and no treatment effects were seen in the behavioural tests, except for a non-significant decreased righting reflex in the 10 ng TMPP/g egg. On histology, a non-significant increase in the number of cerebellar purkinje cells were observed at the highest 1000 ng/g egg group.

Study quality and assessment: Overall the study is well described and assessed to be of high quality. The study provides no evidence for ED MoAs and only very weak evidence for neurodevelopmental effects in chickens.

Crump et al. (2014)

Summary: The present study used chicken eggs to test TMPP (CAS no. 1330-78-5, 93.1% purity) for effects on chicken embryo development. In total 110 fertilised chicken embryos were used; 20 for dimethyl sulfoxide (DMSO) control, 10 for 7.8 ng/g, 20 for 780 ng/g, 8600 ng/g, 43,700 ng/g and 261,400 ng/g, respectively. Pipping success was calculated as the number of pippings by day 22 divided by the number of total fertile eggs per treatment group. Time to pip was also recorded. At day 22, embryos were euthanized and tarsus length, body mass and liver mass was assessed and liver somatic index (LSI = liver mass / body mass * 100) was calculated. Thyroid glands were harvested from the first 6-8 embryos and used for thyroxin (T4) measurement. The hepatic right lobe was removed and used for RNA extraction (n=8/treatment group) of CYP2H1, CYP3A37, deiodinase 1, 2 and 3, insulin-like growth factor 1 (IGF1), liver basic fatty acid-binding protein (LFABP), transthyretin (TTR) and uridine 5'-diphospho-glucuronosyltransferase 9 (UGT1A9). Whole blood was collected for free T4, bile acid and genetic sex determination. Pipping success in TMPP treatment groups were unaffected but an increase in deformities was observed in the embryos in the highest dose group, including reduced tarsus length and increased LSI with discoloured livers. TMPP up-regulated CYP2H1 and CYP3A37, UGT1A9 (not significant) and LFABP and downregulated TTR, as well as affected a number of other genes in the liver. T4 levels in plasma and thyroid glands were unaltered, but bile acid concentration in plasma was increased in the highest dose group.

Study quality and assessment: The study is well-described and assessed to be of high quality. The study provides only very weak evidence of ED MoAs due to effects on TTR gene levels as well as enzymes that could regulate hormone turnover.

Kojima et al. (2013)

Summary: In the present study, the effects of 11 OPFRs, including TMPP (here one of the TMPP isomers tricresyl phosphate (TCP) with CAS no. 78-30-8, >98% purity), on the agonistic and antagonistic activity on human nuclear receptors were studied. A transiently transfected CHO-K1 (Chinese hamster ovary cell line) cell-based transactivation assays were used for testing the agonistic and antagonistic activities of $1 \cdot 10^{-7}$ - $3 \cdot 10^{-5}$ M TCP on estrogen receptor (ER) α , ER β , androgen receptor (AR), glucocorticoid receptor (GR), retinoic acid receptor (RAR) α , retinoic X receptor (RXR) α and thyroid hormone receptor (TR) α and β . Likewise, the simian kidney COS-7 cell-based reporter gene assay was used to test TCP's activity against pregnane X receptor (PXR), peroxisome proliferator-activated receptor (PPAR) α and PPAR γ . All assays were run in 3 independent experiments. TCP showed slight cytotoxicity in both the CHO-K1 and COS-7 cells at the highest concentration of $3 \cdot 10^{-5}$ M. In the CHO-K1 cells, TCP was a weak ER α agonist, an anti-androgen against 5 α -dihydrotestosterone (DHT)-induced AR activity, and antagonized hydrocortisone (HC)-induced GR activity. No effects on ER β , RAR α , RXR α , TR α and TR β activity were measured. In the COS-7 cells, TCP caused PXR agonism and slight PPAR γ agonistic activity but no PPAR α activity.

Study quality and assessment: The description of the material and method section could be more detailed and include more information on the agonism and antagonism modes of the assays. Also, the exact doses are not reported but can be read from the figures. Overall, the study quality is assessed to medium. The study provides moderate evidence for multiple ED MoAs of TCP, including oestrogenic and anti-androgenic activity, as well as GR antagonism and PXR agonism.

Liu et al. (2012)

Summary: The present study investigates the effects of 6 OPFRs, including TMPP (here TCP, CAS no. 1330-78-5, 90% purity), on steroidogenesis in H295R cells, estrogenicity or anti-estrogenicity in MVLN cells, and hormone levels and syntheses in zebrafish. Here only the experiments in the H295R and MVLN cells are presented. H295R cells were exposed to 0.0, 0.001, 0.01, 0.1, 1, 10 and 100 mg/L TCP for 48 hrs. First cytotoxicity was assessed in the MTT bioassay, and the non-cytotoxic doses were further evaluated for effects on hormone production. Next, 0.0, 0.001, 0.01, 0.1, 1 and 10 mg/L TCP was tested for estrogen receptor (ER) binding as well as ER antagonism using 100 nM 17 β -estradiol (E2) in the MVLN luciferase assay. Here also only doses below cytotoxicity were evaluated. All experiments were run in 3 replicates.

TCP caused cytotoxicity at concentrations of ≥ 10 mg/L in both the H295R and MVLN cells. In the H295R cells, TCP increased E2 (≥ 0.01 mg/L) and testosterone (T, ≥ 0.1 mg/L) production, and resulted in an increased E2:T-ratio at 0.01 and 1 mg/L. TCP induced transcription of CYP11A1 (≥ 0.1 mg/L), CYP11B2, CYP19A1 and HSD3 β 2 at 1 mg/L, and down-regulated gene transcription of SULT1E1 and SULT 2A1 at 1 mg/L. In the MVLN cells TCP did not bind to ER but significantly and in a concentration-dependent manner reduced the binding of E2 to ER.

Study quality and assessment: The study is well-described and thorough and assessed to be of high quality. It provides strong evidence of both a steroidogenic and anti-oestrogenic MoA of TCP in human cells lines.

4.10.3.3 In vivo effects with regard to an endocrine mode of action**Bolon et al. (1997)**

Summary: This is an additional study using ovaries from mice tested previously in the NTP Reproductive Assessment by Continuous Breeding (RACB) bioassays for 15 different chemicals, including TMPP (here TCP, CAS no. and purity not reported). The testing and reproductive outcomes of TCP in the RACB assay has been described in Chapin et al (1988) and summarised in an NTP RACB report. In the present study, the ovaries from the exposed and control animals were sectioned and the number of follicles at 3 different stages were counted. Up to five experienced technicians performed the follicle counts, and the variation between pairs of technicians ranged from 0-30%. Then the results of the follicle counts were compared with the reproductive outcomes from the RACB study.

TCP has been shown to cause decreased fertility in both male and female mice (Chapin et al. 1988), but no effects on the follicle counts at the tested TCP doses were seen in the present study.

Study quality and assessment: The study is well-described, although the CAS no. and purity of the chemical is not stated. Also it should be noted that the large interobserver variation of up to 30% might have affected the results. Overall, the study is assessed to be of medium quality. No ovarian toxicity of TMPP was observed in the present study and this effect can therefore not explain the reduction in female fertility in mice seen after TMPP exposure. No other potential MoAs underlying the reduced female fertility were explored and the present study therefore does not provide any evidence of an ED MoA.

Latendresse et al. (1995)

Summary: The study investigated the functional effects of TMPP (here TCP, CAS no. 1330-78-5, no purity reported) and butylated triphenyl phosphate (BTP) induced adrenal and ovarian tissue changes on hormone levels and oestrous cycle in intact, adult female rats. First, female rats were exposed to 0.0 or 0.4 g/kg TCP by a single oral dose for 20 (n=9), 40 (n=6) or 60 days (n=3). Vaginal cytology was taken daily to study the effects of TCP on oestrous cycle. Next, female rats were exposed to 0.0 or 0.4 g/kg TCP by a single oral dose for 20 days (n=12), and blood samples, ovaries and adrenal glands were used after killing the animals. Estradiol, androstenedione and corticosterone concentration in serum was analysed as was cholesterol, high-density lipoproteins (HDL), low-density lipoproteins (LDL), alanine transaminase, albumin and total protein. The TCP exposed rats had normal oestrous cycle. Histopathological changes in the adrenal glands and ovaries were seen and it was hypothesized that these changes could alter steroid hormone production and metabolism. Estradiol levels were elevated but no other effects were seen on hormone levels

Study quality and assessment: Overall the study is thorough, however the purity of the tested compound is not clear and only a single dose of TMPP was tested. Based on this the quality of the study is assessed to be of medium quality. The study provides moderate evidence for effects on female estradiol production or metabolism but the exact MoA(s) behind the elevated estradiol levels is not elaborated. The evidence for female reproductive adverse effect are moderate due to the histopathological changes in the ovaries. It is hypothesized that these may result in altered steroid hormone production or metabolism.

Latendresse et al. (1994a)

Summary: The present study investigates the effect of TMPP (here TCP, CAS no. 1330-78-5, no purity reported) and butylated triphenyl phosphate (BTP) on rat testes, ovaries and adrenal glands. Sexually mature male and female rats were exposed to 0.0 or 0.4 g/kg TCP by a single oral dose for 20, 40 or 60 days (n=3/sex/dose group). After the exposure period, the rats were sacrificed and ovaries, testes, epididymides and adrenal glands were excised and weighed and then undergoing different histological examinations.

No macroscopic changes in the ovaries were seen but at the microscopic level the ratio of interstitial tissue to follicles and corpora lutea was increased and the ovarian interstitial cells from all TCP-exposed female rats were larger than the control group. The weight of testes from the 60 days TCP-exposed rats was decreased, and the seminiferous tubules showed altered morphology in all TCP-exposed animals. The adrenal glands of both TCP-exposed female and male rats were enlarged and had morphological changes. Due to an editorial error in the manuscript in the form of a picture covering text, tables and figures the remainder of the results section cannot be evaluated here.

Study quality and assessment: Apart from the editorial error, the study is well-described. There is only 3 animals per group and only 1 dose of TMPP is tested, and the purity of TMPP is not reported. With these limitations the quality of the study is assessed to be medium. The study provides strong evidence of ovary and testicular toxicity as well as effects on the adrenal glands in rats, but it does not study the underlying MoAs and it is therefore not clear if the adverse effects are due to ED MoAs.

Latendresse et al. (1994b)

Summary: The present study investigates the effect of TMPP (here TCP, CAS no. 1330-78-5, no purity reported) and butylated triphenyl phosphate (BTP) on female and male rat reproductive performance using a modified NTP continuous breeding protocol. First, sexually mature male and female rats were paired and exposed to 0.0 (n=40 pairs) or 0.4 g/kg TCP (n=20 pairs) by a single oral dose for 7 days pre-breeding, 63 days during breeding and 28 days post breeding. Body weight, fertility, litters/pair, live pups/litter, gender and mean weights of pups were registered. Next, the rats were used in an 8 days cross-mating study with either: control males x control females, TCP males x control females or TCP females x control males (n=20/pairs/group). Vaginal plug and smears for sperm as well as oestrous stage was assessed daily. Then all F0 animals were sacrificed and ovaries, testes, epididymides, vagina and uterus, liver and adrenal glands were excised and weighed. The body weights of all exposed rats were significantly decreased as was the number of litters/pair and live pups/litter. In the cross-mating study only the exposed males showed a reduced fertility with no litters produced. No effects on reproductive performance of the exposed females were seen. Both sexes had increased adrenal gland and liver weights. The treated males had lower testicular and epididymal weights, while the ovaries in the treated females had increased weights.

Study quality and assessment: Overall the study is thorough, however the purity of the tested compound is not clear and only 1 dose of TMPP was tested. Based on this the quality of the study is assessed to be of medium quality. The study provides moderate evidence of male reproductive adverse effects of TMPP as well as testicular and ovarian toxicology but does not study the underlying MoAs and it is therefore not clear if the adverse effects are due to ED MoAs.

Chapin et al. (1988)

Summary: This study evaluates the effect of TMPP (here TCP, CAS no. 1330-78-5, purity 74.9%) on reproductive performance in mice and consists of 4 tasks: 1) a range finding 14-day study, 2) a 98-days continuous breeding study, 3) a cross-mating study with F0 animals from 2), and 4) an offspring assessment of F1 animals. In 1), adult mice were exposed to 0.0, 0.437, 0.875, 1.75, 3.5 or 7.0% TMPP via feed for 14 days (n= 8/sex/group), and based on signs of toxicity and mortality the doses for 2) were set. In 2), animals were put in to breeding pairs and exposed to 0.0 (40 breeding pairs), 0.05, 0.01 or 0.2% TMP (corresponding to approximately 62.5, 125, and 250 mg/kg/day, 20 pairs/dose group) via feed for 98 days. After the last litter was reared, the F0 animals were used in a cross-mating study, i.e. task 3, to determine the affected sex in the F0 animals. Three groups were formed: control males paired with control females, control males paired with 0.2% TMPP females, and control females paired with 0.2% TMPP males. Following this the F0 animals were necropsied. The last litters from the control, 0.5% and 0.1% TMPP groups in task 2) were used in task 4) to study the effects on the F1 animals. When the F1 animals were sexually mature they were paired with an opposite sex from the same dose group but another litter and kept until first delivery. In 1) Doses of 1.75% or above resulted in general toxicity and increased mortality. In 2), the highest dose of 0.2% TMPP resulted in hind limb weakness and reduced postpartum weights in the females, and increased number of dead pups, as well as reduced litters/pair, and pub BW. In 3) the fertility of both males and females was affected. The sperm motility and concentration were decreased, and the number of abnormal sperm was increased. Atrophy was seen in the seminiferous tubules. In 4) there was a trend toward lower mating and fertility indexes, and the number of live pups/litter was decreased in the 0.1% TMPP F1 pairs. Sperm motility was decreased and number of abnormal sperm increased in F1

males but the number of sperm s were unaffected. No histopathological changes in the reproductive organs were found.

Study quality and assessment: Overall the study is thorough, but it is not clear how many animals that were used in 3) and 4). Also the exact ages of the animals used in 2) and 3) is not clear from the text but can in most cases be read from the tables. Based on this the quality of the study is assessed to be of medium quality. The study provides moderate-strong evidence of male and female reproductive adverse effects of TMPP as well as effects on offspring reproductive performance but does not study the underlying MoAs and it is therefore not clear if the adverse effects are due to ED MoAs.

Carlton et al. (1987)

Summary: The present study evaluates the reproductive toxicity of TCP. Male Long Evans rats 4-6 weeks old were given 0, 100 or 200 mg/kg TCP (CAS no. and purity not reported) per day by oral gavage for 56 days prior to breeding and during the 10 days breeding period and were then killed and blood, the reproductive tract and sperm were examined (n=12/dose group). Female Long Evans rats age 4-6 weeks were treated with 0, 200, 400 mg/kg per day by oral gavage 14 days prior to breeding, during breeding, gestation and lactation until killed at postnatal day 21, where blood and the reproductive tract were examined (n=24/dose group). One male was mated with 2 females: 0 mg/kg males with 0 mg/kg females, 100 mg/kg males with 200 mg/kg females, and 200 mg/kg males with 400 mg/kg females. Blood and hormone analyses were made on the litters at PD 21. No clinical signs of toxicity were detected in the male and female F0 animals at all doses. The fertility rates of TCP exposed rats were remarkably reduced compared with the controls. The 200 mg/kg TCP exposed male rats showed adverse reproductive effects with reduced epididymis weights, necrosis and degeneration of seminiferous tubules, and reduced sperm concentration, motility and progressive movement, and increased abnormal morphology. Histopathological alterations were found in the ovaries of 400 mg/kg TCP exposed females.

Study quality and assessment: Overall the study is thorough and well-described, although no information on the CAS no. and purity of TCP is reported. The study is assessed to be of medium quality. The study provides strong evidence of male and female reproductive adverse effects of TCP but does not study the underlying MoAs and it is therefore not clear if the adverse effects are due to ED MoAs.

Somkuti et al. (1987a)

Summary: A 14 day dose-range study was conducted on tris-*o*-cresyl phosphate (TOCP, the most toxic isomer of TMPP, 99% purity, no CAS. no. reported) followed by a 63 day sub-chronic study of TOCP and tris-*p*-cresyl phosphate (TPCP, less toxic isomer of TMPP, 97% purity, no CAS. no. reported). In the 14 day study, adult male Fischer were exposed to 0, 100, 200, 400, 800 or 1600 mg/kg TOCP for 14 days (n=8/dose group), and 2 animals/group were prepared for histopathology, and the remaining 6 animals were used for other assays. Next, a 63 day dose-response study was performed by exposing rats to 0, 10, 25, 50, 75 or 100 mg/kg TOCP or 100 mg/kg TPCP daily (n=10/group). Five animals from each dose group were used for histopathology of testes and epididymis, and the remaining 5 animals were used for other assays: activity of nonspecific testes esterase (NSE) and neurotoxic esterase (NTE) was measured in testes, sperm motility, density and morphology were assessed, interstitial testis fluid testosterone levels was measured.

In the 14-day study dose-dependent increase in mortality and cholinergic toxicity were seen in animals exposed to ≥ 200 mg/kg TOCP, and a decrease in sperm density and testicular histopathology were observed in all animals treated with TOCP. In the 63-day study, animals treated with ≥ 50 mg/kg TOCP showed decrease in weight gain and testis:body weight ratio as well as dose-dependent decreases in sperm motility and density and increase in abnormal sperm morphology. Histopathology in testes and epididymis were seen at doses ≥ 25 mg/kg TOCP, and TOCP also resulted in decreased NTE (≥ 50 mg/kg) and NSE (≥ 10 mg/kg) activity. In the TPCP group, only lower sperm density was observed.

Study quality and assessment: Overall the study is thorough and well-described, however it is only the TOCP and TPCP isomers that are tested here. It is not always clear from the material and methods section, which analyses were performed in the 2 studies. Due to these shortcomings, the study is assessed to be of medium quality. The study provides strong evidence of testicular toxicity and male reproductive adverse effects of TOCP in rats but does not study the underlying MoAs and it is therefore not clear if the adverse effects are due to ED MoAs.

Somkuti et al. (1987b)

Summary: In the present study changes in rooster testis histopathology, biochemistry and sperm motility after tris-*o*-cresyl phosphate (TOCP, the most toxic isomer of TMPP, 99% purity, no CAS. no. reported) TOCP oral exposure was studied. Adult leghorn roosters were treated with corn oil (n=10), a single oral dose of 750 mg/kg TOCP and killed after 24 h or 18 days (n=3/group), or 100 mg/kg daily TOCP for 18 days (n=10). Brain, plasma, and left testis were removed quickly after the animals were dead and brain acetylcholinesterase (AChE), testis nonspecific esterase (NSE), testis neurotoxic esterase (NTE), and plasma butyrylcholinesterase (BuChE) were measured. Sperm motility and density were assessed. No clinical signs of toxicity was observed in the single dose 750 mg/kg 24h treatment group, but in the 750 mg/kg 18 days treatment group and the 100 mg/kg daily 18 days treatment group, signs of neurotoxicity was observed. Sperm motility and density were remarkably reduced in the 100 mg/kg daily treated group and in this group 50% of the 10 roosters has disorganized seminiferous epithelium. Inhibition of testis NSE activity was reduced in the 750 mg/kg 24 h treatment group, and the NTE activity was reduced in 750 mg/kg 24 h treatment group and the 100 mg/kg daily 18 days treatment group.

Study quality and assessment: Overall the study is thorough and well-described, however it is only the TOCP isomer that is tested here. The study is assessed to be of strong quality. The study provides moderate evidence of testicular toxicity and male reproductive adverse effects of TOCP in roosters but does not study the underlying MoAs and it is therefore not clear if the adverse effects are due to ED MoAs.

Somkuti et al. (1987c)

Summary: The aim of the present study was to characterize the time course of tris-*o*-cresyl phosphate (TOCP, the most toxic isomer of TMPP, 99% purity, no CAS. no. reported) induced testicular toxicity. Male Fisher 344 rats were exposed to 0 or 150 mg TOCP/kg for 3, 7, 10, 14 and 21 days daily. Body weights and clinical signs were noted during exposure. At the end of exposure, the animals were killed and trunk blood, brain and testes were sampled. A second group of 10 animals

were used in a recovery study and first treated for 21 days, 2 were killed, the rest were allowed 98 days of recovery before they were killed and examined.

No clinical signs of toxicity were observed in any animals, but a decrease in sperm number was seen after 10 days of exposure and sperm motility was reduced after 14 days exposure. The testis to body weight ratio was decreased in rats exposed for 21-days. The testis enzyme nonspecific esterase and neurotoxic esterase activities were reduced at all the time points. No effects on serum LH, FSH or testosterone levels were seen. In the recovery study, the sizes of testes in the TOCP treated groups were decreased and they were devoid of germinal cells indicating irreversible testicular toxicity of TOCP.

Study quality and assessment: Overall the study is thorough and well-described, however it is only the TOCP isomer that is tested here. The study is assessed to be of strong quality. The study provides strong evidence of irreversible testicular toxicity and male reproductive adverse effects of TOCP in rats but does not study the underlying MoAs and it is therefore not clear if the adverse effects are due to ED MoAs.

REACH dossier

Toxicity to reproduction:

All applicant-reported data are from published studies that have already been included here.

Developmental toxicity / teratogenicity:

001 Key | Experimental results

Summary: The applicant reported information originates from an unnamed study report from 2004 and is from a Prenatal developmental toxicity study (EPA OPPT 870.3700). Briefly, adult male and female rats were mated, and females evident of mating were selected for further studies. They were then dosed daily by oral gavage with TCP (CAS no. reported in the dossier, purity not stated) from gestation day 10 to 19 with 0, 20, 100, 400 or 750 mg/kg/day and sacrificed on GD 20. Clinical signs of toxicity, body weights, fertility and gravid uterus weight were some of the endpoints reported. The foetuses were weighted and examined for malformations macroscopically and by histology.

The pregnant dams showed salivation from 100 mg/kg, decreased body weight/ (gain) and lower gravid uterus weights from 400 mg/kg/day. The foetuses in all treatment groups had lower weight with a dose-related response. No malformations in relation to the doses were observed.

Study quality and assessment: The study was performed under GLP and followed the EPA OPPT 870.3700 guideline, but since this is only a summary of a study report an assessment of the quality is not possible. The study however does not provide any evidence for ED related MoAs or adverse effects.

4.10.3.4 Summary of the plausible link between adverse effects and endocrine mode of action

Some ED MoAs for TMPP have been reported in both *in vitro* and *in vivo* studies (Table 1), including effects on steroidogenesis *in vitro* with increased oestrogen, progesterone and testosterone levels and up-regulation of enzymes involved in their production as well as down-regulation of enzymes important for their turn-over. The *in vitro* effects on steroidogenesis and oestrogen production might explain the increased oestrogen level measured in exposed adult female rats (Table 2). PXR agonism, ER α agonism, anti-oestrogenicity and AR antagonism MoAs were found *in vitro*, however in a study from 2016 the ER and AR MoAs could not be confirmed. Overall, there is moderate evidence for ED MoA of TMPP, mainly with regards to effects on steroidogenesis.

A number of studies in rats, mice and roosters, found adverse effects (Table 2) on male fertility with histopathological changes in testes and epididymides and evidence of adversely affected sperm parameters and altered activity of testicular esterases. Reduced sperm motility and increased number of sperms with abnormal morphology were also observed in rat F1 males exposed *in utero* to TMPP in a continuous mating study. Thus, there is strong evidence for adverse reproductive toxicity effects in adult males. There is a biologically plausible link between these male reproductive effects and ED MoA(s) of TMPP, especially effects on steroidogenesis leading to increased oestrogen levels. In female rats, histopathological changes in ovaries have been found with no to weak effects on their fertility. A single study in mice report effects on female fertility after TMPP exposure, but in a retrospective study examining the ovaries from these mice no effects were found in follicle counts. For both sexes, histopathological effects on adrenal glands in rats after TMPP exposure were observed, and these changes might result in modulation of the endocrine system leading to e.g. altered fertility. Weak evidence for adverse effects on the developing foetus have been found with effects on tarsus length and liver mass and gene expression in exposed chicken embryos and inconsistent deformities in rat foetuses.

Overall, the evidence for ED MoA(s) is moderate and the evidence for adverse effects of TMPP is strong, and the link between the adverse effects on reproduction and gonads to ED MoAs is evaluated as moderate. clear

In conclusion, TMPP meet the WHO definition of an endocrine disruptor with adverse effects on male fertility linked to effects steroidogenesis leading to increased oestrogen levels.

Additional literature not included in the evaluation

NTP RACB summary (1997): This is a summary of the NTP Reproductive Assessment by Continuous Breeding testing of TMPP in mice that has been described in the paper by Chapin et al. (1988).

Health Canada (2016): This is a draft screening assessment report that among others assess reproductive toxicity of TMPP and it uses references already included here.

Table 1. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of TMPP.

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Reers et al. (2016)	No ER α , AhR, or AR agonistic or antagonistic activity in two human cancer cells		Low	Weak
Shang et al. (2016)	Effects on basal progesterone production in mouse Leydig tumor cells		High	Moderate
Bradley et al. (2015)		Effects on chicken embryo neurodevelopment: no effects on chick behaviour or neurochemistry after <i>in ovo</i> exposure to TMPP. Non-significant increase in purkinje cell number in cerebellum in the highest exposure group.	High	None-weak
Crump et al. (2014)		Effects on chicken embryo development: the highest dose-level of TMPP resulted in deformities such as reduced tarsus length and increased liver mass, as well as altered liver gene expression with up-regulation of CYP2H1 and CYP3A37, UGT1A9 (not significant) and LFABP, and down-regulation of TTR	High	Weak
Kojima et al. (2013)	ER α and PXR agonism, AR antagonism and very weak PPAR γ agonism. No effects on ER β , TR α , TR β , RAR α , RXR α , and PPAR α activities were measured		Medium	Moderate
Liu et al. (2012)	Effects on steroidogenesis in H295R cells: increased T and E2 production, induction of CYP11A1, CYP11B2, CYP19A1 and HSD3 β 2 and downregulation of SULT1E1 and SULT2A1. Anti-oestrogenic in MVLN cells.		High	Strong

Androgen receptor (AR), estrogen receptor (ER) α and β , aryl hydrocarbon receptor (AhR), glucocorticoid receptor (GR), retinoic acid receptor (RAR) α , retinoic X receptor (RXR) α , thyroid hormone receptor (TR) α and β , liver basic fatty acid-binding protein (LFABP), transthyretin (TTR) and uridine 5'-diphospho-glucuronosyltransferase 9 (UGT1A9), pregnane X receptor (PXR), peroxisome proliferator-activated receptor (PPAR) α and PPAR γ

Table 2. Overview of potential endocrine-related adverse effects of TMPP.

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Bolon et al (1997)	Mice, ovaries from the Chapin et al (1988) study	No effect on follicle counts was seen.	Medium	None
Latendresse et al (1995)	Rats, 3-12/group	Histopathological effects on ovaries and adrenal glands and elevated serum estradiol levels were found in exposed adult female rats.	Medium	Moderate
Latendresse et al (1994a)	Rats, 3/sex/dose group	Histopathological effects on ovaries and testes as well as adrenal glands from both sexes were found.	Medium	Moderate
Latendresse et al (1994b)	Rats, 20/pairs/group	Adverse effects on male fertility as well on ovarian and testes weights.	Medium	Strong
Chapin et al (1988)	Mice, 8-40 males and females/group	Reduced fertility in both sexes was seen in the highest dose group. On necropsy reduced sperm motility and increased number of abnormal sperms were seen in both F0 and F1 males, with only a reduced number of sperms in the F0 males.	Medium	Moderate-Strong
Carlton et al. (1987)	Rats, 12 males and 24 females/ dose group	Males (200 mg/kg): reduced sperm concentration, motility, progressive movement, and increased abnormal sperm morphology, reduced epididymis weight, and necrosis and degeneration of seminal vesicles. Females (400 mg/kg): reduced litter size, histopathological changes in the ovaries	Medium	Strong
Somkuti et al. (1987a)	Rats, 8 males (14-days) or 10 males	Reduced sperm motility and density and increased number of abnormal sperm in the 50, 75 and 100 mg/kg groups. Histopathology in testes and epididymides were seen	Medium	Strong

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
	(63 days)/ dose group	in all treatment groups ≥ 25 mg/kg, and decreases in testicular esterases were also seen as a response to TOCP treatment		
Somkuti et al. (1987b)	Roosters, 10 (100 mg/kg/ day in 18 days) or 3 (750 mg/kg and killed after 1 or 18 days)	Reduced sperm density and motility, and disorganized seminiferous tubules in the 100 mg/kg group, inhibited activity of 2 testicular esterases in the 750 mg/kg 1 d group	High	Moderate
Somkuti et al. (1987c)	Rats, 8 males/time point and 10 males in the recovery group	Reduced sperm motility (≥ 14 days) and density (≥ 10 days), and reduced testis:BW ratio (≥ 21 days), inhibited activity of 2 testicular esterases (≥ 3 days), no effects of LH, FSH and testosterone in serum Recovery study: decreased testis size and absence of germinal cells after 98 days of recovery	High	Strong
REACH dossier	Rats, 25 females/dose group	Maternal toxicity and lower foetal weight was seen in the treatment groups. Inconsistent deformities in the foetuses were seen and these were most likely not related to the treatments	High	None

Luteinizing hormone (LH), Follicle stimulating hormone (FSH)

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2-(4-tert-butylbenzyl)propionaldehyde,

CAS no. 80-54-6

Synonyms: Lilial, Lily aldehyde, lysmeral, butyl methylpropional, butylphenylmethylpropional

The substance 2-(4-tert-butylbenzyl)propionaldehyde (Lilial) (Figure 1) is an aromatic aldehyde used in the industry as an odor agent and for consumers it is used in air care products, cleaning and furnishing care products, laundry and dishwashing products, personal care products and plastic and rubber products. This substance is used in 1 000 - 10 000 tonnes per annum. Lilial is classified in ECHA as a reproductive toxicant (Repr. 1B, May damage fertility or the unborn child).

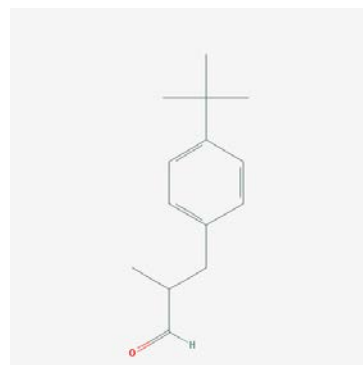


Figure 1: 2D structure from PubChem

4. Human health hazard assessment

4.10.3 Endocrine disruption

4.10.3.1 General approach – human health

4.10.3.2 *In vitro* information indicative of endocrine activity

A.K.Charles and P.D.Darbre (2009)

Summary: The focus of this study is to investigate the estrogenic activity of butylphenylmethylpropional (Lilial), benzyl salicylate and benzyl benzoate in MCF-7 human breast cancer cells *in vitro*. Only data for Lilial will be included in this summary. The estrogen-responsive MCF-7 cell line was used in different assays to test for the estrogenic activity of the substance. The study was performed in five parts as follows 1) Competitive binding assay to the oestrogen receptor (ER) of MCF-7 cytosol, 2) Competitive binding assay to human recombinant ER α and ER β , 3) Assay of stably transfected oestrogen-responsive reporter gene (ERE-CAT) in MCF-7 cells, was used to test the ability of the substance to regulate gene expression, 4) A cell proliferation experiment and 5) Real-time RT-PCR analysis of MCF-7 cells exposed to the substance for 24 hours. Overall the results from the study show that Lilial possess estrogenic activity in MCF-7 cells *in vitro*. In the ER binding assay, Lilial showed a maximal of 47% inhibition of ^3H -oestradiol binding and in the recombinant ER binding assay, inhibition of ^3H -oestradiol binding to ER α was greater compared to the binding of ER β . Lilial induced CAT gene expression (maximal effect of 1.8-fold at 5×10^{-4} M Lilial) although but the expression was not of the same magnitude as with 17 β -estradiol (2-fold at 10^{-8} M). In the cell proliferation experiments Lilial exposure showed a dose-dependent increase in MCF7 cell growth, but not at the same magnitude as for 17 β -estradiol exposure of the cells. The effect on proliferation could be inhibited by the antiestrogenic fluvestrent (Faslodex, ICI182,780), suggesting that the induced cell proliferation was ER-mediated. Finally, expression levels of the oestrogen-regulated gene, pS2, were increased following 24 h exposure to Lilial.

This paper is included in the REACH registration dossier for 2-(4-tert-butylbenzyl)propionaldehyde on toxicity to reproduction, other studies

Study quality and assessment: The study is very well-written and thoroughly described with details on CAS number (80-54-6) and purity ($\geq 95\%$) for the substance used. The assays were carried out in triplicates but cytotoxicity of the substance concentrations used was not evaluated. The findings on gene expression levels and cell proliferation could not be a result of cytotoxicity and the lack of such a test is therefore not assessed to be of importance for these assays. The results in the competitive binding assay in MCF-7 cells, however, could be mistaken for cytotoxicity. The quality is assessed to be high and based on results all pointing to an estrogenic activity of Lilial, it provides strong evidence for an estrogenic ED MoA of Lilial (butylphenylmethylpropional) although less potent than 17β -oestradiol.

REACH registration dossier,

Toxicity to reproduction:

Summary: The information from the “Toxicity to reproduction” section in the REACH registration dossier originates exclusively from unnamed study reports. Based on the available results a short summary is given here. One *in vitro* study was performed in rat Leydig cells with a range of concentrations of Lilial (doses: 33, 10, 3.3, 1, 0.33, 0.1, 0.033, 0.01, 0.0033, 0.001 $\mu\text{g/ml}$). No cytotoxic effect occurred for cells treated with Lilial at concentrations of 0-10 $\mu\text{g/ml}$. Lilial decreased testosterone levels at 10 $\mu\text{g/ml}$ after stimulation with high concentrations of human Chorion Gonadotropin (hCG).

Study quality and assessment: The quality of the study cannot be assessed based on the summary available in the REACH registration dossier. The results indicate that Lilial decreased testosterone levels after stimulation with hCG and provides weak-moderate evidence for an anti-androgen mode of action of Lilial.

4.10.3.3 In vivo effects with regard to an endocrine mode of action

No published studies were found.

REACH registration dossier

Toxicity to reproduction:

Outline: The information from the “Toxicity to reproduction” section in the REACH registration dossier originates exclusively from unnamed study reports. Based on the available results a short summary of the reports has been added below.

Summary: The remainder of the studies were conducted *in vivo* and the animals used include rats (10 studies) mice (1 study), dogs (4 studies), rabbit (1 study) and monkeys (1 study). All were exposed orally by feed or gavage and in four of the rat studies 14 - 35 animals were assigned to each dose group. For the rest of the studies the number of animals per dose group ranged between 2-10. Study

results from experiments with no observation on clinical signs and mortality, mainly include male endpoint data such as decreased mean fraction of motile sperm and mean head count in the cauda epididymis, histopathological findings (epididymis and testis) in dose group 30 mg/kg bw and a dose dependent moderate to severe diffuse (18/20 animals) and focal testicular (11/20 animals) degeneration in rats fed 1700 ppm Lilial. In another study (2006) conducted with rats a dose of Lilial and lysmerylic acid (50 mg/kg) resulted in slight to severe testicular atrophy after 24-96 h of exposure and after 14 days repeated exposure the atrophy of testis were accompanied by 100 % sperm immobility for both substances. These data are in line with data from a study done in 1987 where a dose of 50 mg/kg in 52 days produced decreased weight of testes (75 % of control after 24 days and 60 % of control after 52 days) and a study from 1991 that showed moderate to marked atrophy of testes in rats after 5 days of exposure to 50 and 100 mg/kg bw/d. Histopathological findings of testicular tissue revealed loss of seminiferous epithelium in a study from 1986, again at 50 mg/kg bw/day. On female data, a few studies reported an increase in gestation length, decreased live-born pups and pups delivered at levels of 30 mg/kg bw/day and low offspring weight and other data presented includes endpoints on liver.

Study quality and assessment: The quality of the studies cannot be assessed based on summaries available in the REACH registration dossier. In 4 out of 19 studies the name of the substance used were not given but it must be assumed that Lilial was the substance tested. Overall, the *in vivo* results provide a moderate evidence for male adverse effects on testes after exposure to Lilial. With regard to the studies that investigated female endpoints, the results provide moderate evidence for adverse effect on female reproduction (increased duration on gestation, decreased number of pups born alive, number of pups delivered and reduced birth weight of offspring) after exposure to Lilial.

Developmental toxicity / Teratogenicity:

Outline: The name of the substance used was not given in this study. The study design followed OECD guideline 414 and was conducted under GLP. Rats were exposed orally by gavage, daily from gestation day 6-20 with 25 females per dose group. Results revealed a significant reduction in the mean uterus weight in high dose dams and increased occurrence in skeletal malformations. Overall, data did not contribute with results that are relevant to assess the endocrine disrupting effects of Lilial.

Other studies:

Outline: The following study from the “Toxicity to reproduction, other studies” section in the REACH registration dossier has already been included here (A.K.Charles and P.D.Darbre, 2009) and the summary of their data can be found under the section for in vitro studies.

Repeated dose toxicity – Dermal + Oral:

Outline: The information from the “Repeated dose toxicity” section in the REACH registration dossier comes exclusively from unnamed study reports and in some of the studies the name of the substance used was not given but it must be assumed that Lilial was the substance tested. Based on the available results a short summary of the reports has been added below.

Summary:

All studies are performed *in vivo*, only a few of them follow a guideline (e.g. OECD TG) and about half is conducted according to GLP. In 5 out of 13 studies the substance used was not named and no CAS number could be found. Animals used included mainly rats but mice, dogs, guinea pigs and monkeys were also represented. In the 7 rat studies 3-14 animals was applied to each dose group, in the 3 dog studies 2-3 dogs per dose were used, 5 per dose was included in the two studies with mice and guinea pigs and for the single monkey study 2 monkeys were applied per dose group. The key-study reported that a dose of 50 mg/kg bw/day gave rise to disturbances in spermatogenesis and spermiogenesis, testicular increase in Sertoli cells, increased surface density in Leydig cells and decreased density of spermatozoa, nucleated cells and spermatoceles in the epididymis. In four studies, no treatment related changes were found but the rest of the studies showed data with male endpoints and significant findings was presented for decreased mean weight of testes and seminal vesicles at doses of 50 mg/kg although a note on general toxicity was mentioned (abnormal posture, decreased body temperature, emaciation and pilo-erection). Other data registered was related to liver (inflammation, fatty changes and white spots), kidney (reduced weight), heart (pericarditis) and lungs (emphysema).

Study quality and assessment:

The studies appear to be of low quality when taking into consideration that the number of animals per dose group were between 3-14 and in 5 out of 13 studies no name or CAS number could be found for the substance used, but the quality of the studies cannot be assessed based on summaries available on REACH registration dossier. Overall the studies provided weak evidence for an ED effect of Lilial.

4.10.3.4 Summary of the plausible link between adverse effects and endocrine mode of action

To our knowledge one *in vitro* study (A.K.Charles and P.D.Darbre, 2009) addressing the estrogenic activity of 2-(4-tert-butylbenzyl)propionaldehyde (Lilial) is currently available. The study is of high quality and show that Lilial possess estrogenic activity in MCF-7 human breast cancer cells and it provides a strong evidence for an estrogenic mode of action of Lilial.

The additional information from The REACH registration dossier comes exclusively from unpublished study reports and counts several *in vivo* studies looking at the adverse effect of 2-(4-tert-butylbenzyl)propionaldehyde. Overall the studies appears provide weak to moderate evidence for adverse effects on male reproductive endpoints manifested as atrophy of testes, seminiferous toxicity and decreased motility of sperm. For adverse effects on female reproductive endpoints the studies included in the REACH registration dossier provide moderate evidence for adverse effects on reproduction (increased duration on gestation, decreased number of pups born alive, and number of pups delivered) after exposure to 2-(4-tert-butylbenzyl)propionaldehyde.

The evidence for a plausible link between the strong evidence for ED MoA (*in vitro* estrogenic activity) and the weak to moderate evidence for adverse effects on male reproductive endpoints is evaluate as moderate.

In conclusion, 2-(4-tert-butylbenzyl)propionaldehyde does not meet the WHO definition of an endocrine disruptor, but fulfil the WHO definition of a potential endocrine disruptor. Also 2-(4-tert-butylbenzyl)propionaldehyde fulfil the proposed Danish criteria for being a suspected ED.

Table 1. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of 2-(4-tert-butylbenzyl)propionaldehyde.

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
A.K.Charles and P.D.Darbre (2009)	<p>Over all the results from the study show that Lilial possess estrogenic activity in MCF7 human breast cancer cells <i>in vitro</i>. In the first part, Lilial showed a maximal of 47 % inhibition of ³H-oestradiol binding at 3000000 fold molar and in part two, an inhibition of the binding of ³H-oestradiol to ERα and ERβ was observed at 3000000-fold molar excess with a greater inhibition of ERα (27 %) compared to ERβ. The results from the third part showed that Lilial could induce CAT gene expression, with maximal effect by 5 x 10⁻⁴ M Lilial (1.8-fold) although the expression was not of the same magnitude as with 10⁻⁸ M 17β-estradiol (2.0-fold in each case). For the cell proliferation experiments Lilial exposure showed a dose-dependent increase in MCF7 cell growth after 7 days with a 2.44 \pm 0.06 doublings with 10⁻⁴ M Lilial. Without any addition MCF7 cells gave an average number of doublings in 7 days of 1.84 \pm 0.08 and with addition of 10⁻⁸ M 17β-estradiol the value was 5.23 \pm 0.05. The proliferation effect could be inhibited by 10⁻⁷ M of the antiestrogen fluvestrent (Faslodex, ICI182,780), suggesting that the results measured in the experiment were ER-mediated. The cell density reached near confluence after 35 days with 10⁻⁴ M Lilial and for 10⁻⁸ M 17β-estradiol it was seen after 14 days with. And finally for the last part following 7 days of estrogen deprivation, a 24 h exposure to Lilial showed that it could increase the expression of pS2mRNA using Real time RT-PCR.</p>		High	Strong

Estrogen receptor α and β (ER α , ER β)

References

Charles, A.K. and Darbre, P.D. (2009) 'Oestrogenic activity of benzyl salicylate, benzyl benzoate and butylphenylmethylpropional (Lilial) in MCF7 human breast cancer cells in vitro', *Journal of applied toxicology*.29, pp.422-434, Doi:10.1002/jat.1429

REACH registration dossier: <https://echa.europa.eu/da/registration-dossier/-/registered-dossier/13572/1> (Link used in November 2017)

Salicylic acid (SA), CAS no. 69-72-7

Synonyms: Salicylate, 2-hydroxybenzoic acid, keralyt, acetylsalicylic acid, methyl ester

Salicylic acid (Figure 1) is a Beta hydroxyl acid obtained from the bark and wintergreen leaves of the white willow and encompass bacteriostatic, fungicidal and keratolytic effects. Salicylic acid (SA) is a colorless, crystalline organic carboxylic acid. The substance is used in many skin-care products for the treatment of acne, psoriasis, callouses, keratosis pilaris and warts and is produced and/or imported to EU in 10 000 - 100 000 tpa.

For the studies in this summary, different substance modifications of the substance have been used as a substitute, e.g. Acetylsalicylic acid (Aspirin), sodium salicylate and methyl salicylate.

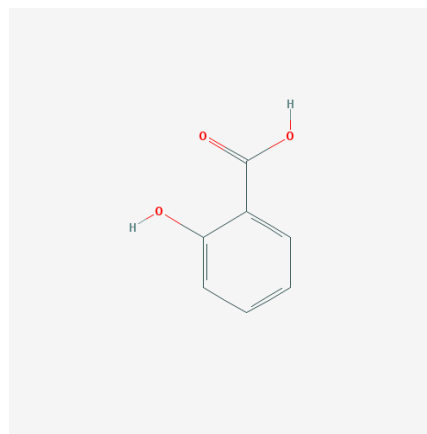


Figure 1: 2D structure from PubChem

Acetylsalicylic acid and methyl salicylate are the results of esterification of salicylic acid and hydrolysis of methyl salicylate produces sodium salicylate. The compounds are closely related and the following statement from WHO has been used as guidance for this summary: “All salicylic acid derivatives except combinations with corticosteroids (M01B) or opioids (N02AJ) are classified in N02BA - Salicylic acid and derivatives, as it is difficult to differentiate between the use of salicylates in rheumatic conditions and other therapeutic uses of salicylates.” (WHO 2017).

4. Human health hazard assessment

4.10.3 Endocrine disruption

4.10.3.1 General approach – human health

4.10.3.2 *In vitro* information indicative of endocrine activity

Mazaud-Guittot et al. (2013)

Summary: The study aimed to investigate the effect of mild analgesics on testis morphology and endocrine function. Three different drugs were used (Paracetamol, Aspirin and Indomethacin) but only data concerning Aspirin will be included in this summary. Human fetal testis (n=62) from gestation week (GW) 7-12 were cultured with exposure to Aspirin at 10^{-4} to 10^{-7} M. Testicular hormone levels of testosterone and Insulin-like factor 3 were measured by Radioimmuno Assay (RIA) and Anti-Müllerian hormone (AMH), prostaglandin D₂ and E₂ (PGD₂/PGE₂) was assayed by ELISA. Testicular cells (germ and Sertoli cells) were counted using the semiautomatic counting CAST grid image analysis software and mesenchymal cells of the interstitial tissue were identified by COUP-TF II staining. For assessment of nuclear receptor-mediated agonist or antagonist activities of Aspirin a reporter cell line expressing androgen, estrogen and peroxisome proliferator-activated γ -receptors was used. The study results showed no change in testis morphology, cell count or number of interstitial

cells. Regarding testosterone production, Aspirin showed a significant dose-response relationship increasing the level in the youngest fetal testis (8-9.86 GW) but not in the older (10-12 GW) and when all ages were pooled, no significant effect was observed. The AMH production was strongly stimulated by Aspirin and the PGE₂ was significantly inhibited. No significant effect was reported on the remaining parameters.

Study quality and assessment: The study is well-described but no CAS number (no.) and purity was reported for the substance used. The study is assessed to be of high quality and provides strong evidence for an endocrine disrupting (ED) mode of action (MoA) from Aspirin exposure.

Albert et al. (2013)

Summary: This study investigates the endocrine disrupting properties of mild analgesics in the adult human testis. Three types of analgesics were used in parallel studies but this summary will only cover the Aspirin part. Tissue used for the study came from prostate cancer patients who had not received any hormone therapy and NCI-H295R steroid producing human cell line. Only testis displaying spermatogenesis (assessed by transillumination) was used. Orchiectomy was performed and testis was transported on ice and processed as followed. The testicular tissue were fragmented and cultured by the procedure of Testis Explants Assay (TEXAS) and the media contained 10⁻⁵ and 10⁻⁴ M of Aspirin (purity > 99%). To determine the levels of hormones in the cultured media, Radioimmuno Assay was applied for the measurement of testosterone and insulin-like growth factor 3 (INSL3), Enzyme-Linked immunosorbent Assay was conducted to measure Inhibin B and for estimation of prostaglandins (PGD₂ and PGE₂) Enzyme Immunosorbent assay was applied. To evaluate gross morphology the testis explants were fixed in Bouin fixative, stained and examined under a light microscope. No change in the gross morphology of the testis was found. Measurements described in the following text include values after 24 h and 48 h of culturing. Testicular testosterone production was decreased but did not reach statistical significance. For the NCI-H295R cell line, exposure to 10⁻⁵ and 10⁻⁴ M Aspirin significantly reduced testosterone level. A reduction was also seen in the levels of INSL3, PGD₂, PGE₂ and Inhibin B production by the adult human testes.

Study quality and assessment: The study is well described and thorough although CAS no. of the substance used could not be found. It is assessed to be of high quality and since data only showed a significant value in NCI-H295R cell line the study provides moderate evidence for ED MoA by direct exposure to Aspirin.

Abend et al. (1991)

Summary: The aim of this study was to examine the feedback mechanism from T3 / T4 on TSH by looking at the role of type II 5'Deiodinase (5'D-II). The study was carried out in 3 parts of which only one part is relevant to this summary. A small *in vitro* investigation was conducted to look at the activity of 5'D-II and it was carried out using 100 and 200 pM [¹²⁵I]-T₄ as substrates. To investigate if sodium salicylate could be a direct inhibitor of 5'D-II, an assay was done with aliquots of normal pituitary homogenates for activity in the presence of 0, 2 and 10 mM sodium salicylates. The results from the study showed that the administrated dose of Salicylate did not inhibit the 5'D-II enzyme activity directly.

Study quality and assessment: The study has some shortcomings – the number of pituitaries used is not given and the CAS no. and purity of the substance used could not be found. The study is assessed to be of medium quality and it did not provide any evidence for an ED MoA on the activity of 5'D-II.

Larsen, P.R. (1972)

Summary: This study was conducted to clarify the effect of SA on the protein binding of the two thyroid hormones (T_3 / T_4) in human serum. The investigation was made up from several smaller studies.

1- The effect of addition of sodium salicylate on the ultrafiltrable fraction of T_4 (UFT₄) and T_3 (UFT₃) *in vitro*: Sodium salicylate was added in increasing amounts to pooled human serum. The concentrations of salicylate used were within the therapeutic (20-30 mg/100 ml) or toxic (60mg/100 ml) range (with humans as a reference for therapeutic and toxic values). A progressive increase in both of UFT₃ and UFT₄ was seen after sodium salicylate exposure..

2- The effect of dilution of human serum containing salicylate on UFT₃ and UFT₄: Two samples of human sera, one control and one salicylate-enriched were progressively diluted and a simultaneously determination of UFT₃ and UFT₄ was conducted. The result revealed a progressive decrease in the effect of salicylate when diluted and for UFT₃ it was significant at 1:2 dilutions.

3- Comparison of the effect of sodium salicylate and barbital on the binding of T_3 and T_4 in human serum and to human serum albumin: Diluted serum or human serum albumin was used in this study and a final concentration of sodium salicylate was established at 15 mg/ml. The result showed an increase of UFT₃ and UFT₄ for both Salicylate and barbital.

4- Effects of sodium salicylate on T_3 / Thyroxin-binding-prealbumine (TBPA): For this study the ultrafiltration system and simultaneous labeling with T_3 -¹³¹I and T_4 -¹²⁵I was used in the presence of 1.3×10^{-6} M TBPA and the result showed that addition of salicylate (15 mg/100 ml) increased both the UFT₃ and the UFT₄.

5- Effects of sodium salicylate on T_3 and T_4 binding to Thyroxine-binding-globulin (TBG): The binding was examined in an assay system described in an earlier study. The result showed that both T_3 and T_4 were displaced from TBG (thyroxine-binding-globulin) by sodium salicylate.

Study quality and assessment: The text is written with addition of several references to earlier studies regarding descriptions of methods used and in the section with results it is a challenge to distinguish result from the study conducted from results obtained from other experiments. No report of CAS no. and purity of the substance used could be found. The study quality is assessed to be medium but the evidence for a thyroid disrupting MoA of SA *in vitro* is strong.

Hansen and Mogensen (1964)

Summary: This study investigated the effect of sodium salicylate on the uptake of ¹³¹I-labelled 1-triiodothyronine by human erythrocytes. For this part of the study, human venous blood was obtained from donor blood and 4 portions of erythrocytes (2 x washed + 2 x unwashed) were prepared for use in a scintillation counter. To all samples ¹³¹I-labelled 1-triiodothyronine was added in the process (concentrations kept below 0,1 µg/ml) and 25 % W/v salicylic acid solution were added in proximately calculated amounts. The result from the study showed that by adding sodium salicylate to

the blood samples an increase in the T₃ uptake was seen in erythrocytes. An additional study was performed where erythrocytes were washed six times and then suspended in physiological saline solution (instead of plasma). No increase in the T₃ uptake was observed under those conditions. According to the authors the effects observed are a result of the influence of salicylate on plasma proteins and not a direct effect on the erythrocytes.

Study quality and assessment: The quality of the study is assessed to be medium. No information on CAS no and purity of the substance used was given and there is no report of any control group. The study provides no evidence for a thyroid disrupting MoA of sodium salicylate through binding of T₃ to erythrocytes *in vitro*.

Wolff et al. (1961)

Summary: The aim of the study was to investigate T₄ displacement from serum proteins in human serum after addition of natrium salicylate. The study consists of 3 experimental parts where only two of them are relevant to this assay. Pooled normal human serum (one batch) obtained from Baxter Laboratory was used in all studies and butanol solutions of thyroxine were added. For the first experimental part natrium salicylate ($3 \cdot 10^{-3}$ M) was added to serum before measurement by reverse flow electrophoresis. For the second part natrium salicylate ($3 \cdot 10^{-3}$ M) was added to the (NH₄)₂CO₃ buffer (pH was adjusted after addition). The displacement was measured at 4 different T₄-concentrations 0.15 µg T₄/ml (0.19 µM), 0.28 µg T₄/ml (0.36 µM), 1.54 µg T₄/ml (2.0 µM) and 2.08 µg T₄/ml (2.7 µM). The result from the first study showed that natrium salicylate tended to displace T₄ from Thyroxine-binding pre-albumin (TBPA) onto thyroxine binding globulin (TBG). However, the result was more reproducible in the second part where natrium salicylate was added to the buffer. In this part it was possible to measure a significant displacement of T₄ from TBPA to TBG at low T₄-concentrations and when the amounts of T₄ was higher, TBG was saturated and then T₄ was displaced and bound to albumin.

Study quality and assessment: The study is well-described but regarding the substance used it is only mentioned that it is of reagent grade but no CAS no. was given. Overall the study is assessed to be of high quality and it provides strong evidence for a thyroid disrupting MoA of natrium salicylate.

4.10.3.3 *In vivo* effects with regard to an endocrine mode of action

Kristensen et al. (2012)

Summary: This study aimed at investigating effects of mild analgesics (paracetamol, indomethacin and Aspirin) on *ex vivo* rat fetal testis development. Only results relevant to Aspirin will be included in this summary. To examine the effects of the mild analgesic, Sprague-Dawley rat testis (gestation day 14.5) was used in a 3 day *ex vivo* organotypic culture system with exposure to Aspirin at doses of 0, 1, 10 and 100 μM . Testosterone, Insulin-like factor 3 (Insl3) and prostaglandin D₂ (PGD₂) concentrations were determined in the medium at 24, 48 and 72 h by Coat-A-Count Total Testosterone Kit (testosterone), Enzyme-linked immune sorbent assay (Insl3) and Prostaglandin D₂-Mox EIA Kit (PGD₂). Leydig cells were prepared and counted by systematic random sampling in a CAST-grid stereotactic system with a reference to an earlier study. For evaluation of gonocyte apoptosis in the testis, a TUNEL assay with the in Situ Cell Death Detection Kit was conducted and finally gross morphology of the seminiferous tubules was examined. The result from the study showed significantly decreased testosterone levels at all Aspirin concentrations, with 20 – 75 % compared to control and for PGD₂ Aspirin led to a modest decrease in the production at all time-points. No significant data was obtained for gross morphology, leydig cells number and rate of gonocyte apoptosis.

Study quality and assessment: The study is well described and thorough all though there was no report of CAS number. and purity of the substance used. The study is assessed to be of high quality and it provides a strong evidence for an anti-androgenic ED MoA after Aspirin exposure.

Kristensen et al. (2011)

Summary: The study consisted of 3 parts (*in vivo*, *ex vivo* and epidemiology) and the *in vivo* part will be described here. This study had a focus on intrauterine exposure to mild analgesics as a risk factor for development of male reproductive disorders in rats. Pregnant rat dams were exposed to acetylsalicylic acid at doses of 150, 200 and 250 mg/kg/day from GD 13-21 with cesarean section conducted on GD 21. For dose-response analysis, anogenital distance (AGD) in male fetuses was analyzed by the calculated ADG index (AGDi) divided by the cube root of the body. Analysis of testosterone and the production by testes were performed by a method with a reference to an earlier study. The results showed reduced AGD compared to control but due to fetal growth retardation AGD was undetectable in a number of fetuses and statistical results are not presented. A significant reduction of testosterone by testes was measured.

Study quality and assessment: The study is well described although no CAS number and purity of the substance used could be found but overall the quality is assessed to be high. In the article it is noted that no signs of general toxicity were observed (no liver toxicity, normal maternal body weight gain, litter size, live fetuses, resorption, implantation and sex ratio when compared with controls). Thus, the findings regarding testosterone and AGD provide strong evidence of an anti-androgenic MoA after Aspirin exposure.

Kristensen et al. (2011)

Summary: The study consisted of 3 parts (*in vivo*, *ex vivo* and epidemiology) and the *ex vivo* part will be described here. This study had a focus on intrauterine exposure to mild analgesics as a risk factor for development of male reproductive disorders in rats. Testes from male rat fetuses (n=8) obtained by caesarean section on GD 14.5 were incubated with two testes in each experiment, for 3 days in a media with or without Aspirin at concentrations of 1 μ M and 10 μ M. The media concentration of prostaglandin D₂ (PGD₂) and testosterone were measured by Prostaglandin D₂-Mox EIA kit after 24, 48 and 72 h. The result from the study show a dose dependent reduction in testosterone and PGD₂ with a significant result for testosterone at 10 μ M Aspirin at all time points and a significant result for PGD₂ at 48 and 72 h.

Study quality and assessment: The study is well-described although no information on CAS number and purity of the substance used could be found. The quality is assessed to be high and it provides strong evidence for an anti-androgenic MoA.

Gupta et al. (2003)

Summary: The aim of this study was to compare the developmental toxicity of Aspirin (CAS 50-78-2) in rats using selected dosing paradigms. To allow a direct comparison between the responses of Sprague-Dawley (SD) rats (in this study) and Wistar rats (from Kimmel et al.1971), the study design and dose levels were based on the work of Kimmel et al (1971). The study was conducted in two parts with a single dose study and a multiple dose study and in both cases, timed-mated SD rats were assigned. In the first study (single dose) groups of 7 rats were orally exposed (by gavage) to acetylsalicylic acid (ASA) on gestation day 9 (0, 250, 500 or 625 mg/kg), 10 (0, 500, 625 or 750 mg/kg) or 11 (0, 500, 750, 1000 mg/kg). In the second study (multiple doses) groups of 20 rats were orally (by gavage) treated with ASA from gestation day 6 to 17 at concentrations of 0, 50, 125 or 250 mg/kg. On gestation day 21 all rats were killed, and fetuses were examined and following parameters were noted: numbers of corpora lutea, implantation sites, late and early resorptions, viable and dead fetuses, individual fetus weight, placenta weight and finally all fetuses were examined for external and visceral anomalies and developmental variations with focus on ventricular septal defects (VSD) and midline defects (MD). The results from the study showed a high concordance between Wistar and SD rats regarding developmental anomalies with the exception to hydrocephalus in Wistar rats and the VSD in the SD rats. Whether ASA was administrated as a single dose or during the organogenesis (GD 6-17), the malformations were similar. All registrations of malformations are presented in a table and only in the high dose-group are they statistically significant. Hypoplastic testes were seen in 2 out of 137 fetuses and only in the highest dose group and along with ectopic adrenals, ablepharia was only detectable in the multiple dose study and not in the single dose study.

This paper is included in the REACH registration dossier for Salicylic acid on developmental toxicity / Teratogenicity

Study quality and assessment: The study is well-written and thoroughly described and contains details on both animal housing conditions and CAS number of the substance used and it is assessed to be of high quality. The results provide weak evidence for ED-related adverse effects after exposure to Aspirin

Conte et al. (1999)

Summary: The aim of this study was to examine the effect of Aspirin on plasma testosterone, pregnenolone, progesterone, 17OH-progesterone, androstenedione, dehydroepiandrosterone and 17 β -estradiol in response to human chorionic gonadotropin (hCG). Healthy men (n=8) age 20-30 years was examined in a placebo-controlled, single-blinded study and to test the efficacy of Aspirin as a prostaglandin-blocker an additional study was conducted where seminal prostaglandin E₂ (PGE₂) were determined at the same doses and times used in the experimental protocol. All subjects received the same four treatments, separated by an interval of at least 1 month. 1) Placebo Aspirin + hCG, 2) Aspirin + hCG, and for control 3) Aspirin + placebo hCG, 4) placebo Aspirin + placebo hCG. Administration of 1 tablet (800 mg) Aspirin or placebo Aspirin was done orally two times a day for 7 days (i.e. 2 days before and 5 days after administration of hCG or placebo hCG). For the experiment 5000 IU hCG was injected intramuscular (IM) and blood samples were collected after 2, 24, 48, 72 and 96 h and in the same period, all participants were asked to collect semen specimens (by masturbation) for PGE₂ measurements. Parameters measured by radioimmune assay were plasma testosterone, pregnenolone, progesterone, 17OH-progesterone, androstenedione, dehydroepiandrosterone, 17 β -estradiol and PGE₂. The study result showed that Aspirin significantly lowered the seminal level of PGE₂ and significantly reduced the response of testosterone, 17 OH-progesterone, androstenedione and dehydroepiandrosterone to hCG stimulation (assessed by the mean integrated area under the curve). No change was seen with regard to 17 β -estradiol, pregnenolone and progesterone when hCG was administrated.

Study quality and assessment: The study is well-described and thorough with the exception that CAS no and purity of the substance used was not given. The study is assessed to be of high quality and because of the significant results it provides strong evidence that the androgen response to hCG is inhibited by Aspirin treatment.

Davis et al. (1996)

Summary: This study has a two-fold purpose. One part investigates the effect on maternal reproduction in rats after oral exposure to salicylic acid. The other part looks at the structure-toxicity relationship between acetylsalicylic acid (ASA) and salicylic acid (SA) with respect to the effects measured in part one. Sprague-Dawley virgin female rats (n=105) at the age of 63 days was mated and the presence of a copulating plug marked gestation day 0. Randomly assigned animals were divided into dose groups receiving SA at the level of 0 mg/kg/day, 20 mg/kg/day, 80 mg/kg/day, and 200 mg/kg/day. A single group received a dose of ASA at 260 mg/kg/day. All groups were exposed by oral gavage on day 15-21 during gestation and administration of the compound was conducted twice a day (one half morning / one half 6-8 h after). Parameters recorded from the dams were body weight (measured on day: 0, 6 and 15-21), duration of gestation, labor time (time between the first and last born) and gross examination of uterus and ovaries (post-mortem). Registrations from pups included examination for external abnormalities, number of viable/non-viable, sex determination and weight. The results of the study showed that the groups exposed to SA 200 mg/kg/day and ASA 260 mg/kg/day had a delay in the onset of labor, an increase in labor time and a significantly increase in maternal perinatal mortality. Regarding the treatment-associated fetotoxicity only the group exposed to ASA 260 mg/kg/day showed a significant increase in stillborn pups and peripartum death. Additionally, ASA and SA were well-tolerated in all treatment groups No substantial potency difference between the salicylate congeners, ASA and SA could be established only the differences in toxicity profile were evident.

Study quality and assessment: The study is well-described but no information about CAS no. and purity could be found regarding sodium salicylate and acetylsalicylic acid. Overall the quality of the study is assessed to be high and from the result found it provides a moderate evidence of adverse effect on maternal reproduction and fetotoxicity including prolongation of labor and gestation after SA exposure.

Abend et al. (1991)

Summary: The aim of this study was to examine the feedback mechanism from T₃/T₄ on TSH by looking at the role of type II 5'Deiodinase (5'D-II). The study was carried out in 3 parts of which only one part is relevant to this summary. Three groups of male Sprague-Dawley rats were exposed to 1) Intraperitoneal injection of 40 nM NaOH vehicle (control), 2) Intraperitoneal injection of 2 µmol/100g 3-methyl-4',6-dihydroxy-3',5'-dibromo-flavone (EMD 21388) and 3) 30 mg/100 g sodium salicylate administered by oral gavage. All rats were sacrificed 1 h after exposure by decapitation and trunk blood and pituitaries was collected. From serum, values of thyroid-stimulating hormone (TSH), total T₄, total T₃ and free T₄ was measured by Radioimmunoassay (RIA) and the level of salicylates was determined by a modification of Trinder's method with a reference to an earlier paper. The results from the study showed that administration of salicylates significantly decreased the serum total T₄ concentration.

Study quality and assessment: Although sodium salicylate was not the main focus of the study, the findings are relevant to this summary because a significant decrease in the serum total T₄ concentration was observed (value of free T₄ is not given for salicylate). In general the article has a number of shortcomings for instance, the age and number of animals used is not given in the text, only body weight is mentioned and no information on CAS number and purity of the chemical used could be found. Overall the study is assessed to be of medium quality and it provides moderate evidence for a thyroid disrupting MoA after exposure to sodium salicylic acid.

Overman and White (1983)

Summary: The aim of this study was to compare the teratogenic effect of methyl salicylate in hamsters after oral and topical exposure. Virgin female hamsters were individually mated and the pregnant rats were grouped into either an oral or topical treatment group. For Oral exposure (by intubation) two dose levels were established at 0 mg/100 g bw (control) and 175 mg/100 g bw. For the topical application four groups were established at levels of 0 mg/100 g bw (control, shaved and treated with saline solution and washed after 2 h.), 0 mg(control, anesthetized with Nembutal and shaved), 350 mg/100 g bw (applied to a clipped area and washed after 2 h), 525 mg/100 g bw (applied to a clipped area and washed after 2 h). All exposures to methyl salicylate were conducted once at 7 days and 9 hours into the pregnancy and at day 9-12 all animals were sacrificed. To monitor if the topical and oral doses were received by the animals, blood samples were obtained on a regular basis by heart puncture under light ether anesthesia and centrifuged for spectrophotometrically reading and to a 50 µl blood sample, 1 ml of modified Trinder reagent was added which precipitates protein and gives a color if salicylate is present. To determine if salicylate also reached the embryos an assay was done with a pooled sample of fetuses (oral treatment group) and compared to maternal salicylate levels from blood samples taken at the same time (assayed by the same procedure). Morphological examinations of the embryos were done on day 9 of gestation. The study result showed similar embryo malformations in both treatment groups and included both the cranium and spina where

failure of closure involved the midbrain region. The monitoring result showed a higher plasma concentration and a faster peak of salicylate after oral exposure than after topical treatment and regarding the comparison of salicylate levels in fetus and mother, it showed that salicylate was reaching the fetus but in lower concentration.

Study quality and assessment: The study is well-described but more information on housing conditions, CSA no. and purity of the substance used would have been preferred. The number of maternal animals used and the size of each litter are not given in the text or any table but the number of litters can be found in table 1. No statistics were conducted. Overall the study is assessed to be of medium quality. The study provides moderate evidence for developmental adverse effects on skeletal malformations after oral and topical exposure to methyl salicylate and no evidence for ED related adverse effects.

Didolkar et al. (1980)

Summary: This study examines the effect of Aspirin (acetylsalicylic acid) on spermatogenesis in rats. The study examined two age-groups of male albino rats (n=12), Norwegian strain. Group one: Immature male rats, age 21-24 days and Group two: Adult male rats of proven fertility - both groups received doses of Aspirin at 0 mg/100 g bw (control) and 5 mg/100 g bw by oral administration once daily for 30 days. All animals were killed 12-13 h after the last exposure and testis was excised, blotted and weighed. Samples from testis were collected for histology, assays for hyaluronidase, β -glucuronidase and sorbitol dehydrogenase were conducted and testicular nucleic acids were extracted and estimated. The result from the study shows that Aspirin caused a significant ($P < 0.05$) decrease in testicular weight in the group of immature rats. A decrease in the activity of testicular enzymes was observed for hyaluronidase and sorbitol dehydrogenase in both groups. Regarding spermatogenesis, for both groups, Aspirin caused an impairment of the later stages by a decrease in numbers of spermatids and morphologically it was registered that the nuclei of the pachytene spermatocytes were increased (statistically insignificant).

Study quality and assessment:

The study is well-described but assessed to be of medium quality due to the lack of information regarding CAS no. and purity of the substance used, and there is no information on general toxicity in the animals. The study provides strong evidence for adverse effects on spermatogenesis after Aspirin exposure.

Balasubramanian and Ramakrishnan (1979)

Summary: The aim of the study was to investigate the effect of acetylsalicylic acid (Aspirin) individually and in combination of prostaglandins (PGs) on carbohydrate and thyroid metabolism in rats. An *in vivo* and an *in vitro* study were performed, but the *in vitro* part and parts of the *in vivo* study focused on glucose and glycogen and will not be included here. The following groups were established: Control (*ad libitum* feeding), control (*pair-fed*), Aspirin 30 mg/day for 6 weeks (Chronic), Aspirin 60 mg as single dose (acute group), PGF_{2 α} 100 μ g intraperitoneal, PGE₂ 100 μ g intraperitoneal (IP), Aspirin (60 mg, single dose) + PGF_{2 α} , Aspirin (60 mg, single dose) + PGE₂ and a withdrawal group with 2 exposure-free weeks following exposure to 30 mg/day for 6 weeks. Aspirin was administered through the diet and the exposure to radioactive iodine (Na¹³¹I) and (U-14C) glucose (5 μ Ci) was administered by intraperitoneal injection. Parameters measured for the *in vivo*

part included %uptake of injected radioactive iodine in thyroid and serum protein-bound iodine. The result from the study showed a decrease in percentage uptake of injected Na^{131}I and plasma protein-bound iodine (PBI) by the thyroid gland in the groups exposed to Aspirin (acute and chronic) and Aspirin + PGs but no effect on the parameters was seen in the $\text{PGF}_{2\alpha}$ and PGE_2 groups. In the group where Aspirin was withdrawn from the diet the two parameters were restored to normal levels.

Study quality and assessment: The description of the study has a number of shortcomings starting with the section of materials and methods where information regarding treatment, size of the groups, housing conditions and procedures for preparation and administrations of PG, radioactive iodine and IP injected glucose is referred to earlier studies. No report of CAS no. and purity could be found for the substance used. Based on the lack of information the study is assessed to be of low quality but it provides moderate evidence for a thyroid disrupting MoA of Aspirin

Beall and Klein (1977)

Summary: This study was designed to determine if maternal food restriction would enhance the teratogenic effects of salicylic acid. Charles River, CD rats were mated and day 0 of pregnancy was determined by sperm in vagina. From a group of 49 pregnant rats four groups of similar size were established and received I) Food *Ad Libitum* (control), II) Food *Ad Libitum* + 250 mg/kg acetylsalicylic acid adm. orally by gavage (0.5 ml/100 g bw) suspended with vehicle (2,5% aqueous Tween 80) from day 7-10 of pregnancy, III) Restricted food (6 g/day) from day 6-15 after mating along with vehicle (control) and IV) Restricted food + 250 mg/kg acetylsalicylic acid administration orally by gavage (0.5 ml/100 g bw) from day 7-10 of pregnancy. Food consumption was measured on a daily basis, all rats were weighed every 3 days and on day 21 after mating all rats were killed. Parameters investigated included offspring abnormalities, number of live pups, sex, bw of the pups, litter size and resorption sites. For examination of skeletal defects two-thirds of the pups from each litter were fixed and stained with alizarin red and for soft tissue examination the rest were fixed in Bouin's solution and examined by serial slicing. The result from the study showed statistical significant values for increase in resorption sites for group IV, reduced mean body weight of pups in group II and increase in developmental defects (rib abnormalities, craniorachischisis and umbilical hernia, eye defects) in both Aspirin-treated groups. The combination of food restriction and exposure to Aspirin increased the incident of abnormalities from 24.4 % in group II (=32 pups) to 66.3 % (=59 pups) in group IV.

Study quality and assessment: The study is well-described but more information on housing conditions and the age of the rats would have been preferred and there was no report of CAS no. and purity of the substance used. The study is assessed to be of medium quality and it provides a moderate evidence for developmental adverse skeletal and soft tissue effects induced by Aspirin.

Wilson et al. (1977)

Summary: This study investigates the embryo toxicity and comparative distribution of acetylsalicylic acid in pregnant rats and rhesus monkeys. For all animals, gestation day 0 was assigned when sperm was found in vaginal lavage. First, two studies on embryotoxicity were performed. In the rat study, a weight adjusted volume of acetylsalicylic acid (suspended in 0.3% aqueous solution of carboxymethyl-cellulose) was administrated orally (by gavage) twice a day on gestation day 9-12 at doses of 0 (control), 100, 150, 175 and 200 mg/kg (2-8 litters/dose gr.). Embryo removal was

conducted at 1, 2, 4, 8 or 17 h after last exposure on GD12 or they were allowed to continue their pregnancy and removed on GD20. Blood samples for preparation of plasma were taken by cardiac puncture under light ether anesthesia at 1, 2, 4, 8 and 17 h after exposure. For the pregnant monkeys (n=8) acetylsalicylic acid was administered orally (by gavage) twice a day on gestation day 23-32 at doses of 100 and 150 mg/kg (no report of a control group). Blood sample for serum preparation was taken by venipuncture at day 4, 5 or 10 at 1, 2, 4, 8 and 17 h after gavage. Hysterectomy was performed at same intervals after the last gavage. For both groups of animals, parameters measured were plasma binding, and concentration of salicylic acid in plasma, placenta and embryonic tissues and finally all fetuses were examined for external, visceral and skeletal abnormalities. Monkey embryos were additionally examined for heart beat. To compare the distribution of acetylsalicylic acid in rats and monkeys, an additional rat study was performed and similarly to the monkey study 100 mg/kg was chosen as the low dose and 150 mg/kg was set as the high dose in this rat study. The study result from the rat part showed a significant effect on intrauterine death, growth and malformation (cardiac, brain and skeletal) at doses of 150 and 200 mg/kg. Maternal plasma concentration was significantly higher in non-pregnant than pregnant rats at 100 mg (2 and 17 h after dosing) and 150 mg/kg (after 2 h) and the teratogenic (maternal) dose of 150 mg/kg resulted in an embryo concentration greater than 60 µg/g maintained for minimum 16 h/day. In monkeys both exposure doses (100 and 150 mg/kg) resulted in a slight increase in intrauterine death and transitory growth retardation. Maternal plasma concentration was not consistent but showed a significant difference between non-pregnant and pregnant animals at 100 mg/kg with a significant higher concentration in non-pregnant at 2, 4 and 8 h after dosage. The mean embryo concentration never exceeded 36 µg/g in monkeys. When the two groups of animals were compared, the highest value of unbound salicylate was found in rat plasma.

Study quality and assessment: Overall the study is well described although information on CAS number, and purity of substance used was not available and more information on housing conditions would have been preferred. The study is assessed to be of medium quality and since the exposure doses were well tolerated by the maternal animals at levels below 200 mg/kg the study provides high evidence for adverse effects on embryonic development, growth and survival in rats and moderate evidence for adverse effects on embryonic growth and survival in monkeys.

Tuchmann-Duplessis et al. (1975)

Summary: The aim of the study is to look at the effects of prenatal administration of acetylsalicylic acid (ASA) in rats. Two groups of pregnant rats (COBS CD Charles River) were established and randomly divided in two dose groups (n=16/group), 0 mg/kg/day and 200 mg/kg/day. ASA, suspended in 1% tragacanth gum, was administered by gastric intubation twice a day starting on day 15 until the end of pregnancy. Registrations were made on gestation length, parturition time and effects on offspring (stillborn and death). The results revealed a statistical significant difference between the two groups with a prolongation of pregnancy in treated dams. The parturition time was also prolonged and in the treated group 2 out of 16 dams died due to an extended period of contractions. No significant observations were registered regarding effect on offspring survival. The dams tolerated the treatment well with no mortality or change in behavior during exposure.

Study quality and assessment:

The study is well-described although the purity of the tested substance is not reported and more information on housing conditions would have been preferred. Overall the study is assessed to be of medium quality and it provides strong evidence for adverse effects on gestation length and parturition.

Larsen, P.R. (1972)

Summary: This study was conducted to clarify the effect of SA on the protein binding of the two thyroid hormones triiodothyronine (T₃) and thyroxine (T₄). An *in vivo* and an *in vitro* experiment was conducted. In the *in vivo* part of the study Aspirin was administered to humans (n=2) for a period of 8-10 days in quantities sufficient to obtain a serum salicylate level of 20-25 mg/100 ml. Three baseline determinations were obtained during a 6 day control period prior to the study. During the period of treatment samples of serum were collected every other day and the free T₃ and free T₄ was estimated by ultrafiltration (UF). The results from the study showed an immediate and persistent increase in the UFT₃ and UFT₄ in both humans.

Study quality and assessment: The study is not described in a structured way and it contains several references to earlier studies for description of methods used. There was no report of batch number of the Aspirin used. The *in vivo* part is assessed to be of medium quality. Although only two subjects were assigned to the study, it provides moderate evidence for thyroid ED MoA after Aspirin exposure.

Collins et al. (1971)

Summary: The aim of this study was to investigate the effect of methyl salicylate on rat reproduction. The study included a main part and a supplemental study but due to a mixed-compound exposure in the supplemental study, the result was found to be non-relevant and only the main part will be included in this summary. Osborne-Mendel rats were divided in groups of 20 pair (litter mated) and fed methyl salicylate through the diet for 100 days prior to mating at the levels of 0, 500, 1500, 3000 and 5000 ppm. Two litters, F_{1a} and F_{1b} were produced by F₀ and on day 4 all litters were reduced to include maximum 10 pups per litter. At weaning F_{1b} were pair-housed and mated (20 pairs per group). Same procedure was followed for the following generations. Parameters investigated were fertility index (number of litters cast/number of females exposed to mating), Litter size, number of live born, viability index (number of live-born/total number born), surviving from day 0-4, survival index (number alive at day 4/number born alive), number of progeny weaned at day 21, weight of weanlings and abnormalities by external examination. Autopsy and histopathological examination (liver and kidney) was only performed on the weanlings from the third generation. The results from the study revealed significant findings at dose levels of 3000 and 5000 ppm regarding a decrease in the average litter size, number of live-born progeny, number of survivors to day 4 and number of survivors to day 21. The decrease in number of live-born appeared to be dose-related. At the lower dose levels only a non-significant decrease was observed.

This paper is included in the REACH registration dossier for salicylic acid on toxicity to reproduction.

Study quality and assessment: The study has a number of shortcomings. For example, the details on diet preparations, the description of the 3 generation study and the reason for the choice of concentrations refers to earlier studies and is only roughly described in the text. No report of CAS number and purity of the substance used could be found. Overall the study is assessed to be of medium quality and it provides moderate evidence for reproductive adverse effect after exposure to methyl salicylate during pregnancy.

Hansen and Mogensen (1964)

Summary: This study investigated the effect of sodium salicylate on the uptake of ¹³¹I-labelled 1-triiodothyronine by human erythrocytes. Human patients (n=9) were given 1 g sodium salicylate three times a day for 4 days and serum concentrations of salicylic acid were measured. From a 20 ml venous blood sample, 4 portions of erythrocytes (2 x washed + 2 x unwashed) were prepared for use in a scintillation counter. To all samples ¹³¹I-labelled 1-triiodothyronine was added in the process (concentrations kept below 0.1 µg/ml). The study result showed an increase of triiodothyronine (T₃) uptake in erythrocytes of patients given salicylate.

Study quality and assessment: The the study is assessed to be of medium quality. No information on CAS number and purity of the substance used was given and there is no report of any control group. The evidence for a thyroid disrupting MoA is moderate.

Warkany and Takacs (1959)

Summary: The aim of the study was to conduct an experimental production of congenital malformations in rats by salicylate exposure. The study was composed of two experimental parts. In the first part 116 female rats were mated and the presence of sperm in vagina marked the first day of pregnancy. One single dose of 0.1-0.5 cc (cubic centimeter) methyl salicylate was administrated subcutaneously on gestation day (GD) 9, 10 or 11. In the second study 43 pregnant rats received a single dose of 60-180 mg sodium salicylate also administrated subcutaneously on gestation day 9, 10 or 11. For control group, 105 females were used. The results obtained in the first part (methyl salicylate) showed a weight reduction in most of the pregnant females of 15-25 g after treatment, 26 of the 116 dosed animals died and 47 had fetal resorbtions. The last 43 pregnant rats were sacrificed on gestation day 21 and 298 progeny were obtained. At examination 45 of the offspring were externally visible malformations and in the remaining 253 pups that appeared externally normal, 75 showed skeletal abnormalities. In the second study (sodium salicylate) 6 of the 43 rats died (all dosed more than 120 mg) and 24 had fetal resorbtions. The remaining 13 were sacrificed on GD 21 and 100 progeny were obtained. At examination 15 were externally abnormal and in the group of 85 that was externally normal, 11 had skeletal abnormalities. In both groups malformations that were encountered were eye defects, fascial clefts, hydrocephaly, exencephaly, gastroschisis and irregularities of the vertebrae and ribs. In the control group of 105 female rats, 59 produced a litter and 484 progeny were obtained. No externally malformations were observed with the exception of 3 which had “wavy ribs”

Study quality and assessment:

The study has a number of shortcomings starting with no information on CAS number or purity of the two substances used. More information on housing condition, and the allocation of rats into dose groups would have been preferred and from the information given in the article it seems that the doses given is administrated with no consideration to body weight. Moreover, all progeny were pooled for

each group and litter effects were not taken into account. All data is only described in the text and not presented in any tables and no statistical analysis was conducted. The study quality is assessed to be low and due to the number of dead dams and resorptions it provides a weak evidence for adverse effect on fetal development.

Epidemiology study

Kristensen et al. (2011)

Summary: This study consisted of 3 parts (*in vivo*, *ex vivo* and epidemiology) and the epidemiology part will be described here. The focus of the study was to evaluate maternal use of mild analgesics during pregnancy in relation to congenital cryptorchidism in humans. A prospective birth cohort study was conducted in collaboration between the university hospital of Copenhagen (Rigshospitalet and Hvidovre Hospital) and the Turku University Central Hospital in Finland with the use of a self-administrated questionnaire (assessing the use of mild analgesic by indication, name, dosage, and gestational week of administration), completed by 2297 women from both countries and a computer-assisted interview over the telephone (addressing the use of analgesic), where 491 of the Danish mothers participated. Following criteria were established to obtain a genetically well-defined population: both the parents and grandparents of the unborn child should have been born and raised in Finland or Denmark with a maximum residence abroad of 10 years for the grandparents and father and 3 years for the mother. A total of 2521 mothers entered the Danish part of the study and 1071 boys were examined, of those 5 were excluded as dependent cases and 26 excluded due to missing data. From Finland a total of 2728 mothers participated were 1499 boys were examined and from that group 25 were excluded as dependent cases along with 4 due to missing data. The assessment of the testicular position in the newborns was performed by trained pediatricians. In the Danish part, findings in the self-administrated questionnaire indicated that many mothers strongly under-reported their use of analgesic unless they were specifically asked and for that reason only the results from the computer-assisted telephone interview were taken into account. The data from that part, showed that the use of mild analgesic was dose-dependently associated with congenital cryptorchidism and especially the use during the second trimester increased the risk - for acetylsalicylic acid data was reported to be significant. In the finish cohort the same association could not be identified, only a trend was seen in the second trimester.

Study quality and assessment: The study is well-described and is assessed to be of high quality. The study provides strong evidence of adverse effects of acetylsalicylic acid on male sexual development leading to congenital chryptorchidism.

Other studies

In addition to the above described studies a number of experiments have been conducted where the results are not directly related to an endocrine disrupting (ED) action but a possible contribution cannot be excluded.

Schardein et al (1969) presented a study with focus on male/female fertility (rats), the teratogenic potential (rats and rabbits) and the effect of treatment in the perinatal and postnatal period (rats). It should be noted that treated animals in all groups showed moderate to severe reduction of weight gain. Skeletal malformations, reduced litter size and reduced viability of the pups were noted and for the dams treated with aspirin in large doses (> 210 mg/kg) all pregnancies resulted in resorption of all fetuses.

Cappon et al. (2003) conducted a study with focus on comparing the developmental toxicity of Aspirin (acetyl salicylic acid) in rabbits when it was administered throughout organogenesis or during sensitive windows of development. A repeated dose study was conducted on GD 7-19 with doses of 125, 250 and 350 mg/kg and a single dose study was conducted on day 9, 10 or 11 with dose levels of 500, 750 or 1000 mg/kg. On GD 29 cesarean sections were performed and an examination of fetuses was done with focus on external, visceral and skeletal development but the results from the study showed no malformations associated with the exposure to Aspirin.

Erikson (1970) investigated the role of dosage and the frequency of administering on the prenatal effect in rats produced by salicylate. Late pregnancy effect in the fetuses included superficial liver and gastric hemorrhage and vessel abnormalities and increased death.

REACH Registration Dossier

Toxicity to reproduction :

Outline:

Following studies and reviews from “Toxicity to reproduction” in the REACH registration dossier has already been included here (*Collins et al.1971 and Chapin and Sloane 1997*) and summaries of their data can be found under the section for *in vivo* studies or reviews. The applicant also reported data from several additional studies and study reports that were not possible to retrieve in a search conducted up to the 15/11-2017. Based on information available in the REACH registration Dossier a short summary of all the additional studies has been added below.

Summary:

The studies did not add significant value or new information to the ED MoA or endocrine-related adverse effects on salicylic acid. In general the studies show a dose-related decrease in the average litter size and pup weight, effects on offspring viability and some studies investigated the effect on male/female reproduction but without any significant findings.

Study quality and assessment:

The quality of the unavailable studies cannot be assessed based on summaries available on REACH registration dossier. They all report to have a minimum of 20 animals/ dose group and a few is performed under GLP and follow a guideline. In the experiments that observe some changes, half of them report that the findings were significant so in general the studies are assessed to provide a moderate evidence for adverse effect on reproduction.

Developmental toxicity / Teratogenicity :

Outline:

Following studies and reviews from “Developmental toxicity / Teratogenicity” in the REACH registration dossier has already been included here (*Cappon et al.2003, Gupta et al.2003, Eriksson et al.1971 and Shardein et al.1969*) and summaries of their data can be found under the section of *in vivo* and *in vitro* studies. The applicant also reported data from several additional studies and study reports where some of them were available but did not provide any relevant data on ED endpoints and others were not possible to retrieve in a search conducted up to the 15/11-2017. Based on information available in the REACH registration Dossier a short summary of all the additional studies has been added below.

Summary:

Overall the studies did not add significant value or new information to ED MoA or endocrine-related adverse effects on salicylic acid. Most of the studies were conducted according to OECD guideline 414 and the study design of the remaining studies did not appear to follow a guideline. In general the studies provided information on decreased litter size and neonatal body weight, increased labor time, increased resorptions, skeletal abnormalities (vertebral and costal bones), hydrocephalus, delayed ossification, internal abnormalities (kidney), subcutaneous, liver and gastric hemorrhage and a reduction in fetal liver glycogen. A few studies presented new data showing that increasing dietary zinc reduced the teratogenic effects of salicylate, but in patterns different to strain (Wistar or Sprague-

Dawley) and from that suggesting that marginal zinc deficiency in certain pregnant women might increase the possibility of salicylate teratogenesis (Hackman et al.1984) but it was also concluded that Zinc deficiency did not cause any lesions detectable in fetal kidneys (Gossrau et al.1988). The new results presented do not contribute with results that are relevant for the ED MoA of Salicylic acid.

Study quality and assessment:

The quality of the studies cannot be assessed based on summaries available on REACH registration dossier but the largest part of the studies contained 15 animals or more per dose. Overall the additional part of the studies does not provide information relevant to the ED effect of Salicylic acid.

Other studies:

Outline:

A large part of the studies presented under “Other studies” in the REACH registration dossier were not possible to retrieve in a search conducted until the 15th of November 2017 and studies with endpoints that were considered to be of non-endocrine disruptive character has not been included here (abnormality of tail, facial malformations, decreased heart beat and focus regarding mouse palatal)

Repeated dose toxicity – Dermal + Oral:

Outline:

Overall the studies under “Repeated dose toxicity” in the REACH registration dossier, presents data that show an effect on the kidneys, liver, skin (dermal lesions) and bones (density and lesions). Data did not contribute with results that are relevant for the ED effect of Salicylic acid.

Reviews

Reviews on SA have been evaluated in the search for additional relevant studies not found in the literature search (Kristensen et al. 2016; Lapczynski et al. 2007; Belsito et al. 2007; SCCNFP 2002; Chapin and Sloane 1997). One of the reviews is included in the REACH registration dossier for Salicylic acid on toxicity to reproduction (Chapin and Sloane 1997).

4.10.3.4 Summary of the plausible link between adverse effects and endocrine mode of action

The androgenic activity is also investigated *in vitro* and *ex vivo* and the studies showed decreased testosterone production after acetylsalicylic acid exposure in all cases except one. The three studies showing a decrease includes investigations in H295 cells (Albert et al.2013) and *ex vivo* rat fetal testicular tissue (Kristensen et al. 2012 and Kristensen et al. 2011). However, *ex vivo* studies in human testis did not show clear reductions in testosterone production after acetylsalicylic acid exposure. One study showed a reduction in testosterone levels in adult testis but it did not reach statistical significance (Albert et al.2013) and another study found results showing that Aspirin stimulated testosterone production in testis from gestation week 8-9 but no effects were seen in older fetal testis (Mazaud-Guittot et al. 2013). It is unclear whether the differences in effects on *ex vivo* testosterone levels are due to species differences or if there is a difference in either the methods used or the sensitivity of the testis at different time-points. Taken together, both the *in vitro* and the *ex vivo* data provide moderate evidence of an anti-androgenic mode of action of acetylsalicylic acid. The human studies available show that Aspirin significantly inhibit the androgen response to hCG stimulation in humans (Conte et al. 1991) and a significant decrease in testicular weight together with a decrease in the activity of testicular enzymes and an impairment of the later stages in the spermatogenesis were found by (Didolkar et al.1980). The adverse effects observed in human testis are likely related to the anti-androgenic MoA of acetylsalicylic acid. When all results are taken into consideration the data provide moderate evidence for an anti-androgenic ED MoA and adverse effects after exposure to acetylsalicylic acid.

The ED MoA of Salicylic acid has been investigated in several *in vivo*, *ex vivo* and *in vitro* studies (Table 1). Starting with the results from *in vitro* studies concerning the thyroid function, some of the studies found that exposure to salicylates increased the free fraction of T₃ and T₄ (Larsen et al.1972). Going further into details, other studies found that salicylate affects the binding capacity between T₄ and TBPA (Wolff et al.1961) and that T₄ then is displaced to TBG (Wolff et al.1961). A similar action of salicylate on the binding of T₃ to TBPA and TBG was shown (Larsen et al. 1972) and it appears that this leads to an increased binding to erythrocytes *in vivo* (Hansen and Mogensen, 1964). Available human studies provide results that back up the *in vitro* findings. A human study showed an increase in free T₃ and T₄ after Aspirin exposure and this was in line with their *in vitro* results (Larsen et al.1972). Furthermore, studies showed a decrease in total serum T₄ concentration (Abend et al. 1991) and increased uptake of T₃ by erythrocytes after salicylate treatment in humans (Hansen and Mogensen, 1964). Together, both the *in vitro* and the human studies provide strong evidence of a thyroid disrupting mode of action of salicylates and when all data are taken together, they provide strong evidence of a thyroid disrupting MoA of salicylates. No studies investigated endpoints relevant for evaluation of adverse effects related to thyroid disruption were found (Table 2).

In conclusion, salicylates meet the WHO definition of an endocrine disruptor with anti-androgenic ED MoA leading to adverse effects.

Table 1. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of Salicylic acid (SA).

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In Vitro</i>	<i>In Vivo</i>		
Albert et al 2013	The production of testosterone by human testis revealed a decreased level but did not reach statistical significance. In the NCI-H295R cell line, exposure to 10 ⁻⁵ and 10 ⁻⁴ M Aspirin significantly reduced testosterone production. A reduction was also seen in the levels of INSL3, PGD ₂ , PGE ₂ and Inhibin B production.		High	moderate
Mazaud-Guittot et al. 2013		Aspirin showed a significant dose-response relationship by increasing the level of testosterone in the youngest fetal testis (8-9.86 GW). Anti-Müllerian Hormone production was strongly stimulated. PGE ₂ was significantly inhibited	High	Strong
Kristensen et al. 2012		Decreased testosterone levels in rat fetal testis were found at all Aspirin concentrations. For PGD ₂ , Aspirin led to a modest decrease in the production at all time-points	High	Strong
Kristensen et al. 2011		The results showed reduced AGD compared to control but due to fetal growth retardation AGD was undetectable in a number of fetuses and statistical data are not presented. A significant reduction of testosterone was measured.	High	Strong
Kristensen et al. 2011		Dose dependent reduction in testosterone and PGD ₂ production in rat fetal testis with a significant result for testosterone at 10 µM Aspirin at all time points and a significant result for PGD ₂ at 48 and 72 h.	High	Strong

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In Vitro</i>	<i>In Vivo</i>		
Conte et al. 1999		Aspirin significantly lowered the seminal level of PGE ₂ and significantly inhibited the androgen response of testosterone, 17 OH-progesterone, androstenedione and dehydroepiandrosterone to hCG stimulation in humans.	High	Strong
Abend et al 1991	The result did not provide evidence for the administrated doses of salicylates to directly inhibit enzyme activity.		Medium	none
Abend et al 1991		Administration of salicylates significantly decreased the serum total T ₄ concentration.	Medium	Moderate
Balasubramanian and Ramakrishnan 1979		Decreased percentage uptake of injected Na ¹³¹ I and plasma PBI by the thyroid gland in the groups exposed to Aspirin (acute and chronic) and Aspirin + PGs.	Low	Moderate
Larsen et al. 1972	Five smaller studies all confirmed the endpoint that addition of salicylate to human sera caused an increased in free T ₃ and T ₄	Increase in free T ₃ and T ₄ after administration of Aspirin to humans (n=2)	medium	In vitro – Strong In vivo – Moderate
Hansen og mogensen 1964	Increased uptake of ¹³¹ I-labelled 1-triiodothyronine by human erythrocytes after addition of sodium salicylate to human donor blood.	Increased uptake of ¹³¹ I-labelled 1-triiodothyronine by human erythrocytes in blood from humans exposed to sodium salicylate	Medium	Moderate
Wolff et al.1961	At low T ₄ concentrations in human serum most of the T ₄ was displaced from TBPA onto TBG by addition of natrium salicylate. In the situation where T ₄ was present in higher amounts TBG became saturated and T ₄ was further displaced to albumin.		High	Strong

Insulin-like growth factor 3 (INSL3), Prostaglandin D₂ (PGD₂), Prostaglandin E₂ (PGE₂), anogenital distance (AGD), hCG, protein-bound iodine (PBI), triiodothyronine (T₃), thyroxine (T₄), thyroxine-binding pre-albumin (TBPA), thyroxine binding globulin (TBG).

Table 2. Overview of potential endocrine-related adverse effects of Salicylic acid (SA):

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Gupta et al. 2003	Rats	The results from the study showed a high concordance between Wistar and SD rats regarding developmental anomalies with the exception to hydrocephalus in Wistar rats and the VSD in the SD rats. Whether acetylsalicylic acid was administered as a single dose or during the organogenesis (GD 6-17), the malformations were similar. Hypo-plastic testes were seen in 2 out of 137 fetuses and only in the highest dose group	High	Weak
Davis et al. 1996	Rats n=105	The results of the study showed that the groups exposed to 200 mg/kg/day SA and 260 mg/kg/day ASA had a delay in the onset of labor, an increase in labor time and a significantly increase in maternal perinatal mortality. Regarding the treatment-associated fetotoxicity only the group exposed to ASA 260 mg/kg/day showed a significant increase in the number of stillborn pups and peripartum death.	High	Moderate
Overman and white 1983	Hamsters	Failure of closure of the neural tube resulting in cranium bifidum and/or spina bifida	Medium	None
Beall and Klein (1977)	Rats n=49	Increase in resorption sites for group IV, reduced mean body weight of pups in group II and increase in developmental defects (rib abnormalities, craniorachischisis and umbilical hernia, eye defects) in both Aspirin-treated groups. The combination of food restriction and exposure to Aspirin increased the incident of abnormalities from 24.4% in group II (=32 pups) to 66,3% (=59 pups) in group IV.	Medium	Moderate
Wilson et al. 1977	Rats (n= 4-8) Monkeys (n=8)	The study result from the rat part showed a significant effect on intrauterine death, growth and malformation (cardiac, brain and skeletal) at doses of 150 and 200 mg/kg and for the monkey part the results showed that both exposure doses (100 and 150 mg/kg) resulted in a slight increase in intrauterine death and transitory growth retardation.	High	Rat study : Strong Monkey study: Moderate

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Tuchmann-Duplessis et al. (1975)	Rats n=32	The results revealed a statistical significant difference in the two groups with a prolongation of pregnancy for the treated dams. The parturition time was also observed to be prolonged and in the treated group 2/16 dams died due to extended period of contractions.	Medium	Strong
Collins et al. 1971	Rats 3.generations	Significant findings was observed at dose levels of 3000 and 5000 ppm regarding a decrease in the average number of litter size, number of live-born progeny, number of survivors to day 4 and number of survivors to day 21. The decrease in number of live-born appeared to be dose-related. At the lower dose levels only a non-significant decrease were observed.	Medium	Moderate
Didolkar et al. 1980	Rats n= 24	The result from the study shows that Aspirin caused a significant decrease in testicular weight in the group of immature rats. A decrease in the activity of testicular enzymes was observed for hyaluronidase and sorbitol dehydrogenase in both groups. Regarding spermatogenesis, for both groups, Aspirin caused an impairment of the later stages	Medium	Strong
Warkany and Takacs 1959	Rats n=159 pregnant rats	Abnormalities (external or skeletal) were observed in 146 out of 398 progeny. Out of 159 maternal rats, 32 died and 71 resorbed their fetuses after exposure	low	weak

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REACH registration dossier: <https://echa.europa.eu/da/registration-dossier/-/registered-dossier/14544/1>

(Link used in November 2017)

Fenitrothion, CAS no. 122-14-5

Synonyms: Phenitrothion, Sumithion, Nitrophos, Metathion, Metathionine.

Fenitrothion (C₉H₁₂NO₅PS) (Figure 1) is an organophosphate insecticide and acaricide and the usage is assumed to be below 100 tonnes per annum. Fenitrothion oxon is the active metabolite. Fenitrothion is classified as very toxic to aquatic life with long lasting effects.

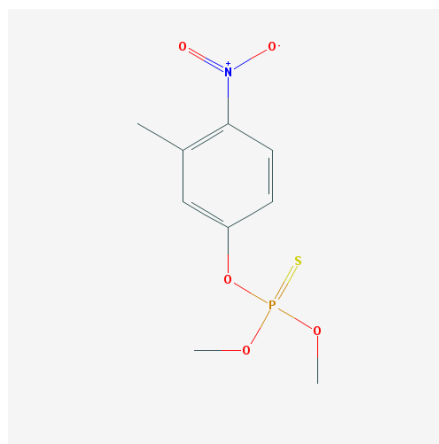


Figure 1. 2D structure from PubChem

4. Human health hazard assessment

4.10.3 Endocrine disruption

4.10.3.1 General approach – human health

4.10.3.2 *In vitro* information indicative of endocrine activity

Suzuki et al. (2013)

Summary: The aim of the study was to identify an organophosphate target in male mouse testis and epididymis. The activity-based protein profiling (ABPP) approach was used. An *in vitro* screen of enzyme activity in testis exposed to fenitrothion oxon, the active metabolite of fenitrothion, was performed in addition to an *in vivo* study. Fenitrothion oxon inhibited the enzyme (fatty acid amide hydrolase) responsible for hydrolysing anandamide.

Study quality and assessment: The *in vitro* part of the study is briefly described in conjunction with testicular enzyme activity analysis from the *in vivo* study. It is unclear how many testis samples were used to extract testicular membrane proteome for the *in vitro* study and whether they were from control animals. The study is assessed to be of medium quality. The study does not provide evidence of endocrine mode of action of fenitrothion.

Okubo et al. (2004)

Summary: The study investigated the estrogenic activity of 20 pesticides. Fenitrothion was tested in the estrogen receptor (ER)-dependent MCF-7 proliferation assay. In addition, the anti-estrogen activity of fenitrothion was investigated by testing for its ability to inhibit cell proliferation induced by 17β-estradiol. Finally, affinity for human ERα and AR were also tested in a competition assay. Fenitrothion suppressed 17β-estradiol-mediated cell proliferation, indicating an anti-estrogenic effect at 100 to 1000 μM. Moreover, fenitrothion showed capacity to bind to AR.

Study quality and assessment: The study is well described, triplicates were performed of each experiment and cytotoxicity was tested. The study is assessed to be of high quality. The study provides strong evidence of an anti-estrogenic mode of action of fenitrothion

Sohoni et al. (2001)

Summary: The aim of this study was to investigate the oestrogenic, androgenic and anti-androgenic activity of fenitrothion and other organophosphate pesticides in *in vivo* and *in vitro* assays. An *in vitro* assay with yeasts stably transfected with human androgen receptors (hAR) and human oestrogen receptors (hER) and with a reporter gene was used to identify active ligands to androgen and oestrogen receptors. Fenitrothion produced a (androgen agonistic) dose-response curve similar to dihydrotestosterone, but fenitrothion was 6000 times less potent compared to dihydrotestosterone. Anti-androgenic activity was seen for fenitrothion with potency close to flutamide.

Study quality and assessment: The overall purpose and methods are described but detailed information on exposure concentrations, incubation time, incubation temperature etc. is missing and more information on purity and CAS-number of the substance would be preferred. Moreover, the main text refers to figures that are missing in the paper. The study is assessed to be of low quality. The study provides moderate evidence of an endocrine disrupting mod of action of fenitrothion acting as an androgen receptor agonist and antagonist.

Tamura et al. (2001)

Summary: This study investigated the interaction of fenitrothion with the human androgen receptor. An *in vitro* assay, a Hershberger *in vivo* assay and neurotoxicity assessment were performed. Interaction with the androgen receptor (AR) was assessed in HepG2 human hepatoma cells transfected with human AR and AR-dependent luciferase reporter gene. The cells were exposed to several concentrations of fenitrothion (10^{-8} - 10^{-5} M) in the presence or absence of dihydrotestosterone (DHT). The activity was measured by luciferase activity determination and 3 replicates of the experiments were performed.

Dihydrotestosterone-dependent AR activity was reduced by fenitrothion in a concentration-dependent manner. Higher concentrations of fenitrothion combined with DHT led to a parallel displacement of the DHT dose-response curve to the right with no depression of the maximal response. These results indicated that fenitrothion is a competitive AR antagonist. Moreover, the highest concentrations of fenitrothion showed slight AR agonist activity.

Study quality and assessment: The study is well-described and assessed to be of high quality. The study provides strong evidence of an anti-androgenic mode of action of fenitrothion.

Berger and Sultatos (1997)

Summary: The aim of the study was to investigate if P450-dependent metabolism of the steroids 17 β -estradiol and oestrone is affected by fenitrothion exposure. *In vivo* studies were performed to measure microsomal concentrations of cytochrome P450 (CYP450). Kinetic analysis was performed *in vitro* to characterise the mechanisms behind the inhibition of oestrogen hydroxylation by fenitrothion.

Co-incubation of hepatic microsomes with fenitrothion and 17 β -estradiol suggested competitive inhibition of metabolisation (2-hydroxylation) of 17 β -estradiol by fenitrothion. Pre-incubation of the mouse hepatic microsomes with fenitrothion before addition of 17 β -estradiol suggested inactivation of certain forms of CYP450. All in all the data indicate that fenitrothion is a mixed inhibitor of 2-hydroxylation of 17 β -estradiol, both serving as a substrate and an irreversible inhibitor of CYP450.

Study quality and assessment: The study has some limitations. Purity and CAS-number of the substance and information of the number of animals used for isolation of microsomes in the *in vitro* part of the study are not given. Detailed information on the microsomal tissue of origin and what (hepatic microsomes) was exposed to fenitrothion in the *in vitro* assay are not found in the main text but in the figure legend. However, the assays seem to be well performed, e.g. with 3-6 determinations. The study is assessed to be of medium quality. The study provides moderate evidence of an estrogenic mode of action of fenitrothion based on inhibiting effects on enzymatic activity on 17 β -estradiol.

Clos et al. (1994)

Summary: The aim of this study was to investigate if fenitrothion was able to modify the testicular capacity to metabolize xenobiotics. Animal studies were conducted and an *in vitro* study was additionally performed to investigate whether a metabolic activation is necessary before loss of testicular cytochrome P-450. Testicular microsomal enzymes with and without NADPH were incubated with fenitrothion at different concentrations (50 μ M-200 μ M).

Cytochrome P-450 was not affected by fenitrothion when NADPH was omitted from the testicular microsomal fraction, but a decrease of cytochrome P-450 was observed (at 100, 150 and 200 μ M fenitrothion) when NADPH was added to the medium. Similar responses were observed in hepatic microsomal fraction.

Study quality and assessment: The *in vitro* study is described under the results section and not in the methods. The method is briefly described, not mentioning details on incubation time and temperature or the medium used. The study is assessed to be of low quality. The *in vitro* part of the study does not provide data relevant for assessing an endocrine mode of action of fenitrothion.

4.10.3.3 In vivo effects with regard to an endocrine mode of action

Saber et al. (2016)

Summary: The objective of the study was to investigate the protective effects of quercetin on testicular toxicity in rats induced by fenitrothion. Adult male rats (4-5 months old) were dosed by oral gavage (6/group) with fenitrothion (20 mg/kg/day), quercetin or a mixture of fenitrothion and quercetin for 70 days. Blood samples for hormone analysis (luteinizing hormone (LH) and testosterone) and testis samples (for gene expression and histopathology) were collected at necropsy. Semen quality (sperm motility, count and morphology) was assessed. Gene expression levels of steroidogenic genes and oxidative stress genes were measured. In the following only data on the fenitrothion treated animals will be described.

All sperm parameters were affected by fenitrothion. Sperm count and motility were both decreased and the percentage of sperm abnormalities were increased in the fenitrothion treated group compared to controls. The serum testosterone and LH levels were reduced compared to controls. Expression of steroidogenic genes (3 β -HSD6, 17 β -HSD3 and Nr5A1) was down-regulated in testis of the treated rats as well as antioxidant gene expression levels. Histopathological assessment of testes revealed degenerative changes in the testis of treated rats (focal testicular degeneration, congestion, interstitial oedema, vacuolation of germinal epithelium, absence of spermatogenesis, focally necrotic seminiferous tubules, spermatid giant cells).

Study quality and assessment: The study is well-described, but CAS no is not mentioned. The substance is called “Fenitrothion 50% EC” and it is unclear whether that reflects the purity of the substance. Fenitrothion was dissolved in corn oil and it would therefore have been preferred that the control group had been dosed with corn oil rather than saline. The choice of using saline rather than corn oil is probably because quercetin was dissolved in saline. However, corn oil has been used as a control solution in many other published studies without giving rise to effects similar to the ones reported in this study. Moreover, identical effects as the ones reported in this study have been described in the literature for fenitrothion and it is therefore highly plausible that the findings observed in this study are related to the treatment with fenitrothion. The quality of the study is assessed to be of medium quality. The study provides strong evidence of adverse effects on male reproductive organs and strong evidence of an anti-androgenic mode of action of fenitrothion.

Ito et al. (2014)

Summary: The aim of the study is to investigate the association between fenitrothion spermatotoxicity and the endocannabinoid signalling system in male reproductive organs. It is hypothesized that testis toxicity is mediated through hydrolysing enzymes affecting substrates for the cannabinoid type-1 receptor (CB1R). Adult male rats (9 weeks old, n=8-10) were exposed orally to 5 or 10 mg/kg/day of fenitrothion 4 days a week for 9 weeks. After euthanasia, blood samples were collected and testes and epididymides were dissected for sperm analysis (sperm count, motility and abnormalities) and sperm ATP levels were analysed. *In vitro* assays for assessment of the enzymatic system hydrolysing substrates for CB1R were performed (i.e. analysis of the activity of the enzymes, the testicular proteome and the testicular substrate levels).

Body weight was decreased in the highest exposure group and clinical signs of neurotoxicity were observed in the fenitrothion treated groups. Sperm motility and the proportion of normal sperm were reduced in the fenitrothion treated animals (5 and 10 mg/kg). The sperm ATP levels were diminished by fenitrothion and may explain the change in motility. Enzyme activity (fatty acid amide hydrolase) and the endocannabinoid levels (anandamide) were reduced. Significant linear regression was observed between enzyme activity (fatty acid amide hydrolase) and sperm motility and morphology.

Study quality and assessment: Some of the methods used in the study are not described in the methods section or are described to a too limited extent in the results section. The administration route is poorly described (exposure is oral, but it is unknown whether it is through gavage, diet or other) and more information on the housing conditions and purity and CAS-number of the substance would have been preferred. The methods used for measuring plasma cholinesterase, are not described and inconclusive, preliminary data are included on histological assessment of testis although the methods are not described. The methods used for testosterone (plasma and testicular) and follicle stimulating hormone levels are insufficiently described. The results of those data are thus unreliable and are not included in the above summary or in the evaluation of the effects of fenitrothion. The methods used for the remaining endpoints are properly described and the study is assessed to be of medium quality. The study provides strong evidence of adverse effects on spermatids (motility and morphology). The study provides moderate evidence of an enzyme-mediated spermatotoxic mode of action of fenitrothion, but it is unknown whether this can be related to an endocrine disrupting mode of action.

Suzuki et al. (2013)

Summary: The aim of the study was to identify an organophosphate target in male mouse testis and epididymis. The activity-based protein profiling (ABPP) approach was used. Male mice (n= 6-7 per group) were exposed orally to 50 or 100 mg/kg/day of fenitrothion for 10 days. Testes and cauda epididymides were weighed and frozen. Testicular membrane proteome was analysed for enzyme activity and hydrolysis activity of enzyme substrates (anandamide and mono-oleoylglycerol) was assayed in testes and cauda epididymides. Finally, testicular anandamide levels and brain acetylcholinesterase activity were measured. Enzymatic activity (of fatty acid amide hydrolase) in testes and cauda epididymides was decreased in both treatment groups compared to controls. Brain acetylcholinesterase activity was reduced.

Study quality and assessment: The method used for measurement of brain acetylcholinesterase activity is briefly mentioned in a figure legend and is insufficiently described. The remaining methods are well described; however, information on the purity and CAS-number of the substance would have been preferred. The study is assessed to be of medium quality. The study provides weak evidence of adverse effects on male reproductive organs based decreased testicular enzyme activity in the absence of changes in testis and epididymis weights.

Taib et al. (2013)

Summary: The aim of the study was to determine the effects on sperm, testis and biochemical and oxidative stress markers in rat testes. Adult male rats (n= 10/group) were exposed to 20 mg/kg fenitrothion for 28 days (5 days a week) through oral gavage. At necropsy, blood samples were collected for biochemical analysis, weight of testes and epididymides was recorded and sperm parameters (viability, concentration and morphology) were assessed. Testes were evaluated for histopathological changes in classical H&E slides and for ultrastructural changes with transmission electron microscopy. Weight gain was reduced in the treated rats. Relative testis weight was increased and both absolute and relative epididymis weights were decreased compared to controls. Sperm parameters were all affected and showed lower viability and density and abnormal morphology. In concordance with these results, testis histology showed degenerative changes (e.g. degeneration of germ cells and Leydig cells, disarrangement of spermatogonia and cellular debris in lumens of seminiferous tubules) and electron microscopy showed ultrastructural changes (lipid droplets, increased number and vacuolation of mitochondria and less condensation of chromatin in late spermatids).

Study quality and assessment: The study is well-described, and thorough. However, more information on the CAS-number of the substance and on housing conditions of the animals would have been preferred. The study is assessed to be of medium quality. The study provides strong evidence of male reproductive adverse effects.

Struve et al. (2007)

Summary: The objective of the study was to investigate if prenatal exposure to fenitrothion affects the development of the sexually dimorphic nucleus of the medial preoptic area in the brain. Time-mated female rats (8-10 weeks old) were dosed daily by oral gavage from gestation day (GD) 12 until GD 21 with 20 or 25 mg/kg/day of fenitrothion (n=5 controls and 6 dams/fenitrothion treatment group). Adult offspring were euthanized when females were 60-65 days old and when males were 96-105 days old. At necropsy, body and brain weights were measured. Brains were assessed for the volume of

the nucleus of the medial preoptic area of the hypothalamus (SDN-POA). Other endpoints assessed are published in Turner et al 2002. Maternal toxicity was observed. The number of pups born alive was decreased in the 25 mg/kg treatment group and a single litter in this group was dead at birth. Moreover, signs of neurotoxicity were observed in dams at GD 19 in exposed dams. In controls, the SDN-POA volume was larger in males compared to females, as expected. In treated animals, the SDN-POA volume was increased whereas in females it was decreased compared to controls of the same sex.

Study quality and assessment: The study is well described but stage of oestrous cycle of adult female pups is not assessed before necropsy. The quality of the study is assessed to be of medium quality. The study provides weak evidence of neurological adverse effects in pups after *in utero* exposure to fenitrothion.

Okahashi et al. (2005)

Summary: The aim of this study was to provide a comprehensive evaluation of the anti-androgenic activity of fenitrothion in the reproductive and endocrine systems. A one-generation toxicity study was performed with dietary exposure (0, 10, 20, 60 ppm) of the parental generation (12 males and 12 females per group) from 10 days prior to mating until end of lactation and the pups (2 pups per sex per litter) were exposed from weaning until the age of 10 weeks. Body weight, food consumption and oestrous cycle were monitored in the female parental rats and reproductive parameters (e.g. fertility, gestation period and live pups per litter) were recorded. Age and body weight at preputial separation and vaginal opening was recorded in the offspring. Moreover, the anogenital distance (AGD, on postnatal day (PND) 0, 21 and 35) and nipple retention (on PND 12 and 49) was recorded in the offspring. Parental animals were euthanized at weaning of the pups, implantation sites were counted and several organs were weighed (including adrenals, ovaries, uterus, testis, epididymides, levator ani-bulbocavernosus muscles and glans penis) and organs were evaluated histologically (pituitary gland, thyroid glands, uterus, ovaries, testes, epididymides, prostate and seminal vesicle). In the ovaries, the number of follicles and corpora lutea was recorded and cholinesterase activity was analysed in the brains. Selected F1 animals were euthanized on PND 70 and the remaining pups were euthanized PND21 and uterus was weighed. On PND 70 organs weights and microscopic analysis was performed as for the parental generation. Sperm analysis (sperm count, motility and morphology) was performed in parental and F1 males. The intake of fenitrothion was higher during lactation (1.32, 2.68 and 7.75 mg/kg/day for the three dose-group, respectively) compared to pregnant and non-pregnant non-lactating animals (0.62-0.87, 1.22-1.82, 3.75-5.58, respectively). No effects on reproductive parameters or on reproductive organs were found in the parental generation and no effects in any of the evaluated endpoints in the offspring showed effects of exposure to fenitrothion during development.

Study quality and assessment: The study is well described and is assessed to be of high quality. However, it should be noted that the purity of fenitrothion used in this study was low (94.60%) compared to the other studies assessed here (most above 99%). No signs of adverse effects were observed on reproduction or development. The study provides especially data to determine the anti-androgen activity of fenitrothion, but no effects were found of fenitrothion on these endpoints. Based on this study, the evidence of adverse effects related to an anti-androgenic mode of action of fenitrothion is weak. Similarly, the evidence of adverse effects related to an estrogenic mode of action of fenitrothion is weak based on endpoints assessed in female adults.

Turner et al. (2002)

Summary: The aim of the study was to investigate the androgenic activity of fenitrothion on male sexual development. An animal study (rats) with *in utero* exposure to fenitrothion by oral gavage from gestation day (GD) 12 to 21 was conducted in two blocks. In the first block time-mated dams were exposed to 0, 5, 10 or 15 mg/kg/day (6 controls and 7 per treated group). In the second block, the dams were treated with 0, 20 or 25 mg/kg/day (5 controls and 6 per treated group). The dams were assessed for neurobehavioral effects in an abbreviated functional observational battery (e.g. tremors, muscle tone and acoustic response). In the offspring, anogenital distance (AGD) was measured and nipple retention, vaginal opening, preputial separation, cryptorchidism and hypospadias were recorded. Maternal body and organ (liver, kidney and uterus) weights were recorded at necropsy on postnatal day (PND) 21. Female and male offspring were euthanized on PND 60-65 and PND 96-105, respectively. Body and organ (liver, kidneys, adrenal glands, brain, ovaries, uterus, testes, epididymides, seminal vesicles with coagulating glands, vas deferentia, prostate and levator-ani bulbocavernosus muscles) weights from offspring were recorded. Some organs (testes, epididymides, prostate, liver, kidney and adrenal glands) were fixed and evaluated histopathologically. Dams from the two highest dose-groups exhibited signs of maternal toxicity (decreased weight gain) and adverse clinical signs of cholinergic stress (effects on gait, open-field tremors and acoustic response) and the severity of the clinical signs increased with subsequent days of dosing. However, no effects were observed on gestation length or the number of implantations. Effects were seen on the number of live pups per litter (in the 25 mg/kg dose-group) as a result of post-implantation loss. Decreased AGD (on PND1) and increased nipple retention was observed in the male offspring in the 25 mg/kg dose-group. In the female offspring, decreased liver and kidney weights were found in the 5 and 15 mg/kg dose-groups, respectively. However, these changes were not considered dose-related as no changes in organ weights were found in the higher exposure groups.

Study quality and assessment: The study is well described including much relevant information on housing conditions, rationale for choice of doses and methods used for e.g. measurement of AGD. However, the CAS-number of fenitrothion is not given and the stage of oestrous cycle at necropsy of sexually mature female offspring was not recorded. This may have led to a higher variation in female reproductive organ weights and histological morphology thus potential effects may not be detected and the relevance of the data is limited. The study is assessed to be of medium quality and provides strong evidence of anti-androgenic mode of action of fenitrothion.

Sohoni et al. (2001)

Summary: The aim of this study was to investigate the oestrogenic, androgenic and anti-androgenic activity of fenitrothion and other organophosphate pesticides in *in vivo* and *in vitro* assays. A prepubertal assay in young male rats (35-36 days old, 10 per group) exposed orally to fenitrothion for 20 days was performed in two blocks. Diethylstilbestrol (DES) was used as a positive control. At termination the day after the last dose, body weight was recorded and liver, epididymides, seminal vesicle and prostate were weighed. A Hershberger assay was performed in castrated male rats (6 weeks old, 5 per group) dosed orally with fenitrothion daily for 10 days. Testosterone propionate (subcutaneous injection) was used as an androgenic and flutamide as an antiandrogenic positive control. At termination, one day after the last dose, body weight was recorded and liver, kidneys, cowper's glands, levator ani muscle, seminal vesicle and prostate were weighed. Finally, an uterotrophic assay was performed in immature mice dosed subcutaneously for 3 days with

fenitrothion. At termination, uterus was weighed (blotted and dry weight). Decreased body weight was observed in peripubertal male rats exposed to fenitrothion or DES. Weights of prostate and seminal vesicles were decreased in both fenitrothion and DES treated rats, but the effect of fenitrothion was only significant in the first block. Liver weight was decreased in both fenitrothion dose-groups in both blocks but not in DES treated animals. No effects of fenitrothion were observed in the Hershberger or uterotrophic assays.

Study quality and assessment: The paper is easily read and understandable due to fluent English and one of the strengths of the study is the use of positive controls/ reference compounds in all the assays. However, the study has some limitations given that the main text refers to figures that are missing in the paper and description of housing conditions and the exact age and strain of the immature female mice used in the uterotrophic assay are not given. There is no rationale why different vehicles (hydroxypropyl methoxycellulose (HPMC) and arachis oil) are used in the different experiments. The study is assessed to be of medium quality. The study provides weak evidence of an anti-androgenic mode of action of fenitrothion.

Tamura et al. (2001)

Summary: This study investigated the interaction of fenitrothion with the human androgen receptor. An *in vitro* assay, a Hershberger *in vivo* assay and neurotoxicity assessment were performed. The Hershberger assay was performed in 7 weeks old castrated male rats (n=8 per group) dosed by oral gavage with 0, 15 or 30 mg/kg fenitrothion concurrently with subcutaneous administration of testosterone propionate. Flutamide (50 mg/kg/day) was used as an anti-androgenic positive control. At necropsy, organs (including the reproductive organs: prostate, glans penis, seminal vesicle with coagulating gland, levator ani-bulbocavernosus muscles) were weighed. Serum testosterone was measured.

Body weight in the high-dose animals and weight gain in the two highest exposure groups were decreased after 7 days of treatment. Weights of ventral prostate, seminal vesicle and levator ani-bulbocavernosus muscles were reduced after fenitrothion treatment (in 15 and 30 mg/kg dose-groups).

Study quality and assessment: The study is well-described and assessed to be of high quality. The study provides strong evidence of adverse effects of fenitrothion on male reproductive organs.

Ashby and Lefevre (2000)

Summary: The aim of the study was to add data to the database for the peripubertal male rat assay, to determine the optimal exposure period to the agents and to assess the relative sensitivity of changes in reproductive organ weights and delays in preputial separation. Several test protocols were performed, but only one smaller study with focus on the influence of initial body weight on final organ weight and the day of preputial separation included exposure to fenitrothion. Immature male rats 35-36 days old (approximately 10/group) were dosed by oral gavage with 5 or 15 mg/kg fenitrothion for 20 consecutive days. The body weight at study start, the day of preputial separation and body weight at preputial separation and at 55 days of age were recorded.

Decreased body weight at 55 days of age but no delay in preputial separation was found for fenitrothion.

Study quality and assessment: The experiment with fenitrothion is briefly described and information on number of animals and dosing interval is described in a table summarizing the data. Information on housing conditions is missing and the CAS-number and purity of the chemical are not given. The study is assessed to be of medium quality. The study showed no effect on preputial separation and does thus not provide evidence of adverse effects on male reproductive development.

Sunami et al. (2000)

Summary: The aim of the study was to evaluate the reliability and feasibility of the 5-day Hershberger assay in young mature male rats and to investigate if fenitrothion has an androgen receptor (AR)-mediated mechanism using this assay. The androgenic and anti-androgenic activity of fenitrothion was assessed in the Hershberger assay. Castrated male rats (10 weeks of age, 6 per group) were dosed by oral gavage to 0.75, 1.5 or 3 mg/kg/day for 5 days to evaluate the androgenic activity of fenitrothion. A similar experiment where the rats were treated with testosterone propionate was used to determine the antiandrogenic activity of fenitrothion. Serum androgen levels were measured and organs (ventral prostate, seminal vesicles with coagulating gland and levator ani-bulbocavernosus muscles) were weighed. Other effects of toxicity were additionally evaluated, such as body weight gain, liver weights and cholinesterase activity in the brain.

No effects on reproductive organ weights were observed for either of the two experiments with fenitrothion, with or without treatment with testosterone propionate. The results suggest that fenitrothion does not have an AR-mediated mechanism *in vivo*.

Study quality and assessment: The study is well-described and the study is assessed to be of high quality although the CAS-number of fenitrothion is not given. The study showed no effects demonstrating an androgenic or anti-androgenic mode of action of fenitrothion and thus the study provides no evidence for endocrine disrupting mode of action of fenitrothion.

Berger and Sultatos (1997)

Summary: The aim of the study was to investigate if P450-dependent metabolism of the steroids 17 β -estradiol and estrone is affected by fenitrothion exposure. Several experiments were performed using intraperitoneal injection of fenitrothion in male mice. In an acute toxicity test animals were killed 4 hours after exposure to 0, 7, 15, 30, 70 or 500 mg/kg fenitrothion (n=4-5 per group). Time course studies were also performed dosing the mice with 70 mg/kg fenitrothion and isolating microsomes from livers 0, 4, 8, 16 and 24 hours after dosing. Concentration of hepatic microsomes was measured.

Effects on microsomal activity were seen at doses below those giving rise to clinical neurological signs (symptoms of cholinergic crisis observed at 500 mg/kg). A decrease in the capacity to 2- and 4-hydroxylate 17 β -estradiol was observed in the mouse hepatic microsomes after exposure to fenitrothion, based on increased production of 2-OHE2 and 4-OHE2 from 7 mg/kg fenitrothion and more. In contrast, 16 α -hydroxylation was increased by fenitrothion exposure based on increased 16 α -OHE1 production (from 7 mg/kg and above) and increased estriol production (from 30 mg/kg and above). The maximal effects on microsomal activity were seen at 4 and 8 hours after dosing in the time course studies with attenuation of the effect later on. In the case of 2-OHE2, production was subsequently increased compared to controls 24 hours post-treatment.

Study quality and assessment: The study has some limitations. Purity and CAS-number of the substance are not given and no information on housing conditions is described besides the name of the feed. Additionally, it is not described if a section of the liver or the whole organ is used for isolation of hepatic microsomes. They refer to other articles without a brief description of the method on how the microsomes were isolated from the livers. Details on the number of animals used and the microsomal tissue of origin are not described in the main text but can be found in the figure legends. In contrast, the incubation procedures and methods used for microsomal protein measurement are well described. The study is assessed to be of medium quality. The study provides moderate evidence of an estrogenic mode of action of fenitrothion based on effects on the enzymatic products from CYP450-dependent hydroxylation of estradiol in the hepatic microsomes.

Berlinska and Sitarek (1997)

Summary: Fenitrothion was dosed daily by oral gavage to female rats from gestation day 6-15 (3, 15, 30 and 45 mg/kg). Some dams were found dead in the two highest dose-groups. Several findings were found in dams in the 30 mg/kg dose-group including but not restricted to decreased weight gain, early resorptions and postimplantation losses were found in the 30 mg/kg group as well as fetal effects including delayed ossification, enlarged cerebral ventricles and decreased fetal body weight and length. In the 15mg/kg dose-group, maternal toxicity was observed as decreased liver weight and fetal toxicity was observed as enlarged cerebral ventricles.

Study quality and assessment: The article is in polish but an English abstract was available. The quality of the study is not assessable based on the abstract only. The study provides strong evidence of adverse effects on female reproduction and foetal development based on changes such as early resorptions, delayed ossification and decreased foetal body weight. However, the relation to an endocrine mode of action is weak.

Clos et al. (1994)

Summary: The aim of this study was to investigate if fenitrothion was able to modify the testicular capacity to metabolize xenobiotics. Male rats were exposed orally to fenitrothion in an acute (single dose of 165 mg/kg), a subacute (55 mg/kg for 3 days) or a chronic (5.5 mg/kg for 30 days) study. The testicular microsomal system (CYP-450, cytochrome b₅ and NADPH cytochrome c reductase activity) and plasma testosterone levels were determined. The effects on the microsomal system were compared to the microsomal system in the liver. Moreover, plasma testosterone levels were measured.

Acute and subacute exposure to fenitrothion inhibited testicular cytochrome P-450 (around 50% compared to controls). Decreased CYP-450 concentration was also seen in livers of rats after acute or subacute exposure. Plasma testosterone levels were markedly decreased after acute and subacute exposure but not after chronic exposure to fenitrothion.

Study quality and assessment: The study has some limitations. The exact number of animals used is not given, but based on the table summarizing the results on microsomal enzymes, an estimation of 8 animals per group can be made (each value is a mean of 4 samples, each sample consisting of pooled tissue from 2 rats). Moreover, very few details on housing conditions are described and it is not clear whether the control group was dosed with the vehicle, corn oil, or not. The study is assessed to be of low quality. The study provides strong evidence of an anti-androgenic mode of action of fenitrothion.

Holmes and Boag (1990)

Summary: The aims of the study were (i) to gather information useful for interpretation of results from field studies and (ii) to determine the usefulness of an automated system for collection of behavioural data in evaluating neurobehavioral effects. Behavioural and reproductive effects were evaluated in zebra finches exposed orally (catheter down the oesophagus to the crop) to fenitrothion (0, 1.04 or 3.80 mg/kg, n=16 pairs per group). The birds were fasted 10-12 hours before treatment and 10-15 minutes after dosing. Pairs were assessed for their breeding success, distributed among the groups and united the day before treatment. Reproductive parameters (number of eggs, nestlings and fledglings) were monitored and activity (perch hopping) was recorded.

Effects were observed on activity levels but not on reproduction.

Study quality and assessment: The study has some limitations that may affect the sensitivity of endocrine related endpoints on reproduction. The feed given to the birds included alfalfa sprouts and soy bean oil was used as vehicle in controls, both of which contain phytoestrogens. Control animals were thus exposed to estrogenic substances and this may potentially mask possible effects on these endpoints. Moreover, more information on housing conditions and CAS-number would have been preferred. The study is assessed to be of low quality. The study showed no effects on reproductive parameters and does thus not provide evidence of adverse effects on reproductive function.

Lehotzky et al. (1988)

Summary: The aim of the study was to investigate the prenatal effects of fenitrothion. Mated female rats (6 per group) were dosed by oral gavage from gestation day 7-15 with 0, 5, 10 or 15 mg/kg Sumithion (50% fenitrothion) fenitrothion per day. The number of pups per litter was recorded and the day of eye- and ear opening was recorded. Ten males from each dose-group were used in a battery of behavioural tests. The animals were tested on days 26, 36 and 104 post-partum.

Treated animals had reduced horizontal activity in the highest exposure-group on day 104. On days 26 and 104, the highest exposure group performed worse than the other groups in the motor coordination test (rotorod). The two highest exposure-groups showed more conditioned responses and shorter latencies compared to controls during the first days of testing in the conditioned escape test. Also, the two highest treatment groups spent more time in the social interaction test compared to controls.

Study quality and assessment: The study is well described, but the substance used for the study contained 50% fenitrothion, which is a much lower concentration/ purity compared to the substances used in other studies. It is not stated what the other half of the substance is consisting of. The study is assessed to be of medium quality. The study provides strong evidence for behavioural effects of fenitrothion. However, the relation to an endocrine mode of action is weak.

Draft Assessment Report

In the draft assessment report for fenitrothion, 6 reproductive toxicity studies are included. One of these studies is described above (Sunami et al. 2000), but the remaining studies were not possible to retrieve from PubMed or a Google search (05/12-2017, 15:51 GMT+1) (Hoberman 1990; Morseth 1987; Morseth 1986a; Morseth 1986b; Kohda et al. 1975). According to the draft assessment report, these studies count a multigeneration study in rats, 3 developmental toxicity studies in rats and rabbits and a dominant lethal assay in mice. In general, the studies showed reduced body weight in dams and offspring. Neither evidence of foetotoxicity nor any evidence of impaired reproductive performance was recorded. Reduced lactation indices were reported in a study with rats exposed to 120 ppm (corresponding to 7.4 mg/kg/day) and abortions were observed in another study with rabbits exposed to 30 mg/kg/day which was considered related to maternal toxicity. It is stated in the summary that the multigeneration and developmental toxicity and long-term studies do not reveal findings suggestive of an endocrine disrupting mode of action of fenitrothion.

Study quality and assessment: The quality of the studies cannot be assessed based on the summaries available in the draft assessment report. The additional studies included in the draft assessment report not already assessed above do not add significant data on endocrine disrupting adverse effects or on an endocrine mode of action of fenitrothion.

4.10.3.4 Summary of the plausible link between adverse effects and endocrine mode of action

Several studies showed effects indicating an androgenic and/or anti-androgenic mode of action of fenitrothion (Table 1). *In vitro* studies either found that fenitrothion has the capacity to bind androgen receptors (AR) or that it acts as an androgen giving rise to dose-response curves similar to dihydrotestosterone in the assays suggestive of an androgenic mode of action (Okubo et al. 2004; Sohoni et al. 2001). Some studies suggest that fenitrothion may additionally have anti-androgenic activity similar to flutamide or act as a competitive AR antagonist (Sohoni et al. 2001; Tamura et al. 2001). Overall, the studies provide moderate evidence of an androgenic and anti-androgenic mode of action of fenitrothion *in vitro*.

One study has investigated the anti-androgenic mode of action of fenitrothion *in vivo* and the data support the findings from the *in vitro* studies. Decreased serum testosterone levels and down-regulation of testicular steroidogenic genes were found (Saber et al. 2016). A Hershberger assay was performed in three studies but they found conflicting results although two of the studies used the same dose of fenitrothion (15 mg/kg). One study found no effects of fenitrothion (Sohoni et al. 2001) whereas another study found reduced male reproductive organ weights (Tamura et al. 2001). This could be explained by a larger group size (n=8 versus n=5/group) and the use of a lower dose of dihydrotestosterone (0.05 mg/day versus 0.12 mg/day in the latter study). The third study tested low levels of fenitrothion (up to 3 mg/kg) in the Hershberger assay compared to the two other studies (Sunami et al. 2000). Based on these studies, there is strong evidence of an anti-androgenic mode of action of fenitrothion *in vivo*.

In contrast to the few studies on *in vivo* mode of action, there are several animal studies showing adverse effects on male reproductive organs, male reproductive development, plasma testosterone levels and on sperm parameters, all suggesting an anti-androgenic mode of action of fenitrothion (Table 2). Decreased sperm count, sperm motility and sperm abnormalities were observed in adult

male rats, a finding retrieved in different laboratories (Saber et al. 2016; Ito et al. 2014; Taib et al. 2013). One study investigated plasma testosterone levels and found a reduction after fenitrothion treatment (Clos et al. 1994). Decreased seminal vesicle weight and increased weight of testis with degenerative changes has been reported (Taib et al. 2013) although other studies did not see such effects (Suzuki et al. 2013; Okahashi et al. 2005). The absence of effects in the latter studies may be due to differences in species (mice versus rats), fewer animals per group (6-7/group versus 10/group) and lower doses (60 ppm corresponding to 7.75 mg/kg during the highest exposure period versus 20 mg/kg). Finally, effects on male development such as decreased anogenital distance and increased nipple retention in male offspring exposed *in utero* (Turner et al. 2001) are known to be related to an anti-androgenic mode of action and support the previous findings in *in vitro* and *in vivo* studies suggesting an anti-androgenic mode of action of fenitrothion. All in all, the *in vivo* studies provide strong evidence of adverse effects that can be related to an endocrine disrupting mode of action of fenitrothion and there is a strong link to an anti-androgenic mode of action.

The mechanisms related to the effects observed on sperm motility and morphology have been investigated in a study (Ito et al. 2014) and they found decreased sperm ATP levels, reduced enzyme activity (fatty acid amide hydrolase) and the lower endocannabinoid levels (anandamide) in treated animals. A correlation between the enzyme activity and sperm motility and morphology was found. Another study supported the findings on activity of this enzyme *in vivo* and *in vitro* (Suzuki et al. 2013) and these changes appear to be linked to the observed effects on motility and morphology (Ito et al. 2014) and low ATP levels may explain the effects on sperm motility, but it is unclear whether these mechanisms could be activated through endocrine disruption. Aside from this, other *in vivo* adverse effects were found to be consistent with an anti-androgenic mode of action and agree with the findings *in vitro* and *in vivo* an anti-androgenic mode of action of fenitrothion. There is strong evidence of a link between the adverse effects observed *in vivo* and the mode of action found *in vitro* and *in vivo* studies.

The estrogenic effects have been investigated to a lesser extent compared to the anti-androgenic activity of fenitrothion (Tables 1 and 2). Two *in vitro* studies suggest an anti-estrogenic mode of action of fenitrothion in MCF-7 cells (Okubo et al. 2004) and a competitive inhibition of the metabolism of 17 β -oestradiol (Berger and Sultatos 1997). However, these effects may be mediated through another pathway than through estrogen receptor (ER), as binding to the human ER was not demonstrated (Sohoni et al. 2001). There is weak evidence that fenitrothion has an anti-estrogenic mode of action *in vitro*.

A mode of action of fenitrothion involving oestrogen levels is supported by *in vivo* studies showing decreased cytochrome P450 activity and ability to hydroxylate 17 β -oestradiol in hepatic and testicular microsomes after fenitrothion exposure (Clos et al. 1994; Berger and Sultatos 1997). In the uterotrophic assay, no effects were observed indicating that fenitrothion does not act as an oestrogenic agonist (Sohoni et al. 2001). There is thus weak evidence for an anti-estrogenic mode of action of fenitrothion *in vivo*.

In vivo studies did, however, not show adverse effects supportive of an anti-estrogenic mode of action of fenitrothion. No *in vivo* studies showed effects on weight or histopathology of female reproductive organs after adult or *in utero* exposure to fenitrothion (Okahashi et al. 2005; Turner et al. 2002). There is weak evidence of adverse effects of fenitrothion on female reproduction *in vivo*. All in all, the available *in vitro* and *in vivo* data suggest that fenitrothion may have an anti-androgenic mode of action but more studies focused on oestrogen sensitive endpoints are necessary to elucidate this further.

Thyroid disrupting effects of fenitrothion has not been investigated. However, a single study with exposure during gestation showed behavioural changes in male rat offspring. Female offspring were not tested and it is not known whether the behavioural changes are sex-specific. The behavioural changes could be related to the endocrine disrupting modes of action described above or it may be related to thyroid disruption. Based on the few available data on behaviour, it is not possible to determine whether it is related to endocrine disruption or not.

Summary and conclusions

In vitro and *in vivo* studies demonstrate that fenitrothion has an anti-androgenic mode of action. This was shown both on the receptor level (binds to AR), blood hormone level (testosterone) and at gene expression level in testis (steroidogenic genes) and *in vivo* adverse effects (effects on male reproductive organs and male development) suggestive of an anti-androgenic mode of action supported these findings. There are less data supporting an androgenic mode of action of fenitrothion. The available data suggest that fenitrothion may have anti-estrogenic mode of action, but more *in vivo* studies with focus on oestrogen sensitive endpoints are necessary to determine if fenitrothion gives rise to adverse effects related to such a mode of action.

There is strong evidence of an anti-androgenic mode of action of fenitrothion leading to adverse effects on male reproductive organs and male development. In conclusion, fenitrothion fulfil the WHO definition of an endocrine disruptor.

Table 1. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of fenitrothion.

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Saber et al. 2016		The serum testosterone and luteinizing hormone (LH) levels were reduced. Expression of steroidogenic genes was down-regulated in testis of the treated rats.	Medium	Strong
Ito et al. 2014		Fatty acid amide hydrolase activity and anandamide levels were reduced. Significant linear regression was observed between fatty acid amide hydrolase activity and sperm motility and morphology.	Medium	Weak
Suzuki et al. 2013	Fenitrothion oxon (active metabolite of fenitrothion) inhibited the enzyme responsible for hydrolysing anandamide.	Enzymatic activity (of fatty acid amide hydrolase) in testes and cauda epididymides was decreased in both treatment groups compared to controls.	Medium	None
Okubo et al. 2004	Fenitrothion suppressed 17 β -estradiol-mediated cell proliferation in MCF-7 cells, indicating an antiestrogenic effect of the pesticide. Moreover, fenitrothion showed capacity to bind to AR.		High	Moderate
Sohoni et al. 2001	Fenitrothion produced a (androgenic) dose-response curve similar to dihydrotestosterone in yeast cells with integrated AR, but fenitrothion was 6000 times less potent compared to dihydrotestosterone. Anti-androgenic activity was seen for fenitrothion with potency close to flutamide.	No effects of fenitrothion were observed in the Hershberger or uterotrophic assays.	Low (<i>in vitro</i>)- Medium (<i>in vivo</i>)	Moderate (<i>in vitro</i>)- Weak (<i>in vivo</i>)
Tamura et al. 2001	Dihydrotestosterone-dependent AR activity was reduced by fenitrothion in a concentration-dependent manner. Higher concentrations of fenitrothion combined with DHT led to a parallel displacement of	In a Hershberger assay (n=8 per group), body weight in the high-dose animals and weight gain in the two highest exposure groups were decreased after 7 days of treatment. Weights of ventral prostate, seminal vesicle and	High	Strong (<i>in vitro</i>) – Moderate (<i>in vivo</i>)

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
	the DHT dose-response curve to the right with no depression of the maximal response. These results indicated that fenitrothion is a competitive AR antagonist.	levator ani-bulbocavernosus muscles were reduced after fenitrothion treatment.		
Sunami et al. 2000		No effects of fenitrothion were observed on reproductive organ weights in the Hershberger assay with or without co-administration of testosterone propionate.	High	Weak
Berger and Sultatos 1997	Co-incubation of hepatic microsomes with fenitrothion and 17 β -estradiol suggested competitive inhibition of metabolism of 17 β -estradiol. Pre-incubation of the hepatic microsomes with fenitrothion before addition of 17 β -estradiol suggested inactivation of certain forms of CYP450.	A decrease in the capacity to (2- and 4-) hydroxylate 17 β -estradiol was observed in the mouse hepatic microsomes after exposure to fenitrothion from 7 mg/kg and above. In contrast, 16 α -hydroxylation was increased by fenitrothion exposure (from 7 mg/kg and above). The maximal effects on microsomal activity were seen at 4 and 8 hours after dosing and the effects were attenuated later on. In the case of 2-OHE ₂ , production was subsequently increased compared to controls 24 hours post-treatment.	Medium	Moderate
Clos et al. 1994	Cytochrome P-450 was not affected by fenitrothion when NADPH was omitted from the testicular microsomal fraction, but a decrease of cytochrome P-450 was observed (at 100, 150 and 200 μ M fenitrothion) when NADPH was added to the medium. Similar responses were observed in hepatic microsomal fraction.	Acute (165 mg/kg) and subacute (55 mg/kg) exposure to fenitrothion inhibited testicular cytochrome P-450. Decreased CYP-450 concentration was also seen in livers of rats after acute or subacute exposure. Plasma testosterone levels were markedly decreased after acute and subacute exposure but not after chronic (5.5 mg/kg) exposure to fenitrothion.	Low	Strong

Androgen receptor (AR), dihydrotestosterone (DHT), luteinizing hormone (LH)

Table 2. Overview of potential endocrine-related adverse effects of Fenitrothion.

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Saber et al. 2016	Rats, n=6/group	Adult male rats treated with Fenitrothion for 70 days showed decreased sperm count and motility and the percentage of sperm abnormalities were increased. Histopathological assessment of testes revealed degenerative changes in the testis of treated rats.	Medium	Strong
Ito et al. 2014	Rats, n=8-10/group	Body weight was decreased in adult males in the highest exposure group and clinical signs of neurotoxicity were observed in the fenitrothion treated groups. Sperm motility and the proportion of normal sperm were reduced in the fenitrothion treated animals (5 and 10 mg/kg). The sperm ATP levels were diminished by fenitrothion. Enzyme activity (fatty acid amide hydrolase) and the endocannabinoid levels (anandamide) were reduced by fenitrothion.	Medium	Strong
Suzuki et al. 2013	Mice, n=6-7/group	No effects on male reproductive organ weights were found. Brain acetylcholinesterase activity was reduced.	Medium	Weak
Taib et al. 2013	Rats, n=10/group	Weight gain was reduced in the treated adult male rats. Relative testis weight was increased and both absolute and relative epididymis weights were decreased compared to controls. Sperm parameters showed lower viability and density and abnormal morphology. Testis histology showed degenerative changes and electron microscopy showed ultrastructural changes of testes.	Medium	Strong
Struve et al. 2007	Rats, n=5 controls and 6 dams/ treatment group	In offspring exposed <i>in utero</i> , the volume of the nucleus of the medial preoptic area of the hypothalamus was increased whereas in females it was decreased compared to controls of the same sex.	Medium	Weak
Okahashi et al. 2005	Rats, n=2 pups/ sex/ litter, 10-12 litters/ group	No effects on reproductive parameters or on reproductive organs were found in the parental generation and no effects in any of the evaluated endpoints in the offspring showed effects of exposure to fenitrothion during development.	High	Weak

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Turner et al. 2002	Rats, n=5-7 dams/group	Decreased AGD and increased nipple retention was observed in the male offspring in the 25 mg/kg dose-group.	Medium	Strong
Sohoni et al. 2001	Rats, n=10/group	Weights of prostate and seminal vesicles were decreased in fenitrothion treated rats, but the effect of fenitrothion was only significant in the first block.	Medium	Weak
Ashby and Lefevre 2000	Rats, n=10/group	Decreased body weight at 55 days of age but no delay in preputial separation was found in young male rats	Medium	None
Sunami et al. 2000	Rats, n= 6/group	No effects of fenitrothion were observed on reproductive organ weights in the Hershberger assay with or without co-administration of testosterone propionate. The results suggest that fenitrothion does not have an AR-mediated mechanism <i>in vivo</i> .	High	Weak
Berlinska and Sitarek 1997	Rats, n=unknown	Fetal toxicity was observed as enlarged cerebral ventricles. No effects were observed in the lower dose-groups (3 mg/kg).	Not assessable	Weak
Clos et al. 1994	Rats, n=probably 8/group	Plasma testosterone levels were markedly decreased after acute and subacute (high doses) exposure but not after chronic (lower dose) exposure to fenitrothion.	Low	Strong
Holmes and Boag 1990	Finches, n= 16 pairs per group	Effects were observed on activity levels in adults but not on reproduction.	Low	None
Lehotzky et al. 1988	Rats, n=6/group	The male offspring exposed <i>in utero</i> had reduced horizontal activity in the highest exposure-group on day 104. On days 26 and 104, the highest exposure group performed worse than the other groups in the motor coordination test (rotorod). The two highest exposure-groups showed more conditioned responses and shorter latencies compared to controls during the first days of testing in the conditioned escape test. Also, the two highest treatment groups spent more time in the social interaction test compared to controls.	Medium	Strong

Anogenital distance (AGD)

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Bifenthrin, CAS no. 82657-04-3

Synonyms: Cyclopropanecarboxylic acid, (2-methyl-3-phenylphenyl)methyl (1R,3R)-3-[(Z)-2-chloro-3,3,3-trifluoroprop-1-enyl]-2,2-dimethylcyclopropane-1-carboxylate (IUPAC Name)

Bifenthrin is a pyrethroid insecticide. Bifenthrin has 3 sites of isomerism and can therefore exist as 8 potential enantiomers. Each of the four pairs of enantiomers is present as racemic mixture. The cis-Z isomers are the predominant species comprising minimum 98% total Bifenthrin (Figure 1) ((ECHA 2011). In 2012, it was decided in EU that biocidal products with bifenthrin of product type 18, insecticides, acaricides and products to control other arthropods shall no longer be placed on the market with effect from 1 May 2013 because of an unacceptable risk for the aquatic compartment.

The decision was based on a RAC opinion proposing harmonised classification and labelling at Community level of bifenthrin (ECHA 2011). Bifenthrin was evaluated in the US EPA EDSP Tier 1 screening assays. The results of these assays are described under “other information used”

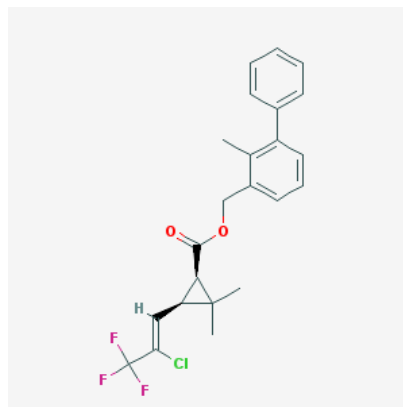


Figure 1. 2D structure from PubChem

5. Environmental hazard assessment

5.6.2 Endocrine Disruption

5.6.2.1 General approach – environment

The peer reviewed literature was investigated by use of Web of Science including all databases. Search terms included bifenthrin + endocrine, bifenthrin + fish, bifenthrin + amphibian, bifenthrin + vitellogenin. A google search including the search terms bifenthrin + endocrine, bifenthrin + fish, bifenthrin + amphibians and bifenthrin + vitellogenin were also performed and revealed the US EPA EDSP tier 1 screens of which the amphibian metamorphosis assay (AMA) and the fish short term reproduction assay (FSTRA) are discussed. Studies solely investigating effects of bifenthrin in invertebrates were not taken into account due to lack of endocrine specific endpoints. Studies using mixtures of chemicals where the effects could not be directly related to bifenthrin were also not taken into account. A summary of the EDSP tier 1 screens including in vitro screens and mammalian screens are included. Also, abstracts from relevant mammalian tests published at Toxnet are included.

5.6.2.2 *In vitro* information indicative of endocrine activity.

Beside the study below, *in vitro* information is included when such assays were conducted in combination with *in vivo* environmental assays. An overview of the *in vitro* assays can be seen in Table 2.

Zhao et al. (2014)

Summary: The authors tested the effects of bifenthrin enantiomers on human trophoblast cells (JEG-3 cell line) *in vitro*. Biomarkers included cell viability, hormone secretion, and steroidogenesis gene expression. The interactions of BF enantiomers with the estrogen receptor were predicted. The study showed that progesterone and human chorionic gonadotropin secretion were induced below cytotoxic levels. Also, gene expression of the progesterone receptor was increased significantly. An upregulation of gene-expression of GNRHI, GNRHRI, CYP17 and CYP19 was seen – especially for the S-BF enantiomer which generally affected the endocrine markers more than R-BF. This difference in effect was also shown by molecular docking of enantiomer ER binding affinity.

Study quality and assessment: The study is well described and evaluated to be of high quality. The link between the gene-expression data, molecular docking and progesterone secretion to the estrogenic pathway is highly plausible.

5.6.2.3 *In vivo* effects with regard to an endocrine mode of action

Bertotto et al. (2017)

Summary: In this study, the authors investigated the hypothesis that BF impairs dopaminergic (DA) and estrogenic regulation in fish and this was tested in both embryonic and one-month old juvenile zebrafish (*Danio rerio*) with exposure to measured concentrations of 0.34 and 3.1 µg/L BF for 96 hours. Effects on the estrogenic pathways were investigated by analysis of gene expression (qRT-PCR) of estrogen receptor α (ER α), ER β 1, ER β 2, LH β , FSH β , vitellogenin (VTG), cytochrome P450 cyp19a1a and cyp19a1b. Effects on the dopaminergic pathways were investigated by gene expression of tyrosine hydroxylase (TH), dopamine receptor 1 (DR1) and 2A (DR2A) and dopamine active transporter (DAT). Levels of E2 were measured by ELISA. Dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) concentrations were measured by LC-MS/MS. For embryonic exposure, 20 viable embryos (3 hpf) were placed in 10 ml of the working solution. Treatments consisted of vehicle control (0.1%EtOH), 0.15 µg/L BF, and 1.5 µg/L. For qPCR assays, each replicate pool consisted of 20 embryos with 5 to 12 replicate pools per treatment. For the E2 ELISA assay, each replicate pool consisted of 80 embryos (20 embryos per petri dish) with 6 to 7 replicate pool per treatment. For measurements of DA and its metabolites, 200 embryos (20 embryos per petri dish) were used for each replicate pool, with 3 to 4 replicate pool per treatment group. Water was sampled before and after exposure to determine measured values of BF. For juvenile exposure, fish were transferred to glass beakers at 30 dpf. Juveniles were exposed to vehicle control, 0.15 and 1.5 µg/L BF nominal concentrations, for 96 hours. No significant difference was identified in hatch per day, morphological deformities or survival in embryos or juveniles compared with controls in both zebrafish stages. Significant decreases of TH (31 fold) and DR1 (33 fold) transcripts, and HVA levels as well as ratios of HVA/DA and HVA+DOPAC/DA in zebrafish embryos were observed after BF treatment. Significant 2-fold and 4.5-fold increases in the expression

of ER β 1 were observed in juveniles exposed to 0.15 $\mu\text{g/L}$ and 1.5 $\mu\text{g/L}$ BF respectively. A significant increase in the DOPAC/DA ratio was also noted. E2 decreased significantly in a dose-dependent relationship in the embryonic exposures and a trend toward increasing E2 was seen in the juvenile exposure. These results show a possible anti-estrogenic effect of BF in embryos, and estrogenicity in juveniles.

Study quality and assessment: The study is very detailed and is assessed to be of high quality. The results on the dopaminergic pathway of BF exposure are quite clear because of more than 30-fold decreases in gene transcription of key genes but the link to the estrogenic effects are unclear although plausible. The effects on the estrogenic pathway in embryos and juveniles are well established and of medium-high plausibility. In this study, there are no endocrine specific adverse endpoints so the link to adversity is weak.

Decourten & Brander (2017)

Summary: In this study, the authors investigated the effects of 1 ng/l BF exposure (measured concentration, 0.89 ng/l) at either 22 °C or 28 °C in a multigenerational study with inland silverside (*Menidia beryllina*). Parents (P) were exposed for 14 days prior to the initiation of spawning trials. Embryos in the F1 generation were exposed to EDCs until 21-days post hatch (dph), reared to adulthood (~35 days) in clean water at elevated temperatures, and spawned. F2 juveniles were not exposed to BF. Five replicates of 10-13 were used for F0. For the spawning assays, 8–15 adult fish (depending on exposure mortality) for the F0 generation and 9–18 fish for the F1 generation were used. F1 sex ratios were significantly influenced by elevated temperature but not by BF alone. The authors observed fewer viable offspring (larvae) at 22 °C and increased developmental deformities in the F1 and F2 generations, with a greater impact on F2 juveniles caused by BF exposure. Also, fewer eggs were produced by F1 at 22 °C.

Study quality and assessment: The study is very detailed and is assessed to be of medium-high quality all though the authors only used one BF exposure group and the exposure concentration of 0.89 ng/l bifenthrin is very low. Also, the exposure to 1 ng/l EE2 did not affect sex ratio at 22 °C. It is not discussed if the phenotypic sex in the inland silverside is reversible and if the 35-day depuration period could affect the sex ratio result. Beside sex ratio, no endocrine specific endpoints were investigated so the potential adverse effects on fecundity cannot be directly linked to ED effects of BF. The link to e.g. anti-estrogen and anti-androgen effects of BF and its metabolites is weak.

Li et al. (2017)

Summary: In this study, the effect of BF on the testicular development in the marine rockfish *Sebastes marmoratus* was investigated after 50 days of exposure. Three concentrations of BF (1, 10 & 100 ng/L) as well as a solvent control group (4 $\mu\text{l/l}$ acetonitrile) were tested with groups sizes of 25 fish per concentration. Endpoints and biomarkers included GSI (gonadosomatic index), T and E2 hormone levels and caspase 3 activity in testes, CYP19A & B gene expression, histological examination of testes and cell apoptosis in testes (TUNEL assay). BF from 1 ng/l caused a change in the percentage of spermatocytes, spermatogonia, and sperm with an increase in both spermatocytes and spermatogonia while the percentage of sperm cells decreased (n=3). The levels of T and E2 decreased from 10 and 1 ng/l BF respectively (n=6). CYP19A gene expression levels decreased in both brain and testes whereas CYP19B expression increased in both organs. The marker for apoptosis; Caspase-3 activity increased in a dose dependent manner with significance from 10 ng/l BF and this

apoptotic effect was confirmed in the TUNEL assay from 1 ng/L BF. The concentrations of BF were quantified in the liver of 4 fish per group.

Study quality and assessment: The study is assessed to be of medium-high quality. The study investigated the effect of chronic (50 days) exposure of BF to adult male *S. marmoratus*. The study design and biomarker examination is clearly described: Fish were randomly sampled from two replicates of 25 fish for the different biomarkers. Only 3-6 fish per exposure group were though used to investigate single endpoints. The link between decreased E2 and Cyp19A is strong but decreased T is not linked to decreased CYP19A. The apoptotic effects cannot be directly linked to an endocrine mechanism. Decreased sperm cell number were based on only 3 fish per group but could be moderately linked to decreased T.

Brander et al. (2016)

Summary: The effects of BF were investigated in two separate experiments with the euryhaline fish *Menidia beryllina*: A 14-day exposure of juvenile inland silverside *M. beryllina* and a 21-day reproduction study with adult silverside. Also, a 96-h acute toxicity range finding study were performed to determine BF LC₅₀. The main objective of the studies was to assess the molecular and physiological responses of BF with focus on ED-related effects. The authors evaluated responses of juvenile fish exposed to BF using a specific microarray, quantitative real-time polymerase chain reaction (qPCR) on 26 ED-related genes, and a specific ELISA to the egg-coat protein choriogenin. The approach was to get information on mechanisms of toxicity and linked changes at higher biological levels of organization. Test concentrations in the juvenile experiment were 0.5, 5 and 50 ng/L BF. The effects on reproduction were investigated in a 21-day adult assay exposed to 0.5 ng/l BF. Several thousand genes were investigated in the microarray and a number of estrogen-related genes were downregulated – especially at 0.5 ng/l. The ELISA on the choriogenin showed a dose related decrease. The reproduction was significant reduced (less fertilized eggs per female) at 0.5 ng/l BF. All experiments were conducted with 4 replicates of 10 fish per concentration and controls. The experiments were semi-static with 80% daily renewal of water. Chemical analysis of BF concentrations in the test water were conducted at the beginning of the experiment and the measured concentrations were <0.6 ng/l (detection limit), 14.6 ng/l and 62.9 ng/l.

Study quality and assessment: The study is very detailed and is assessed to be of high quality. The authors link the different effects to an anti-estrogenic effect of BF and an estrogenic effect of the metabolite 4-hydroxy BF dependent on the test concentration. Estrogenic effects were dominant at the low concentration of 0.5 ng/L whereas anti-estrogenic effects were more dominant at the higher concentrations which the authors hypothesise is caused by enzyme saturation. The decrease in the choriogenin is probably endocrine mediated whereas the decreased reproductive capacity could or could not be endocrine mediated. The link between the altered gene expressions of estrogen related genes and the decrease in choriogenin is moderate. The link to the reproductive decrease is also moderate because this endpoint is not endocrine specific.

Crago & Schlenk (2015)

Summary: The effects of BF (unknown mix of R-Cis-BF and S-Cis BF) on the dopaminergic pathway and E2 levels were investigated in juvenile rainbow trout (*Oncorhynchus mykiss*) for 96 h and 14 days in a semi-static system with 50% water exchange daily. Stock solutions of BF were analysed but BF was not measured in test tanks. A reference to previous measurements of BF in a similar

experiment in the same laboratory was given (Forsgren et al., 2013). The authors measured changes in plasma E2 and hepatic VTG mRNA expression as well as in dopaminergic pathway genes (TH, DR2A, and GNRH2) in the brain of juvenile rainbow trout. For the 96 h exposure, 9 tank replicates of 1 fish were used per concentration and for the 2 weeks exposure, 7 replicate tanks of 1 fish per concentration were used. Fish were exposed to either dechlorinated tap water with <0.01% ethanol as solvent, 0.15 µg/l BF or 1.5 µg/l BF. Neither weight change nor mortality was observed in any of the experiments. After both 96 h and 2 weeks exposure there was a trend of increase in plasma E2 and after 2 weeks also a significant concentration-dependent 2.3 fold increase in the relative expression of VTG mRNA was observed in fish exposed to 1.5 µg/l BF. For the dopaminergic pathway, after 96 h exposure, there was a 3.5 fold increase in the relative expression of TH mRNA in fish exposed to 1.5 µg/l BF. After 2 weeks exposure, TH transcripts were only identified in the control but not in the BF groups. There was no difference between TH expression in controls at 96 h and 2 weeks. A massive 426-fold decrease in the relative expression of DR2A was observed in the 1.5 µg/l BF group after 96 h. This was followed up by a 269-fold decrease in DR2A expression after 2 weeks exposure to 1.5 µg/l BF.

Study quality and assessment: The study is very detailed and is assessed to be of high quality. The authors suggest that the effects of BF on the estrogenic pathways in rainbow trout are partly caused by modulation of the dopaminergic pathways by BF where the present results indicate that the BF affects negatively the dopamine pathway and thereby decreases the dopaminergic inhibiting of GnRH2. In this study, there are no endocrine specific adverse endpoints so the link to adversity is weak but link to BF effect on dopaminergic and estrogenic pathways are moderate.

Crago et al. (2015)

Summary: In a larger study design where mixtures of pesticides and alkylphenols was tested, the authors also tested the effect of 1 and 5 ng/l BF on the VTG-gene expression in adult male fathead minnow (*Pimephales promelas*). The fish were exposed for 7 days with 3 replicates of 3 fish per exposure concentration. No mortality was observed during the experiment and. Nominal concentrations were used, based on measured stock solutions and a daily 50% water exchange was performed. No statistical difference between BF exposure and control values of VTG-mRNA expression was observed in this study.

Study quality and assessment: The study is very detailed and is assessed to be of high quality. The lack of change in VTG-gene expression is different from other studies but it should be taken into account that the results are based on 3 fish in three replicates only. No link to endocrine effects by BF exposure in this study.

Weston et al. (2015)

Summary: in this study, juvenile rainbow trout (*Oncorhynchus mykiss*) or chinook salmon (*Oncorhynchus tshawytscha*) were exposed for 5 days for river water including up to 14.6 ng/l BF for the rainbow trout exposure and 7.8 ng/l for the chinook exposure. Seven replicates of 1 rainbow trout or 7 replicates of 6 chinook salmon were used. No effect on VTG or steroid hormone levels were seen (11-KT, T, E2).

Study quality and assessment: All though the study is well described and the quality is evaluated moderate (low N), the exposure system with river water and varying BF concentrations make it

difficult to judge in relation to ED effects of BF. The number of fish per exposure is quite low (7x1 for rainbow trout and 7 pooled replicates of 6 fish for Chinook salmon. The BF is also not described in terms of enantiomer composition so is not possible to link effects and lack of effects to BF.

Brander et al. (2012)

Summary: The authors evaluated the in vivo concentration-dependent ability of BF (1, 10, 100 ng/L) and permethrin to induce choriogenin (an estrogen-responsive protein) in inland silverside *Menidia beryllina*. The choriogenin in vivo response (quantified by specific ELISA) were then compared with an estrogen receptor responsive in vitro assay—chemical activated luciferase gene expression (CALUX) using a human ovarian carcinoma (BG-1) cell line. The study was 14-d static aqueous exposure (with daily water renewal) using 65- to 70-d-old juvenile fish. Four replicates of 10 fish were used except an EE2 control with two replicates. All three BF concentrations caused significant induction of choriogenin compared to methanol control (0.01%). This result indicates, according to the authors that BF is estrogenic at the low ng/l level and 1 ng/l BF was a more potent inducer of choriogenin than 1 ng/l EE2. Estrogenic effects were dominant at the low concentration of 1 ng/l whereas anti-estrogenic effects were more dominant at the higher concentrations. This could be caused by difference in estrogenicity between the parent BF and metabolite(s) which was also demonstrated by the results of the CALUX assay.

Study quality and assessment: The study is detailed and assessed to be of high quality. BF concentrations were measured analytically and had recoveries within 80-110% of nominal values. Overall the experiments indicate that BF is estrogenic in vivo but anti-estrogenic in the BG-1 cell line. This is probably caused by an estrogenic metabolite that does not occur in the in vitro assay. The link between estrogen agonism at low BF concentrations and increased choriogenin concentrations is high. The link between BF estrogen antagonism and the CALUX assay is moderate.

DeGroot & Brander (2014)

Summary: In this study, it was investigated whether the in vivo estrogenic effects of bifenthrin (BF) could be caused by the metabolite 4-hydroxy BF because BF is documented to be anti-estrogenic in vitro, (ER-CALUX assay). Juvenile (60 DPH) Inland silverside (*Menidia beryllina*) were exposed for 7 days (semi-static) to 10 ng/l BF, 10 ng/l 4-hydroxy BF and 10 ng/l BF + 25 µg/l piperonyl butoxide (PBO, a P450 inhibitor). Five replicates of 8 fish were used for all concentrations. The study revealed that 4-hydroxy BF caused significantly higher estrogen-mediated choriogenin protein levels than BF/PBO-exposed fish. Choriogenin was quantified by use of a *M. beryllina* specific ELISA. Exposure with BF alone was between these groups and not significantly different from either. The authors suggest that the metabolites are the main contributors to BF in vivo estrogenicity.

Study quality and assessment: the study is precise and well-written and assessed to be of high quality. The links between estrogen agonism of 4-hydroxy BF and increased choriogenin concentrations is high. The suggestion that it is metabolization of BF to 4-hydroxy BF that causes the in vivo estrogenicity is well documented by the data on inhibition of the estrogenic effect with the P-450 inhibitor PBO and the link is high.

Forsgren et al. (2013)

Summary: In this study, Juvenile male and female steelhead (*Oncorhynchus mykiss*) were exposed to bifenthrin (BF) for two weeks (0.1 & 1.5 µg/l) in either freshwater or 8 or 16 ppt saltwater at semi-static conditions with water renewal every 48 h. N per replicate was 5 but the number of replicates were not given. For male fish, BF did not affect Plasma 11-KT and E2 levels in freshwater or saltwater. GSI was reduced in freshwater but not in saltwater. Testicular tissue was also unaffected. T plasma levels were not affected by BF exposure except at 8 ppt saltwater where it was reduced. For females, BF exposure in freshwater caused significant increase in Plasma E2 levels and ovarian follicle diameter whereas saltwater acclimated fish exposed to the same concentrations of BF had significantly reduced E2 levels and smaller follicles as well as ovarian follicles undergoing atresia. Plasma 11-KT levels in female fish in freshwater were not significantly different. Plasma 11-KT was significantly elevated in fish treated with 1.5 µg/L BF in 8ppt whereas both BF treatments significantly elevated 11-KT in 16ppt with no difference observed between the treatments. BF treatment in freshwater significantly reduced plasma T levels. There was no significant difference in T levels in fish in 8ppt treated with BF. Fish treated with BF at 16 ppt had significantly lower plasma T levels.

Study quality and assessment: Overall, the study is not very well described: The fish mortality and the number of test replicates are not described. A total of 148 fish were used for GSI analysis and with 9 treatment in total it could be estimated that 4 replicates were used per exposure concentration including about 20% mortality but this is speculative. The measured concentrations of BF varied highly from nominal and were generally 20-50% of nominal. Therefore, the study is assessed to be of low to moderate quality. Opposite effects on hormones and gonads of BF exposure dependent on the salinity of the test water and fish sex make the linking between BF exposure and effects difficult.

Riar et al. (2013)

Summary: the authors exposed two strains of the salmonid *Oncorhynchus mykiss* (rainbow trout and steelhead) acclimated to freshwater and 8 g/L and 17 g/L salinity to 0.1 and 1.5 µg/l BF (99.1% purity, Z-cis-bifenthrin isomer mixture) for 14 days. For each exposure concentration 3 replicate tanks of 5 fish were used. The exposure system was semi-static with change of water every 2 days. Potential biotransformation of BF was measured in liver microsomes. The authors inform that the NADPH-dependent biotransformation of BF was lower in rainbow trout than in rat supersomes and human microsomes. Significant mortality was observed for freshwater acclimated rainbow trout exposed to 1.5 µg/l BF (1.07 ug/L measured). Beside some non-endocrine related endpoints, plasma VTG and plasma steroid hormones (11-KT, T and E2) were measured. No significant effects were seen in VTG levels in any exposure. Also 11-KT and T were not changing. The only significant change was an increase in plasma E2 levels for steelhead acclimated to freshwater.

Study quality and assessment: The study is detailed and well described and the quality is high. The authors suggest that the lack of biotransformation of BF to the more estrogenic metabolite 4-hydroxy BF could explain the lack of VTG induction in the present study. The ratio between the 1R-Cis BF and 1S-Cis BF was not known. The amount of the 1S-Cis enantiomer is important because other studies have shown that this enantiomer of BF is up to several orders of magnitude more ED potent than the 1R-Cis enantiomer. This study does not provide a link between observed effects and endocrine specific mechanisms.

Tu et al. (2016)

Summary: In this study, embryonic zebrafish (blastula stage) were exposed semi-statically to 1, 3 and 10 µg/L of bifenthrin (BF) or λ-cyhalothrin (λ-CH) until 72 h post fertilization with daily water renewal. Embryos were exposed to BF in 1 L beakers with 500 mL of exposure solution and 500 embryos. Three replicates per control and test concentration were used. The following biomarkers and endpoints were investigated: Body condition, bioaccumulation, thyroid hormone levels and transcription of related genes along the HPT axis. After exposure, 430 larvae from each replicate were collected and divided into three groups: 30 for RNA extraction, 100 for BF analysis, and 2x150 for the Thyroid assay. The deviations between measured and nominal concentrations were 18 to 31.7% at T0 and 43 to 51% at T24. Body weight was not affected by BF exposure groups and no BF-related mortality or malformations occurred. BF markedly accumulated in the larvae up to 538 ng/g. BF caused a decrease in thyroxine (T4) and an increase in triiodothyronine (T3) levels. Most of the HPT axis-related genes (including CRH, TSHβ, TTR, UGT1ab, Pax8, Dio2 and TRα) were significantly upregulated in the presence of BF. Molecular docking analyses revealed that at the atomic level, BF binds to thyroid hormone receptor (TRα) protein.

Study quality and assessment: The study is detailed and well described. The gene-expression data are based on pooled fish with N=3 and some of the effects on the expression on single genes could be a little overinterpreted. Overall the study is assessed to be of moderate to high quality. Looking at the effects on all genes related to the thyroid axis in combination with the molecular docking model, the link between BF exposure and HPT-interference is moderate to high.

Schlenk et al. (2012)

Summary: In this study, Evaluation of estrogenic activity of BF (1 and 5 ng/L) and other chemicals was characterized by *in vitro* bioassays using rainbow trout hepatocytes (*Oncorhynchus mykiss*) and *in vivo* studies with Japanese medaka (*Oryzias latipes*). Adult male Japanese medaka were held in 1 L glass jars (3 fish per jar in three replicates per treatment) under static conditions for 7 days. Biotransformation of BF to estrogenic metabolites was not observed in liver microsomes. Also, no VTG-gene expression or protein induction were observed when hepatocytes or male medaka were exposed to BF at 1 ng/L or 5 ng/L.

Study quality and assessment: The study is well described. The number of fish per replicate is low (N=3) and the pooling of replicate fish before VTG protein analysis is generally not recommended because it removes inter-replicate variability and decreases the power of the assay. The BF concentration in the *in vivo* and *in vitro* studies were $98 \pm 4\%$ of nominal concentrations (no further details) which is surprisingly close to nominal concentrations compared to other studies. The study is assessed to be of medium to high quality. There was no link to endocrine effects of BF at the tested concentrations. Of 1 and 5 ng/L.

Beggel et al. (2011)

Summary: This study investigated sublethal effects of BF (ref. std. grade 99% purity, 97% Cis-isomer) on larval fathead minnow (*Pimephales promelas*) exposed for 24 h to 0.09, 0.18, 0.31 or 0.46 µg/l BF (0.07, 0.14, 0.24, or 0.35 µg/l measured concentrations). Fish were sampled after 24 h, 48 h (24 h recovery) and 7 days (6 days recovery) with 30 fish (10 per replicate) per timepoint. Transcriptomic responses were assessed by RT-QPCR and combined with individual effects on swimming performance of the larvae. Each treatment consisted of 9 replicate 600 mL Pyrex beakers

containing 250 mL test solution and 10 fish larvae. Swimming performance was tested by touching the tail fin to trigger escape responses for the duration of 1 min. The distance travelled by the fish was recorded and used as a measure for swimming performance. Significant transcriptomic responses were observed at 0.07 µg/l BF (lowest observed effect concentration, LOEC) but mostly followed a biphasic rather than a linear dose–response with increasing concentration. By PCA (Principle Component Analysis) Genes were clustered in: (A) detoxification, general stress response, (B) neuromuscular function, energy and (C) endocrine function. Transcript patterns for genes involved in detoxification, neuromuscular function and energy metabolism were linked to an impairment of swimming performance at ≥ 0.14 µg/L. With increasing treatment concentration, a significant down-regulation was observed for genes coding for cyp3a, aspartoacylase, and creatine kinase. Metallothionein was up-regulated. The description of VTG-gene regulation is unclear because it is written that BF induced a significant down-regulation of vitellogenin gene expression (7-8 fold) after 24 and 48 h but no difference after 6 days recovery. But it is also written that: The egg yolk precursor

vtg, a well-known biomarker of exposure to estrogenic compounds, was significantly up-regulated and later that: During a 6-day recovery period, down-regulation of vitellogenin was observed at lowest exposure concentrations. Based on the data it seems correct that a down regulation of VTG-gene expression was seen after 24 h and 48 h and no effect was seen after 6 d recovery

Study quality and assessment: The study is detailed and generally well described but the description of VTG-gene regulation is unclear as discussed above. The study is assessed to be of medium quality. The short exposure period of 24 h is a limitation in assessing the link between the gene responses to BF and the effect on swimming behaviour. Endocrine gene responses can though not be linked directly to behaviour and is therefore weak.

Wang et al. (2010)

Summary: In this study, the estrogenic potential of specific enantiomers of three pyrethroid pesticides including 1R-Cis-BF and 1S-Cis-BF were investigated *in vitro* in a yeast assay with human ER α responsive β -galactosidase induction. Beside the yeast assay, a program for ligand docking (Autodock 1.4.5) was employed to dock pyrethroid enantiomers including Cis-BF into the ER- α site. Pyrethroid enantiomers were separated by a HPLC-procedure before use in the yeast assay and complete resolution was obtained for Cis-BF by this method. The relative induction efficiency (RIE) defined as the ratio of maximal enzymatic activity of samples to that of E2 (E2 set to 100) was 64.9 for 1S-Cis-BF and 0.11 for 1R-Cis-BF. The EC₅₀ for 1S-Cis-BF was $10^{-7.1}$ mol/l compared to $10^{-10.1}$ mol/l for E2. The EC₅₀ for 1R-Cis-BF could not be determined due to low activity. The docking results show that hydrogen bonds were found in 1S-cis-BF but not 1R-cis-BF and hydrophobic interactions with the glycine amino acid No 512 in ER α could be the reason for the difference in affinity of R and S-Cis-BF.

Study quality and assessment: The study is detailed and well described and is assessed to be of high quality. The yeast assay show clear estrogenic action of the BF enantiomer 1S-Cis-BF via ER α -interaction. Both enantiomers were inducing β -galactosidase in the yeast assay but 1S-Cis BF was much more potent than 1R-Cis-BF The difference is argued to be caused by different binding affinity to ER α . The link between an estrogenic mechanism of the enantiomers and yeast cell β -galactosidase induction *in vitro* is highly plausible.

Jin et al (2009).

In this study, among several non-endocrine endpoints, zebrafish embryo–larvae were exposed to BF and Vtg-1 mRNA levels were measured using reverse transcription real-time PCR in pools of 7 larvae in three replicates. Results showed that, at the only exposure level of 150 µg/l, the Vtg-1 gene was clearly induced in larval zebrafish exposed to BF for 72 h, approximately 5.7-fold relative to the negative control. The effect increased further after 96 h exposure.

Study quality and assessment: The study is well described and is assessed to be of medium quality in relation to the endocrine effect because the concentration of BF is quite high in the Vtg-1 gene study (150 µg/l BF) and caused different other effects as malformations, increased hatching rates and increased swimming rates. The induction of the VTG gene is though plausibly linked to an estrogenic mechanism of BF.

Wang et al. (2007)

Summary: In this study, the estrogenic potential and uptake of the 1R and 1S-Cis enantiomers of BF were investigated *in vitro* in the human MCF-7 cell proliferation assay and *in vivo* in a 10-day semi-static exposure of adult male Japanese medaka (*Oryzias latipes*) to 10 ng/l BF. Significant differences in estrogenic potential were observed between the two enantiomers in the MCF-7 cell proliferation assay where the 1S-Cis BF was the more potent inducer of maximum cell proliferation (at 10⁻⁹ mol/l compared to 10⁻⁸ mol/l for 1R-Cis BF and 10⁻⁹ mol/l for E2). The difference could be due to different metabolization of the enantiomers. The cell proliferation caused by BF enantiomers could effectively be blocked by ICI 182,780 indicating a direct ER-interference. In the medaka experiment, VTG was induced by both enantiomers but the response to 1S-cis-BF was about 123 times greater than that to the R enantiomer (N=5). This was not caused by a difference in uptake because the uptake of 1R-Cis BF was higher in both liver and other tissue.

Study quality and assessment: The study is detailed and well described and is assessed to be of high quality. The MCF-7 assay show clear estrogenic action via ER-interference because the effect was blocked by ICI 182,780. Both enantiomers induced VTG above control levels with 1S-Cis BF being more than 100 times as potent as 1R-Cis BF. This difference is argued to be caused by different steric accessibility for enzymes. E.g. hydroxylation via a cytochrome P450 monooxygenase mediated conversion to more estrogenic potent metabolites. The link between an estrogenic mechanism of the enantiomers and MCF-7 cell proliferation *in vitro* and VTG induction *in vivo* is highly plausible.

Additional information used:

US EPA Tier 1 EDSP (Endocrine Disrupter Screening Program).

BF was included in the Tier 1 screening program with the screening assays shown in Table 1

Table 1. US EPA Tier 1 screening assays for bifenthrin.

Chemical: Bifenthrin		PC Code: 128825
Guideline	Assay	MRID
890.1100	Amphibian Metamorphosis Assay (Frog)	48675901
890.1150	Androgen Receptor Binding (Rat Prostate)	48615001
890.1200	Aromatase Assay (Human Recombinant)	48615002
890.1250	Estrogen Receptor Binding	48615003
890.1300	Estrogen Receptor Transcriptional Activation (Human Cell Line HeLa-9903)	48615004
890.1350	Fish Short-Term Reproduction	48675902
890.1400	Hershberger (Rat)	48615005
890.1450	Female Pubertal (Rat)	48669401
890.1500	Male Pubertal (Rat)	48669401
890.1550	Steroidogenesis (Human Cell Line – H295R)	48615006
890.1600	Uterotrophic (Rat)	48615007

The Amphibian metamorphosis assay (AMA) and the fish short term reproduction assay (FSTRA) will be discussed in more details below:

AMA: The AMA (OECD 231) protocol was followed. Exposure concentrations were 13.3, 50.6 & 235 ng/l BF. Biological Endpoints: Day 7: NF stage (Nieuwkoop & Faber), wet weight, SVL (snout-vent-length), HLL (hind-limp-length), normalized HLL. Day 21: NF stage, wet weight, SVL, HLL, normalized HLL, thyroid histopathology. There were significant differences between the negative and solvent control groups in growth parameters, which showed a consistent pattern of promotion in the solvent control relative to the negative control. BF significantly delayed ($p < 0.05$) median NF developmental stage at 21 days in the high treatment group (median NF=57) relative to the solvent control (median NF=58.5). No effect in relation to negative control. Day 21 HLL (Hind Limp Length) and normalized HLL were significantly decreased ($p < 0.05$) by 22.1 and 18.7%, respectively, in the high treatment group relative to the solvent control. Mild to moderate thyroid gland atrophy was noted in the high treatment group (9/20) relative to the negative control (4/15) and solvent control (4/20).

These effects were evaluated by the US EPA to be inconsistent with a direct, thyroid-related delay and may be more reflective of treatment-related growth inhibition because no effects were seen on the incidence of follicular cell hypertrophy or hyperplasia, which are expected to be more commonly affected in the presence of a thyroid related developmental delay. Due to lack of detailed knowledge to the AMA effects interpretation, it is difficult for the authors of the present document to challenge this conclusion. Our opinion is though, that a lack of thyroid interference by BF in amphibians cannot be concluded by the present study alone.

FSTRA: The 21-day assay (comparable to OECD 229) was performed with fathead minnows (*Pimephales promelas*) under flow-through conditions with 6-month old fish in 20 spawning groups of 2 males and 4 females. Fish were exposed to BF (93.6% purity) at mean measured concentrations of 0, DMF <20 µL/L, 17, 75, and 402 ng BF/l. There was a 100% male mortality in the high dose and erratic swimming in the females but with no significant mortality in females. There were statistically significant effects on fecundity in females, with a 60% increase at the mid concentration and a 74% reduction at the high concentration. No statistically significant reductions ($p > 0.05$) in fertilization success were seen. There was an increase of 51% in female GSI at the high concentration. There was

a significant increase in oocyte atresia in 13/15 female in the high treatment group (compared to 4/15 fish in the negative control). There were no other statistically significant differences between severity scores or gonadal stages in treated groups compared to the negative or solvent controls for females. No effect on Plasma VTG were seen but a trend toward increasing VTG in female fish was seen at the highest test concentration (P=0.087).

The reason for the gender specific mortality was not discussed. This could be of interest in relation to ED-effects. There are no clear links between effects and endocrine mechanisms because VTG and secondary sex characteristics were not affected. Changes in fecundity could be endocrine mediated but also caused by other mechanisms.

A summary copied from the EDSP Weight of Evidence (WoE) Conclusions on the Tier 1 Screening Assays for the List 1 Chemicals (<https://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-program-tier-1-screening-determinations-and>) is inserted below and sum up the WoE for BF in relation to interaction of BF with the EA&T pathways as evaluated by US EPA:

Executive Summary:

“The Endocrine Disruptor Screening Programs (EDSP) Tier 1 assay battery is designed to provide the necessary empirical data to evaluate the potential of chemicals to interact with the estrogen (E), androgen (A) or thyroid (T) signaling pathways. This interaction includes agonism and antagonism at the estrogen and androgen receptors, altered steroidogenesis, as well as hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary thyroid (HPT) perturbations. In addition to the available Tier 1 data, other scientifically relevant information (OSRI), including general toxicity data and open literature studies of sufficient quality were considered in this weight of evidence (WoE) assessment. In determining whether bifenthrin interacts with E, A, or T hormone pathways, the number and type of effects induced, the magnitude and pattern of responses observed across studies, taxa, and sexes were considered. Additionally, the conditions under which effects occur were considered, in particular, whether or not endocrine-related responses occurred at dose(s) that also resulted in general systemic toxicity or overt toxicity.

On June 11, 2014, the EDSP Tier 1 Assay Weight of Evidence Review Committee (TIWoERC) of the Office of Pesticide Programs (OPP) and the Office of Science Coordination and Policy (OSCP) conducted a weight-of-evidence (WoE) analysis of the potential interaction of bifenthrin with the E, A or T hormone pathways. The TIWoERC conclusions from the WoE evaluation in this report are presented by pathway (E, A and then T) beginning with the results of the Tier 1 in vitro assays followed by in vivo mammalian and wildlife results, then the results of the cited OSRI for mammalian and wildlife studies (40 CFR Part 158 and literature).

Bifenthrin is a member of the synthetic pyrethroid class of insecticide having similar toxicity profiles that elicit neurotoxic behaviors in laboratory animals. This assessment includes studies conducted with two enantiomers: 1S-cis-bifenthrin and 1R-cis-bifenthrin. For the estrogen pathway, bifenthrin was negative in the Tier 1 in vitro assays [estrogen receptor (ER) binding, ER transactivation assay (ERTA), steroidogenesis and aromatase assays]. Although, potential estrogenic and/or anti-estrogenic activity were reported in several non-Guideline in vitro assays, these positive in vitro responses were not supported by the in vivo assays. The Tier 1 uterotrophic assay was negative and, in the female pubertal rat assay, there were no treatment-related estrogen-related effects observed in the absence of overt toxicity. Furthermore, in the fish short-term reproduction assay (FSTRA), all effects in females occurred in the presence of overt toxicity. In the mammalian Part 158 studies, the only estrogen-related effect in the absence of overt toxicity was a slight (9%) decrease in absolute

ovarian weight in the two-generation rat reproduction study. In the avian reproduction studies, the only effect observed was an increase in number of cracked eggs, but no differences in mean egg weight or egg shell thickness or other reproductive parameter. Therefore, there is a lack of convincing evidence for potential interaction with the estrogen pathway. For the androgen pathway, bifenthrin was negative in the Tier 1 in vitro AR binding and steroidogenesis assays. Additionally, the Tier 1 Hershberger assay was negative. In a nonguideline Hershberger assay submitted as OSRI, while decreases in AST weights were reported, chemical purity and numerical AST data were not reported. No treatment-related effects were observed in the male pubertal rat study, in the absence of overt toxicity. No androgen specific effects were observed in the FSTRA, in the absence of overt toxicity, or in the Part 158 wildlife studies. As such, there is no convincing evidence for potential interaction with the androgen pathway.

Based on mammalian Tier 1 assays and the OSRI mammalian studies, bifenthrin does not appear to affect the thyroid or the HPT axis. Developmental findings in the amphibian metamorphosis assay (AMA) included decreased developmental stage and normalized hind limb length at Day 21 (test termination), concurrent with effects on growth (decreased snout-vent length and body weight). Treatment-related differences in thyroid gross pathology and histopathology analysed statistically by the study authors indicated a slight trend ($p = 0.11$) towards mild gland atrophy in the high treatment group relative to the negative control group with a decrease in maximum follicle size ($p=0.024$). Although findings of thyroid gland atrophy are identified in the AMA guideline as diagnostic criteria, the differences between control and treated tadpoles in this study were slight. Moreover, no effects were seen on the incidence of follicular cell hypertrophy or hyperplasia, which are expected to be more commonly affected in the presence of a thyroid related developmental delay. Overall, the thyroid and developmental findings in the AMA with bifenthrin are inconsistent with a direct, thyroid-related delay and may be more reflective of treatment-related growth inhibition. In both the female and male pubertal rat assays, no treatment-related effects were noted for thyroid weight or histopathology. Serum thyroxine (T4) concentration was decreased only in the low-dose groups of both sexes ($\downarrow 16\%$), with an increase in thyroid stimulating hormone (TSH) concentration occurring only in the high-dose males and which was associated with overt toxicity. Furthermore, there were no effects on thyroid weight or histopathology.

Based on weight of evidence considerations, mammalian EDSP Tier 2 testing is not recommended for bifenthrin since there was no convincing evidence of potential interaction with the estrogen, androgen or thyroid pathways.”

Comment to the summary: The summary describe that the assessment includes studies conducted with two enantiomers: 1S-cis-bifenthrin and 1R-cis-bifenthrin but considering the DER (Data Evaluation Record) the bifenthrin description is: Technical bifenthrin of 93.6% purity and the proportion of the 1S and 1R-Cis enantiomers is not referred. This is important because the studies by Wang et al. (2007 & 2010) show a more than 100-fold difference in estrogenic potency between these two enantiomers with 1S-Cis bifenthrin as the more potent. The WoE considerations by the US EPA turns out not to recommend further testing of bifenthrin because of lack of convincing evidence of interaction with EA&T pathways. This is based on that most ED related effects occurred at concentrations where also signs of overt toxicity existed. The authors of the present document do not agree that ED effects occurring in combination with overt toxicity should not be considered. Such ED effects should be evaluated case by case and the plausible mechanism behind the effect should be discussed.

Mammalian tests as summarized from Toxnet (<https://toxnet.nlm.nih.gov/cgi-bin/sis/search/a?dbs+hsdb:@term+@DOCNO+6568>) :

“Developmental or Reproductive Toxicity/ The commercial bifenthrin (BF) contains two cis isomers. In the present study, a dose of 15mg/kg of 1R-cis-BF or 1S-cis-BF was orally administered for 3 weeks to female mice before or during pregnancy. Then, the expression of steroidogenesis related genes which were considered as effective biomarkers of endocrine disruption were analyzed in the male offspring. Maternal exposure to 1S-cis-BF during pregnancy significantly reduced the mRNA levels of peripheral benzodiazepine receptor (PBR) and steroidogenic acute regulatory protein (StAR) in the testes of 3- or 6-week old male offspring. In addition, a significant decrease of cytochrome P450 17alpha-hydroxysteroid dehydrogenase (P450-17alpha) was also observed in the testes of 6-week old male offspring when dams were treated with 1S-cis-BF during pregnancy but not before pregnancy. Moreover, the scavenger receptor class B type 1 (SRB1) and cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc) decreased significantly in the testes of 6-week old male offspring when dams were treated with 1S-cis-BF during and before pregnancy. Thus, oral administration of the maternal mice to cis-BF for 3 weeks, particularly during pregnancy, resulted in endocrine disruption in the male offspring, with the 1S-cis-BF causing more significant alterations than the 1R-cis-BF form.

[Jin Y et al; *Reprod Toxicol* 42: 156-63 (2013)] ****PEER REVIEWED**** PubMed Abstract

/ENDOCRINE MODULATION/ /The objective was/ to study the potential endocrine disrupting effects of bifenthrin (BIF) by using uterotrophic assay and Hershberger assay. In uterotrophic assay, 60 female SD rats were randomly divided into 6 groups, 10 rats per group. Rats in bifenthrin-treated groups were given different doses of bifenthrin (1.47, 4.41 and 13.23 mg/kg BW by gavage for 3 consecutive days). Rats in negative control groups were given corn oil by gavage. Rats in ethinyl estradiol (EE) oral positive control groups were given EE 1.0 ug/kg BW by gavage. Rats in EE injected positive groups were given 0.6 ug/kg BW EE by subcutaneously injection while given corn oil by gavage. At necropsy, the wet and blotted uteri were weighed. The relative uteri weights were calculated, and the histology of uteri was observed. In Hershberger assay, 60 castrated male SD rats were randomly divided into 6 groups, with 10 rats in each group. Rats in BIF-treated groups were given different doses of BIF (1.4, 4.2 and 12.6 mg/kg BW) by gavage. Flutamide (3.0 mg/kg BW) were given to animals in the positive control group by gavage. Rats in the negative control group and testosterone propionate group were given corn oil by gavage for 10 consecutive days. Rats in all groups except the negative control group were also treated with testosterone propionate (TP, 0.2 mg/kg BW) by subcutaneous injection. At necropsy, ventral prostate (VP), seminal vesicle plus fluids and coagulating glands (SVCG), levator ani-bulbocavernosus muscle (LABC), paired Cowper's glands (COW) and the glands penis (GP), liver, kidneys, adrenals were weighed. Serum triiodothyronine (T3) and thyroxine (T4) were determined. In uterotrophic assay, compared with the negative control group, the mean relative wet weight and relative blotted weight of uterine were increased significantly in the female rats given by BIF at 13.23 mg/kg BW for 3 days ($P < 0.05$). BIF resulted in a significant increase of epithelial cell heights of uteri at 4.41 and 13.23 mg/kg BW, as compared with control group. In Hershberger assay, compared with the TP group, mean absolute weight and relative weight of VP and LABC in BIF high dose group were significant decreased. Moreover, total T3 and T4 level were decreased also ($P < 0.05$). ...

[Tan Y et al; *Wei Sheng Yan Jiu* 41 (3): 399-404 (2012)] ****PEER REVIEWED**** PubMed Abstract

ENDOCRINE MODULATION/ Bifenthrin (BF), as a chiral pyrethroid, is widely used to control field and household pests in China. At present, the commercial BF is a mixed compound containing cis isomers (cis-BF) including two enantiomers of 1R-cis-BF and 1S-cis-BF. In the present study, the two individual cis-BF enantiomers were separated by a preparative supercritical fluid chromatography. Then, four week-old adolescent male ICR mice were orally administered 1R-cis-BF and 1S-cis-BF separately daily for 3 weeks at doses

of 0, 7.5 and 15 mg/kg/day, respectively. Results showed that the transcription status of some genes involved in cholesterol synthesis and transport as well as testosterone (T) synthesis in the testes were influenced by cis-BF enantiomers. Especially, we observed that the transcription status of key genes on the pathway of T synthesis including cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc) and cytochrome P450 17alpha-hydroxysteroid dehydrogenase (P45017alpha)) were selectively altered in the testis of mice when treated with 1S-cis-BF, suggesting that it is the possible reason to explain why the lower serum T concentration in 1S-cis-BF treated group. Taken together, it concluded that both of the cis-BF enantiomers have the endocrine disruption activities, while 1S-cis-BF was higher than 1R-cis-BF in mice when exposed during the puberty. The data was helpful to understand the toxicity of cis-BF in mammals under enantiomeric level.

[Jin Y et al; *Environ Toxicol* 30 (7): 746-54 (2015)] ****PEER REVIEWED**** PubMed Abstract”

5.6.2.4 Summary of the plausible link between adverse effects and endocrine mode of action

There is strong evidence from in vitro and in vivo studies that especially the 1S-Cis bifenthrin enantiomer is estrogenic and/or anti-estrogenic (summarized in Table 2). In a yeast assay with human ER α responsive β -galactosidase induction, the relative induction efficiency (RIE) defined as the ratio of maximal enzymatic activity of samples to that of E2 (E2 set to 100) was 64.9 for 1S-Cis-BF and 0.11 for 1R-Cis-BF. In another in vitro study with human MCF-7 cells, 1R-Cis-BF and 1S-Cis-BF were tested. Maximum cell proliferation was at 10^{-9} mol/l for 1S-Cis BF compared to 10^{-8} mol/l for 1R-Cis BF and 10^{-9} mol/l for E2. In this study, ER-interference of bifenthrin enantiomers was confirmed by cell proliferation blockage by ICI 182,780. In an in vivo study, estrogenic effects were dominant at the low concentration of 1 ng/l where the estrogen responsive protein choriogenin was significantly induced, whereas anti-estrogenic effects were more dominant at the higher concentrations. This could be caused by difference in estrogenicity between the parent bifenthrin and metabolite(s). Anti-estrogenicity was demonstrated with the CALUX assay using a human ovarian carcinoma (BG-1) cell line. Several studies show that BF negatively affects the dopaminergic pathway and this could be a part of the estrogenic response of BF because GNRH2 is normally negatively regulated by dopamine.

The mammalian studies referred from Toxnet are not evaluated for quality in this document but it should be noticed that these studies also indicate that the 1S-Cis BF enantiomer is the more potent enantiomer in relation to the estrogenic pathway.

The link from the mechanistic effects described above to effects seen on adverse endpoints like fecundity and other reproductive parameters described in the evaluated studies is difficult to establish because of lack of endocrine specificity of these endpoints.

The challenge with the interpretation of results from most of the evaluated studies is that the enantiomer composition of the bifenthrin used in the studies was not given and mostly a technical bifenthrin solution was used. Several studies reported that bifenthrin did not affect endocrine biomarkers like vitellogenin and in one study also fish sex ratio but these studies did not report the enantiomer composition of bifenthrin. Examples of this are the US EPA tier 1 EDSP screens (e.g. AMA and FSTRA) where a technical bifenthrin of 93.6% purity was used without further information on enantiomer composition.

The link from endocrine mode of action of BF and population relevant adverse effects is weak to moderate because the adverse effects seen on fish fecundity in three studies are not endocrine specific (summarized in Table 3). The effects do though occur at levels below 1 ng/l in two fish species, and overt toxicity is not expected at such concentrations in the affected fish species. The only study that was investigating phenotypic sex ratio did not see any effect after 21 day exposure and a 35 day depuration period. In this study, it was not discussed if estrogenic induced phenotypic sex change in the inland silverside is reversible and if the 35-day depuration period could affect the sex ratio result.

The evidence from the referred studies do not support that technical bifenthrin is an endocrine disrupter according to the IPCS/WHO definition but there is a high concern that the 1S-Cis enantiomer is an ED and that the metabolite 4-hydroxy bifenthrin is an ED.

5.6.2.5 Environmental relevance

Effects related to the estrogenic pathway in fish were reported at concentrations in the low ng/l BF area and are clearly within concentrations measured in the aquatic environment. Reduced T and E2 levels were seen between 1 and 10 ng/l. Decreased fecundity was seen below exposure levels of 1 ng/l in two studies and effects on the oestrogen responsive protein choriogenin was also seen in two studies at these environmental relevant concentrations. It should also be considered that the major metabolite of BF in the environment has been reported to be 4-hydroxy BF which was shown in one study to be more estrogenic potent than BF.

Table 2. Overview of potential endocrine MoA effects of Bifenthrin.

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Decourten & Brander (2017)		Sex ratio study in a multigenerational study with inland silverside (<i>Menidia beryllina</i>). Five replicates of 9-18 fish were used for F1. Parents F0 were exposed for 14 days, F1 from egg to 21 dph and reared for 35 days in clean water. F2 not exposed. One exposure concentration of 0.89 ng/l BF at two temperatures (22 °C and 28 °C) EE2. No effect on sex ratio of BF in F1. No effect of EE2 at 22 °C ↓ in F1 and F2. Deformities ↑ in F1 and F2	Medium-High	Weak
Li et al. (2017)		Testicular development in the marine rockfish <i>Sebastes marmoratus</i> , 50 days of exposure (n=3-6) to 1, 10 or 100 ng/l BF. Number of spermatocytes and spermatogonia ↑. Percentage of sperm cells ↓ CYP19A gene expression levels decreased in both brain and testes whereas CYP19B expression increased in both organs Reduced T and 17β-E2 levels in the testes compared to the control. The levels of T were significantly reduced in the 10 and 100 ng/L groups, and that of E2 were significantly reduced in the all BF treated groups The apoptosis marker Caspase-3 activity increased from 10 ng/l BF (confirmed by Tunnel assay).	Medium-high	Moderate-strong
Bertotto et al (2017)		Dopaminergic and estrogenic regulation investigated in zebrafish embryos and juveniles (96 h exposure). Tyrosine Hydroxylase ↓ (31 fold) and Dopamin Receptor 1 ↓ (33 fold). ERβ1 ↑ in juveniles (0.15 and 1.5 µg/L). E2 ↓ in embryos (trend in juveniles).	High	Moderate-Strong
Brander et al. (2016)	Effects on steroidogenesis in the H295R cell line: ↑ progesterone, ↓ testosterone, aldosterone and cortisol at sub-cytotoxic concentrations	A 14-day exposure of juvenile inland silverside <i>M. beryllina</i> (0.5, 5 and 50 ng/l) and a 21-day reproduction study (0.5 ng/l BF). All studies with 4 replicates of 10 fish per concentration. Estrogen related genes downregulated at 0.5 ng/l (microarray data). Choriogenin ↓ (dose related). Reproduction ↓ at 0.5 ng/l.	High	Moderate-Strong

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Tu et al. (2016)		Embryonic zebrafish exposed semi-statically to 1, 3 and 10 µg/l BF. Three replicates of 500 embryos. T4 ↑, T3↓. CRH, TSHβ, TTR, UGT1ab, Pax8, Dio2 and TRα ↑ Molecular docking show BF binding to TRα.	Medium-high	Moderate
Crago et al. (2015)		VTG-gene expression in adult male fathead minnow (<i>Pimephales promelas</i>). Exposure for 7 days with 3 replicates of 3 fish to 1 and 5 ng/l BF. No effect on VTG	High	Low
Crago & Schlenk (2015)		Dopaminergic and estrogenic pathway and E2 investigated in juvenile rainbow trout (<i>Oncorhynchus mykiss</i>) for 96 h and 14 days. VTG mRNA ↑ (1.5 µg/l). Tyrosine Hydroxylase mRNA ↑ (1.5 µg/l). DR2A ↓ (426-fold after 96 h and 269-fold after 2 weeks exposure to 1.5 µg/l)	High	Moderate
Weston et al. (2015)		Exposure of juvenile rainbow trout and chinook salmon to river water with up to 14.6 ng/l BF for 5 days. No effect on VTG or steroid hormone levels was seen (11-KT, T, E2).	Medium	Low
Degroot & Brander (2014)		Juvenile (60 DPH) Inland silverside (<i>Menidia beryllina</i>) exposed for 7 days (semi-static) to 10 ng/l BF, 10 ng/l 4-hydroxy BF and 10 ng/l BF + 25 µg/l piperonyl butoxide (PBO, a P450 inhibitor). Five replicates of 8 fish were used for all concentrations. Choriogenin ↑ after exposure to 10 ng/l of the metabolite 4-hydroxy BF. P-450 inhibitor PBO removed effect.	High	Strong
Zhao et al. (2014)	BF enantiomers were tested on human trophoblast cells (JEG-3 cell line) in vitro. Progesterone and human chorionic gonadotropin secretion ↑ Progesterone receptor ↑ Gene-expression of GNRHI, GNRHRI, CYP17 and CYP19 ↑ S-BF more potent than R-BF (confirmed by molecular docking of enantiomer ER binding affinity)		High	Strong

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Riar et al. (2013)		<i>Oncorhynchus mykiss</i> (rainbow trout and steelhead) acclimated to freshwater and 8 g/L and 17 g/L salinity exposed to 0.1 and 1.5 µg/l BF (99.1% purity, Z-cis-bifenthrin isomer mixture) for 14 days. Biotransformation of BF – low. No effect on plasma VTG and plasma steroid hormones (11-KT, T and E2) except E2 ↑ in freshwater acclimated steelhead.	High	low
Forsgren et al. (2013)		Juvenile male and female steelhead (<i>Oncorhynchus mykiss</i>) were exposed for two weeks (0.1 & 1.5 µg/l) in either freshwater or 8 or 16 ppt saltwater. Plasma E2 levels and ovarian follicle diameter ↑ in females in freshwater. whereas saltwater acclimated females exposed to the same concentrations of BF had significantly reduced E2 levels and smaller follicles as well as ovarian follicles undergoing atresia. No effect on 11-KT and E2 plasma levels in males.	Low-medium	moderate
Brander et al. (2012)	Estrogen receptor responsive in vitro assay—chemical activated luciferase gene expression (CALUX) using a human ovarian carcinoma (BG-1) cell line. BF was anti-estrogenic in the BG-1 cell line.	Inland silverside <i>Menidia beryllina</i> (65-70 dph) exposed for 14 days (1, 10, 100 ng/L). Four replicates of 10 fish. Choriogenin quantified by specific ELISA. Choriogenin ↑ at all doses. Highest effect at 1 ng/l. Probably metabolite that is estrogenic.	High	Strong
Schlenk et al. (2012)	In vitro bioassays using rainbow trout hepatocytes. No VTG-mRNA induction after 1 and 5 ng/l BF exposure.	in vivo studies with Japanese medaka (<i>Oryzias latipes</i>). Adult male Japanese medaka (3 fish per tank in three replicates per treatment) for 7 days and 1 or 5 ng/l BF. No VTG induction Biotransformation of BF to estrogenic metabolites was not observed in medaka liver microsomes.	Medium	low
Beggel et al. (2011)		Larval fathead minnow exposed for 24 h to 70 or 140 ng/l BF. VTG gene expression was down regulated after 24 and 48 h but no difference after 6 d recovery.	Medium	Low-moderate
Wang et al. (2010)	Yeast assay with human ERα responsive β-galactosidase induction. The Z-Cis BF enantiomers 1R-Cis-BF and 1S-Cis-BF was tested. The relative induction efficiency (RIE) defined as the ratio of maximal enzymatic activity		High	Strong

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
	of samples to that of E2 (E2 set to 100) was 64.9 for 1S-Cis-BF and 0.11 for 1R-Cis-BF. The EC50 for 1S-Cis-BF was $10^{-7.1}$ mol/l compared to $10^{-10.1}$ mol/l for E2. The EC50 for 1R-Cis-BF could not be determined due to low activity.			
Jin et al. (2009)		zebrafish embryo–larvae were exposed to BF (150 µg/l) and Vtg-1 mRNA levels were measured using reverse transcription real-time PCR in pools of 7 larvae in three replicates. VTG1-gene expression ↑ but concentration of BF high	Medium	Moderate-strong
Wang et al. (2007)	Human MCF-7 cell proliferation assay. The Z-Cis BF enantiomers 1R-Cis-BF and 1S-Cis-BF was tested. maximum cell proliferation was 10^{-9} mol/l for 1S-Cis BF compared to 10^{-8} mol/l for 1R-Cis BF and 10^{-9} mol/l for E2. ER-interference was blocked by ICI 182,780	10-day semi-static exposure of adult male Japanese medaka (<i>Oryzias latipes</i>) to 10 ng/l BF. VTG was induced by both enantiomers but the response to 1S-cis-BF was about 123 times greater than that to the R enantiomer (N=5). This was not caused by a difference in uptake because the uptake of 1R-Cis BF was higher in both liver and other tissue	High	Strong
US EDSP tier 1 FSTRA (TG 229)		The 21-day assay (comparable to OECD 229) was performed with fathead minnows (<i>Pimephales promelas</i>) under flow-through conditions with 6-month old fish in 20 spawning groups of 2 males and 4 females. Fish were exposed to BF (93.6% purity) at mean measured concentrations of 0, DMF <20 µL/L, 17, 75, and 402 ng BF/l. There was a 100% male mortality in the high dose and erratic swimming in the females but with no significant mortality in females. There were statistically significant effects on fecundity in females, with a 60% increase at the mid concentration and a 74% reduction at the high concentration. No statistically significant reductions ($p>0.05$) in fertilization success were seen. There was an increase of 51% in female GSI at the high concentration. There was a significant increase in oocyte atresia in 13/15 female in the high treatment group (compared to 4/15 fish in the negative control). There were	High	low

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
		no other statistically significant differences between severity scores or gonadal stages in treated groups compared to the negative or solvent controls for females. No effect on Plasma VTG were seen but a trend toward increasing VTG in female fish was seen at the highest test concentration (P=0.087).		
US EDSP tier 1 AMA (TG 231) (OCSPP Guideline 890.1100)		<p>The AMA (OECD 231) protocol was followed. Exposure concentrations were 13.3, 50.6 & 235 ng/l BF. Biological Endpoints: Day 7: NF stage (Nieuwkoop & Faber), wet weight, SVL (snout-vent-length), HLL (hind-limp-length), normalized HLL. Day 21: NF stage, wet weight, SVL, HLL, normalized HLL, thyroid histopathology</p> <p>There were significant differences between the negative and solvent control groups in growth parameters, which showed a consistent pattern of promotion in the solvent control relative to the negative control.</p> <p>BF significantly delayed (p<0.05) median NF developmental stage at 21 days in the high treatment group (median NF=57) relative to the solvent control (median NF=58.5). No effect in relation to negative control.</p> <p>Day 21 HLL (Hind Limp Length) and normalized HLL were significantly decreased (p<0.05) by 22.1 and 18.7%, respectively, in the high treatment group relative to the solvent control</p> <p>Mild to moderate thyroid gland atrophy was noted in the high treatment group (9/20) relative to the negative control (4/15) and solvent control (4/20)</p>	High	Low-moderate

Table 3. Overview of potential endocrine-related adverse effects of bifenthrin in fish.

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Decourten & Brander (2017)	Inland silverside	In vivo effect on reproduction including decreased fecundity	high	moderate
Brander et al. (2016)	Inland silverside	In vivo effect on reproduction including decreased fecundity	high	moderate
USEPA, EDSP Tier 1 FSTRA	Fathead minnow	In vivo effect on reproduction including increased and decreased fecundity	high	Low-moderate

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Di-n-pentylphthalate (DPP), CAS no.131-18-0

Synonyms: Dipentyl phthalate (DPeP), Diamyl phthalate, Amyl phthalate, Amoil, di-n-Amyl phthalate

Di-n-pentyl phthalate (C₁₆ H₂₆ O₄) is a phthalate ester used as a plasticizer (Figure 1). Since no registration dossiers in REACH on DPP are available, it is assumed that it is produced and/or imported to EU in tonnages less than 100 tpa. In EU (classification) the substance is classified as Rep. Cat. 1B (H360: May damage fertility or the unborn child) and as hazardous to the environment/ acute hazard category 1 (H400: very toxic to aquatic life).

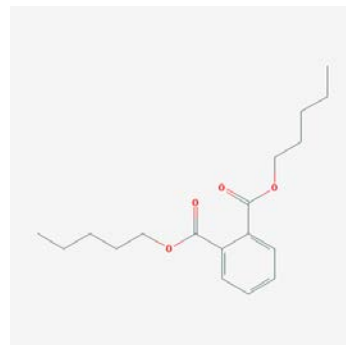


Figure 1: 2D structure from PubChem

4. Human health hazard assessment

4.10.3 Endocrine disruption

4.10.3.1 General approach – human health

4.10.3.2 *In vitro* information indicative of endocrine activity

Yuan et al. (2012)

Summary: In this study, 14 phthalates were tested for their inhibitory action on human and rat testicular 3 β -hydroxysteroid dehydrogenase (3 β -HSD) and 17 β -hydroxysteroid dehydrogenase 3enzymes (17 β -HSD3) (with exposure to 1mM phthalate) and their activity was compared with their structure. The inhibitory effect of a range of concentrations of the phthalate (10⁻⁷-10⁻³ M) was tested on the microsomal enzymes prepared from human and rat testis. The enzymes are important in testosterone synthesis in Leydig cells. The enzyme 3 β -HSD catalyses conversion of pregnenolone to progesterone and 17 β -HSD3 catalyses conversion of androstenedione to testosterone.

Activity of 3 β -HSD was reduced by more than 50% in both rat and human testis and 17 β -HSD3 was also reduced in rat and human testis by DPP. Altogether, the results demonstrated that phthalates with ethanol moieties consisting of 1-2 or 7-8 carbon atoms had no effects on the enzyme activities.

Study quality and assessment: The study is assessed to be of high quality and the study provides moderate evidence of an endocrine mode of action of DPP on testosterone synthesis.

Christen et al. (2010)

Summary: In this study *in vitro* androgen receptor reporter gene assays for androgenic and anti-androgenic activity was performed. Partial inhibition curves were observed for DPP whereas no notable androgenic activity was seen at the concentrations tested (3.3, 67 and 334 μ M DPP).

Study quality and assessment: The study is well described, included triplicates of the assays, included controls and tested concentrations of DPP below cytotoxic levels. This study is assessed to be of high quality. The study provides strong evidence for anti-androgenic mode of action of DPP *in vitro*.

Creasy et al. (1988)

Summary: The effects of mono-*n*-pentyl phthalate (MPP) on Sertoli cell ultrastructure and germ cells *in vitro* was investigated and compared with the effects of DPP on these cell types *in vivo*. Sertoli and germ cell cultures from Sprague-Dawley rat testis was exposed to MPP at 0, 100, 200, 500 or 1000 μM for 3 or 24 hours. Cell morphology was examined under transmission electron microscope and scanning electron microscope and evaluation of the distribution of actin filaments was performed on the cultured cells.

Sertoli cells *in vitro* changed in shape and developed microprocesses between Sertoli cells and germ cells after MPP exposure (200-1000 μM). Reorganization of actin filaments was seen *in vitro* after MPP exposure (500-1000 μM), a change that correlated well with changes in Sertoli cell shape *in vivo* after DPP exposure. Rarefaction of Sertoli cell cytoplasm *in vivo* correlated with mitochondrial hypertrophy *in vitro*.

Study quality and assessment: The study is old and the *in vivo* part of the study is relatively superficially described. The *in vitro* part of the study is described in more details than the *in vivo* part, yet some information on the number of samples and replicates used per group and assessment of cytotoxicity is not described. The *in vitro* part of the study is assessed to be of low quality. The data provide weak evidence that MPP can lead to adverse effects on the testicular Sertoli cells.

4.10.3.3 *In vivo* effects with regard to an endocrine mode of action

Gray Jr et al. (2016)

Summary: The aims of this study were to (1) determine the reductions necessary in testosterone production, testicular testosterone levels and foetal rat plasma testosterone to induce reproductive malformations and (2) determine the reduction in foetal testosterone necessary to induce postnatal adverse effects in the offspring. In this paper two animal studies are described with exposure to DPP by oral gavage of time-mated rats (0, 11, 33, 100, 300 mg/kg/day). In the foetal experiment dams were dosed GD14-18 and animals were euthanized on GD18 (n=7-9 litters per group). Testes and blood samples from fetuses were collected and endpoints for assessment of maternal toxicity was recorded (maternal weight gain, number of resorptions, live fetuses etc.). Testis weight was recorded (n=up to 3 males from 5-6 litters/group), foetal testosterone levels in plasma and testis was measured, the descent of testis was evaluated, the *ex vivo* testosterone production in testis and gene expression of foetal testes was investigated. In the postnatal experiment, dams (n=5 litters per group) were dosed GD 8-18 and necropsy of the offspring was performed when they were 120 days old. Age and weight at sexual maturation of male and female offspring were recorded. At necropsy of female offspring, gross malformations and weights of pituitary, uterus, ovaries, kidneys and livers were recorded. In male offspring gross malformations, length of gubernacular cord and weight of glans penis, seminal vesicle, ventral prostate, testis, epididymis, levator ani-bulbocavernosus muscles, Cowper's glands, kidney and liver were assessed. Testis and epididymis were processed for histopathology and male and female skulls were processed for morphometric analysis.

Assessment of maternal toxicity in the postnatal study showed decreased maternal weight gain and lower pup survival in the highest exposure group (300 mg/kg/day). In the foetal study, delayed testis descent (1 and 13 male pups with undescended testis out of 14 and 13 examined males from the 100 and 300 mg/kg dose-groups, respectively), decreased foetal testis weight (at 300 mg/kg/day) and decreased testosterone levels in plasma and testis (at 33-300 mg/kg/day) and decreased *ex vivo* testicular testosterone production (at 33-300 mg/kg/day) was found. Decreased male anogenital

distance (AGD) and nipple retention was observed in offspring from the two highest exposure groups in the postnatal experiment (2 out of 14 and 9 out of 13 examined males from each 4 litters, in 100 and 300 mg/kg/day, for nipple retention respectively). Malformations of adult male reproductive organs were seen in high numbers in the highest exposure group (50% of examined animals or more; n=13 males examined from 4 litters in the highest exposure group compared to n=30 males examined from 5 litters in controls). The malformations observed were hypospadias, agenesis of vas deferens, agenesis or abnormal seminal vesicle, abnormal testis, agenesis of epididymis, agenesis or elongated gubernaculum and undescended testis. Few males (n=2) had small ventral prostates in the highest exposure group. Weights of reproductive organs (seminal vesicle, epididymis, testes, ventral prostate, levator ani-bulbocavernosus muscles, Cowper's glands and glans penis) were decreased in 120 days old offspring. Decreased body weight and liver weight were found in adult males. Altogether, this study shows that DPP disrupts foetal testicular endocrine function and induces malformations in reproductive organs of male rats.

Study quality and assessment: The study is well-described and thorough. The number of test and control samples (i.e., n=5-9 litters) is adequate for showing clear effects and the study design is relevant. The study is assessed to be of high quality. The study provides strong evidence for an endocrine mode of action on testosterone steroidogenesis. Moreover, the study provides strong evidence for irreversible adverse effects on male reproductive system such as malformations of male reproductive organs, decreased weight of male reproductive organs, decreased AGD and nipple retention in males.

Beverly et al. (2014)

Summary: The objective of the study was to test two hypotheses: (1) that Simvastatin exposure *in utero* leads to lower foetal testicular testosterone production without changes in the expression of genes involved in cholesterol and androgen synthesis and transport; (2) that Simvastatin and DPP in a mixture reduces testosterone levels in an additive manner as they may have different modes of action. Two animal studies were performed, and one of the studies investigated the effects of DPP. In this study, pregnant rat dams were exposed by oral gavage on GD 14-18 to 50 mg/kg/day of DPP (3-4 dams/group) and euthanized GD 18. Samples from dams included blood (for clinical chemistry, e.g. cholesterol, creatinine and bilirubin), weights and gene expression of liver, adrenals and ovaries and histopathology of livers. Samples from offspring included blood (for clinical chemistry), testis for *ex vivo* foetal testicular testosterone production and gene expression of adrenals and testis.

Ex vivo foetal testicular testosterone production was measured on GD 18 and was found to be decreased. Genes involved in cholesterol transport and synthesis, sex differentiation and steroidogenesis were downregulated in the foetal testis.

Study quality and assessment: The study is well-described with few uncertainties on duration of dosing. The number of test and control samples (i.e., n=3-4 litters) is adequate based on power analysis. The study is assessed to be of high quality. The study provides strong evidence for an endocrine mode of action on testosterone steroidogenesis.

Hannas et al. (2012)

Summary: This study had three objectives. Firstly, to determine if phthalates with an anti-androgenic mode of action act through similar mechanisms on the gene expression level, secondly, to determine if the potency for the individual phthalates to reduce testosterone production correlates with their potency to reduce gene expression. Third, test a model prediction of the ability of a mixture of 9 phthalates to reduce gene expression. Several phthalates were tested in this study (DPP, diisobutyl, dihexyl, diheptyl, diisononyl and diisodecyl phthalate) for effects in testis after gestational exposure and evaluation of the relative potency of the tested phthalates. Pregnant rat dams were exposed by oral gavage GD 14-18. Animals exposed to DPP were dosed with 0, 11, 22, 100 or 300 mg/kg/day. A gene array screen on testis from GD18 foetuses was performed for DPP and downregulation was found for several genes involved in steroid synthesis (in testis and adrenals), steroid regulation and steroid transport. DPP was found to be more potent in downregulation of steroid-related genes than the other phthalates tested. This was in good coherence with the high potency of DPP to decrease testosterone production and inducing malformations in the reproductive tract postnatally, as reported in a previous study (Hannas et al. 2011). The data demonstrated that the effects of phthalates on gene expression of androgen-related genes were linked to the reduced testosterone production.

Study quality and assessment: The study is well-described and thorough. The number of test and control samples (one testis from every male foetus in a litter were pooled; n=3-4 litters per group) is adequate. The study is assessed to be of high quality. The study provides moderate evidence for an endocrine mode of action on testosterone steroidogenesis, but when these effects at gene expression level are taken together with data on testosterone synthesis reported in the previous study by Hannas et al. (Hannas et al. 2012), these data provide strong evidence for an anti-androgenic mode of action of DPP.

Hannas et al. (2011)

Summary: The objective was to establish comprehensive dose-response and potency data on foetal and postnatal male reproductive endpoints for DPP. Four different *in vivo* experiments are described. In the first experiment pregnant rat dams were exposed to a single dose of 500 mg/kg DPP on GD 17 (6 dams per group). In the second study, pregnant rat dams were exposed orally to a single dose of 0, 300, 600, 900 or 1200 mg/kg DPP on GD 17 (3 dams per group). In these two first studies *ex vivo* testicular testosterone production in foetuses GD 17 was measured in testis dissected 5-6 hours after maternal exposure. In the third study pregnant rat dams were dosed by oral gavage to 0, 11, 33, 100, 300 mg/kg/day of DPP from GD 14-18 (3 dams per group). Gene expression (of StAR, insl3 and Cyp11a) and testosterone production on GD 18 were assessed in this 5-days repeated-dose study. In the final study on postnatal development pregnant rat dams were dosed by gavage to 0, 11, 33, 100, 300 mg/kg/day of DPP from GD 8-18 (5 dams per group). AGD and NR were evaluated in the last study. Foetal testicular testosterone production was decreased in a dose-related manner and in 5-days repeated-dose study the gene expression levels of the androgen synthesis related genes StAR and Cyp11a and of insl3 were decreased (from 100 mg/kg/day). Decreased AGD (at 100mg/kg/day or more) and increased NR (at 300 mg/kg/day) was found in male offspring exposed to DPP for 10 days *in utero*.

Study quality and assessment: The study is well-described and thorough, although few details on the number of exposed dams in the first study are missing. The study is assessed to be of high quality. The study provides strong evidence for an endocrine mode of action on testicular testosterone

steroidogenesis. The study also provides strong evidence for endocrine-related adverse effects on male sexual differentiation.

Howdeshell et al. (2008)

Summary: The aim was to provide dose-response information on the effects of several phthalates on foetal testosterone production and to investigate if the data could be used to predict effects of a mixture of phthalates on testosterone production. Time-mated rat dams were dosed by oral gavage from GD8-18 with 0, 25, 50, 100, 200, 300, 600 or 900 mg/kg/day of DPP. At study start the number of mated dams was 2-6 per dose-group. On GD18, there were 5, 5, 4, 6, 4, 2, 1 and 2 pregnant dams, respectively. DPP reduced maternal body weight gain GD8-18 at 300, 600 and 900 mg/kg/day and in these dose-groups 100% foetal mortality (resorptions/implantations) was seen. Testosterone production measured in *ex vivo* testis from foetuses exposed to DPP during gestation (GD 8-18) was reduced at 100 and 200 mg/kg/day. The study showed inhibition of foetal testosterone production

Study quality and assessment: This study is well described and includes necessary information such as compound, dosing period and number of animals. The study is assessed to be of high quality. The data provide strong evidence on an anti-androgenic mode of action of DPP on foetal testosterone production.

Hild et al. (2007)

Summary: This study evaluates the time course of acute ultrastructural changes occurring in Sertoli and germ cells after a single dose of CDB-4022 with DPP as a positive control. Four adult male rats per group were exposed orally to a single dose of 0 or 2200 mg/kg DPP and killed after 0, 3, 6, 9, 12, 18 or 24 h after dosing. Controls were killed after 0 or 24 h after dosing. Testes were examined morphologically under light microscope and ultrastructurally in a transmission electron microscope.

Degenerative changes in Sertoli cells, spermatocytes and spermatids were seen after DPP exposure. Spermatogonia appeared unaffected.

Study quality and assessment: The present study is well described and included important information such as animal number, life stage of animals and time point at euthanasia, although more details on CAS-number, exact age of the animals and housing conditions would be preferred. The study is assessed to be of moderate quality. The data from the study provides strong evidence of adverse effects on testes at the cellular level but does not study the underlying modes of action and it is therefore not clear if the adverse effects are due to an endocrine mode of action.

Liu et al. (2005)

Summary: The aim of the study was to identify genes involved in the development of testicular dysgenesis. A selection of phthalates known to affect male reproductive development after *in utero* exposure was used to induce such changes, including DPP. A range of phthalates known not to affect male reproductive development was used as negative controls. Time-mated rat dams (10 controls and 5 per exposed group) were dosed by oral gavage from GD 12-19 to 500 mg/kg/day DPP. Caesarean section of foetuses was performed on GD 19, where AGD was measured and testes were used for gene expression analysis (testis from 1 foetus per litter per group was used for microarray analysis).

For RT-QPCR, 1 testis per litter from 6 litters in the control group and 1 testis per litter from 3 litters in the treated group were used). Immunohistochemistry of testes from control and DBP exposed foetuses was performed.

Male foetuses exposed to DPP *in utero* had reduced AGD compared to controls on GD19. Gene expression levels in GD19 foetal testes exposed to DPP *in utero* was changed for genes involved in regulating steroidogenesis and insulin signalling (e.g. downregulation of Luteinizing hormone/choriogonadotropin receptor (*Lhcgr*), Low-density lipoprotein receptor (*Ldlr*) and Insulin induced gene (*Insig1*)). The phthalates appeared to target Sertoli cells and gonocytes and the interaction between the two.

Study quality and assessment: The study is well described and important information on sample sized, exposure period and age at tissue sampling are described. Although no CAS-number for DPP is mentioned, the study is assessed to be of high quality. The data provide strong evidence of adverse effects on male AGD. The changes in gene expression levels of genes involved in steroidogenesis provide moderate evidence of a mode of action of DPP on steroidogenesis.

Hild et al. (2001)

Summary: In this study, the testicular cell types affected by CDB-4022 were determined. DPP was used as a known Sertoli cell toxicant and estradiol-3-benzoate was used as a positive control of germ cell apoptosis. To study the acute effects on testicular germ cell apoptosis and Sertoli cell function and testicular morphology, fertile male rats were exposed to a single oral dose of DPP (2200 mg/kg, 3 per group) and killed after 3, 6 or 12 hours after dosing. A second study was performed to investigate the acute effects on Sertoli cell function in prepubertal male rats (10 per group) dosed orally with a single dose of 2200 mg/kg DPP and euthanized 24 or 48 hours after dosing. An indicator of the seminiferous tubule fluid (STF) was assessed. Serum inhibin B and epididymal androgen binding protein (ABP) content were assessed in the two studies to evaluate Sertoli cell function. A third study was performed to determine the effects on Leydig cell function and fertility but only exposure to CDB-4022 was investigated.

In the first study (in adult males), an increased percentage of seminiferous tubules with a higher number of apoptotic germ cells (early spermatocytes and spermatogonia) per tubule compared to controls was seen. Morphologically, vacuolization of Sertoli cells and detachment of germ cells was observed. Serum inhibin B was decreased. In the second study (in prepubertal males), STF secretion (by Sertoli cells) was suppressed and serum inhibin B levels and epididymal ABP were decreased. The data indicate that DPP interferes with early stages of spermatogenesis by inducing apoptosis of early stages of germ cells via disruption of Sertoli cell structure and function.

Study quality and assessment: The study is well-described, but more details on housing conditions, age of adult males, CAS-number and purity of DPP would be preferred. The study is assessed to be of moderate quality. The data show decreased Sertoli cell function, disrupted Sertoli cell morphology and apoptosis of germ cells. The study provides strong evidence of adverse effects on testis function and morphology in adult males and decreased function of Sertoli cells in prepubertal male rats after exposure to DPP.

Jones et al. (1993)

Summary: The aim of this study was to investigate phthalate toxicity on Leydig cells structure and functionality. Four different phthalates, including DPP, were investigated *in vivo* and their monoesters, including MPP, were investigated *in vitro*. Adult (6-8 weeks old) male rats were exposed to DPP by daily oral gavage for 2 days (n=3/group). Rats were euthanized 24h after the last dose. Rats were killed and both testes from each rat were used for pathological and ultrastructural examination. *In vitro* tests on isolated Leydig cells from 10 male rats were performed to measure the effects of MPP on testosterone production.

Exposure to DPP showed rarefaction and vacuolization of Sertoli cells, necrosis of germ cells and shedding of spermatocytes and spermatids. No changes were seen in Leydig cell morphology or ultrastructure and *in vitro* secretion of testosterone from Leydig cells was not affected by MPP.

Study quality and assessment: Although the study is old, some important information on number and age of animals and viability of cultured cells *in vitro* is provided. No information on CAS-number or purity is provided and more details on housing of animals would be preferred. The quality of this study is assessed to be of moderate quality. The data provide strong evidence on adverse effects of DPP on the structure of testicular tubular cells (Sertoli cells and spermatogonia) but not on Leydig cells *in vivo*. The study provides moderate evidence that the mode of action of DPP behind the effects on testosterone production is not through the metabolite MPP on Leydig cell function.

Granholt et al. (1992)

Summary: The testicular inflammatory reaction after DPP exposure and correlation with morphological inflammatory changes in the testes was investigated in rats and mice in this study. Rat testis weight and morphology were also assessed. Male rats (40 days old) were dosed by oral gavage to 2.2 g/kg DPP. Rats were killed after 0, 3, 6, 9, 12, 18 or 24 hours after dosing. Control animals were killed 0 or 24 h after dosing.

No changes were seen in testis weight but histological examination showed slight morphological changes in Sertoli cells with rarefaction of the basal cytoplasm.

Study quality and assessment: The present study is poorly described and it is not always readily obvious whether data are assessed from mice or rats. The study is old and there is missing information on CAS-no, number of animals used per treatment groups and animal housing conditions. The study is assessed to be of low quality. The reported data provide weak evidence for adverse effects of DPP on male testis.

Heindel et al. (1989)

Summary: The reproductive toxicity of three phthalates was investigated in this study, where a continuous breeding protocol was followed. The reproductive effects of DPP were investigated in a breeding study and in a cross-over breeding study. In the breeding study, 40 control pairs and 20 pairs/treatment group was used. Male and female mice (11 weeks old) were exposed to 0, 0.5, 1.25 or 2.5% DPP in the feed for 7 days prior mating. The breeding pairs were evaluated for clinical signs, body weight, fertility (based on the number of pairs producing litters), number of litters per pair, live pups per litter, proportion of pups born alive, sex of live pups, pup body weight and food and water consumption. In the 1-week cross-over breeding study, exposed mice (20 pairs/group) were paired

with control mice of the opposite sex to determine the sex affected by DPP. Mice were exposed to 0 or 2.5% DPP in the food during the whole period after mating. Decreased fertility was seen in the breeding study in the 0.5% treatment group; fewer litters/pair, fewer live pups per litter and fewer pups born alive were seen. Breeding pairs exposed to higher levels of DPP were infertile. In the cross-over breeding study, both male and female mice were infertile after treatment with 2.5% DPP. Decreased body weight was seen for male and female mice and decreased absolute and relative organ weights were found for testis, epididymis, seminal vesicles and kidneys. Epididymal sperm count was decreased to a non-detectable level. Prostate weight was comparable to controls and liver weight was increased (absolute and relative). Histopathological changes were seen in testis, where degeneration of seminiferous tubules and interstitial cell hyperplasia was seen. In epididymis, accumulation of fluid and degenerated cells was seen. No effects were seen on oestrous cycle length and no histopathological changes were found in female reproductive organs (ovaries, oviduct, uterus and vagina). Infertility induced by DPP on mating mice was shown to be related to effects in both the male and the female mice, although no morphological or weight-related changes were found in the reproductive organs of exposed female mice. Several changes in male reproductive organs were found.

Study quality and assessment: Although the study is old and the study protocol is complicated, it is well-described and includes important information such as number and age of animals. More information on housing conditions and CAS-number would have been preferred. A large number of animals per group were used. The study is assessed to be of high quality. This study provides strong evidence on adverse effects on reproductive organs and fertility of mice exposed to DPP.

Creasy et al. (1988)

Summary: The effects of MPP on Sertoli cell ultrastructure and germ cells *in vitro* was investigated and compared with the effects of DPP on these cell types *in vivo*. An *in vivo* study with 3 adult (28 days old) male rats per group exposed orally to a single dose of 2.2 g/kg DPP were killed 1, 3, 6, 12 or 24 hours after dosing. Morphology of Sertoli- and germ cells was evaluated under transmission electron microscopy and compared with the findings from the *in vitro* tests with MPP.

Generally seen, Sertoli cell shape resulted in direct apposition of germ cells 6 hours after dosing and necrotic germ cells 24 hours after dosing. Interstitial neutrophil infiltrates were apparent after 12 hours of dosing. Rarefaction of Sertoli cell cytoplasm *in vivo* correlated with mitochondrial hypertrophy *in vitro* and changes in Sertoli cell shape *in vivo* after DPP exposure correlated well with Sertoli cell shape and actin filament organisation changes seen *in vitro* after MPP exposure.

Study quality and assessment: The study is old and the *in vivo* part of the study is relatively superficially described. The most important information such as age and number of animals used, the doses and timing of termination is described, but no details on housing conditions, source of the animals or whether one or two testes per animal was used for the assessment of testicular changes was described. The *in vivo* part of the study is assessed to be of low quality. The data provide moderate evidence that DPP can lead to adverse effects on the testicular Sertoli cells.

Lindström et al. (1988)

Summary: The relationship between spermatogenesis and serum levels of androgen binding protein were investigated in this study by testing if testicular effects are reflected by altered serum levels of androgen binding protein. This was performed in two animal studies with single dose-exposure to DPP at 0, 0.25, 1 or 2g/kg by oral gavage. In the first study, 10 adult Fisher 344 rats per group (approximately 6 weeks of age) were killed 2 days after exposure or each week for 10 weeks. At necropsy, body weight and weight of testis, epididymis, prostate, seminal vesicle, liver and kidney were assessed. Epididymal sperm density and morphology were measured and histological morphology of testis and epididymis was evaluated. Serum androgen binding protein was measured.

In the second study (20 males per group), male fertility after DPP exposure was evaluated by testing their ability to impregnate female rats and assessment of the number of live pups and preimplantation loss. After fertility assessment, the males were killed 14, 18 and 30 weeks after dosing (5 controls and 5 high-dose males at each time-point) to assess testicular recovery histologically.

In the first study Lindström and co-workers found effects on body weight and male reproductive organs. Lower body weight in the two highest dose-groups was seen during the first week after dosing. Testicular weight and epididymal weight were lower than controls in the 1 and 2g/kg dose-groups and remained below controls for all 10 weeks they were studied. Sperm density was lower than controls in all dose-groups and sperm morphology was abnormal in the highest dose-group. Histological evaluation of testis showed effects on germ cells and intertubular spaces. Liver weights were increased 2 days after dosing but returned to control levels after 2 weeks. In the second study, fewer fertile males were found in the high-dose group compared to controls; they had less ability to impregnate female rats, there was an increased number of preimplantation losses after mating and an increased number of dead pups. Finally, 30 weeks after dosing no signs of recovery of the testicular damage was seen.

Study quality and assessment: Although this is an old study much important information is included such as purity of the chemical, rat strain, details on housing conditions and number of animals for each study performed and for the endpoints assessed. However, more information on CAS-number would be preferred. The study is assessed to be of moderate quality. The data from the present study provide strong evidence for adverse effects on testis in adult males after exposure to a single dose of DPP without obvious recovery at the cellular level 30 days after exposure.

Creasy et al. (1987)

Summary: The study investigates the effects of 2.2 g/kg DPP on sexually mature rat testis at different time-points after oral exposure. Two control and three exposed animals (15 weeks of age) were killed 3, 6, 12, 18, 24 or 48 h after dosing and testis were examined under light microscope, electron microscope and transmission electron microscope.

Creasy and co-workers found stage-specific changes in the testicular tubules (morphologically observed as vacuolization and closure of the lumen, increased affinity for stain and vacuolization of tail cytoplasm of elongating spermatids and necrosis of spermatocytes) and degeneration of Sertoli cells. Interstitial inflammatory infiltrate was seen 12 hours after dosing or later. The changes appeared to be reversible.

Study quality and assessment: Although this is an old study, it is well described and includes much important information such as number of animals used, age of animals at study start and detailed

descriptions of the changes observed. Few control animals were included (2 control rats per experiment) and no CAS number or purity was stated for the chemical. All in all the study is assessed to be of moderate quality. This study provides strong evidence for testicular adverse effects in sexually immature rats in the case of continuous exposure.

Gray and Gangolli (1986)

Summary: This paper investigates some features of testicular effects of some phthalates in rats, including effects on Sertoli cells, and effects of phthalate monoesters on testicular germ cell cultures. Some investigations, such as the effects on reproductive organs with co-administration of gonadotropins or the effects of metabolites on testicular cell cultures of Sertoli cells and germ cells were performed on other phthalates but not on DPP or the metabolites of DPP.

Tubular atrophy was seen in testes from 15 weeks old males 24 hours after exposure to 2200 mg/kg/day DPP, but was less severe and developed slower than in immature rats. A single dose of 2200 mg/kg DPP in immature 4-5 weeks old rats (5 per group) showed a complete or close to complete depression of androgen binding protein (ABP) and fluid in rete testis, respectively. The lower single dose of 440 mg/kg DPP decreased fluid and ABP, but not 220 mg/kg. In older rats 10 weeks of age, the highest dose (2200 mg/kg) also decreased fluid and ABP, but not in the same dramatic extent as in the younger rats (reduced to 60% of control values). Repeated dosing of 220 mg/kg for 3 days did not change fluid or ABP production significantly. Mono-*n*-pentyl phthalate (MPP) was shown to cross the blood-testis barrier in very little amounts (levels in rete testis was 5% of plasma levels 25 minutes after phthalate administration, n=3 controls and 2 dosed with MPP). MPP administered *i.v.* for 4 days showed histological testicular changes characteristic of phthalate changes at 50 mg/kg/day but not at 25 mg/kg/day.

Study quality and assessment: The methods used in the study are described in an unstructured way and are confusing. Dosing levels, duration of dosing and number and age of animals used is not described in the methods section and the number of animals is not accessible, if the data are not presented in a table (e.g. for the data on testicular atrophy in males exposed to DPP). The methods used for cell culture are poorly or hardly described. It is unclear whether some of the data described are from the present study or are a description of findings from a previously published study. The study is assessed to be of low quality. The data provide weak evidence of adverse effects of DPP on male reproductive tract.

Creasy et al. (1983)

Summary: The study was performed to identify the primary site of DPP damage and follow the development of the lesion. Moreover, the study aimed at providing indications of the functional integrity of the affected Sertoli cells. Single dose studies and repeated dose studies on DPP are described with focus on testicular effects in sexually immature male Sprague-Dawley rats (3-4 weeks of age). Necropsies of rats were performed 1, 3, 6 or 24 hours after a single dose of 2.2 g/kg DPP or after 3, 4 or 5 days of daily dosing of 2.2 g/kg DPP. One control animal and 3 exposed animals were used for each experiment.

Early degenerative vacuolization of Sertoli cells 3 to 6 hours after oral dosing was seen. After 2-4 days of repeated dosing, degeneration of spermatocytes and spermatids as well as depletion of spermatogonia and germinal cells was seen.

Study quality and assessment: Although this is an old study, it is well described and includes much important information such as purity of the chemical, age of animals at study start and at necropsy and number of animals used. Few control animals were included (1 control rat per experiment) and no CAS-number was stated for the chemical. All in all the study is assessed to be of moderate quality.

This study provides moderate evidence for testicular adverse effects in sexually immature rats.

Foster et al. (1983)

Summary: Steroidogenic enzyme activity was measured in testis and livers from male rats (4 weeks old) exposed orally to DPP or diethyl phthalate daily or as a single dose. Male rats were dosed with a single dose of 7.2 mmol/kg of DPP and killed 0, 1, 3, 6 or 24 h after dosing. Other male rats were dosed daily with 7.2 mmol/kg/day of DPP and killed after 2, 3 or 4 days. Microsomes and cytochrome P450 from testes and livers were extracted. Single doses of DPP decreased enzyme activity of 17- α -hydroxylase and 17-20 lyase in testes. Repeated dosing of DPP for 2 or 4 days increased the activity of 17- β -dehydrogenase in testicular microsomes. 17- α -hydroxylase, 17-20 lyase and 17- β -dehydrogenase are enzymes involved in conversion of progesterone to testosterone. Cytochrome P450 in testes was also decreased by DPP.

Study quality and assessment: The present study is old and there is missing information on CAS-number, number of animals used per treatment groups and animal housing conditions. The study is assessed to be of weak quality. The reported data provide moderate evidence for an endocrine mode of action of DPP through action on the steroidogenic enzymes in testes.

Foster et al. (1982)

Summary: The aim of this study was to determine the localization of zinc in the normal testis and in testis exposed to DPP until reaching atrophy of testis. Single dose and repeated dose studies of 2.2 g/kg DPP by oral gavage in male rats were described by Foster et al (1982). In the single-dose experiments, 3 controls and 3 exposed animals were killed at 0, 1, 3, 6 or 24 h after dosing. In the repeated-dose studies, 3 controls and 3 exposed animals were killed after 2, 3 or 4 days of daily exposure. The weight of testis, prostate and seminal vesicle was measured and a significant decrease in testis weight was found. Morphology of testes was assessed and vacuolization of Sertoli cells and necrosis of spermatids and spermatocytes was found. Analysis of zinc deposits in testicular cells indicated that the testicular damage may be associated to zinc depletion.

Study quality and assessment: Although this is an old study, it is well described and includes much important information such as purity of the chemical, weight of animals at study start and number of animals used. The relative organ weights were reported as % of controls and the life stage (sexually mature or immature) of the animals is unclear. The study is assessed to be of moderate quality. This study provides strong evidence of testicular adverse effects. The data indicate a relation to zinc depletion which may be affect gonadotropins and androgen production in testis, but it is not clear if the adverse effects are due to an endocrine mode of action.

Foster et al. (1980)

Summary: This study was performed to compare the testicular effects of some phthalates with their effects on zinc excretion in the rat. Young adult male rats (70-90 g, 12/group) were exposed to 7.2 mmol/kg/day (2.1 g/kg/day) of DPP by oral intubation for 4 days. Body weight gain, food intake, testis weight, histological testicular morphology and zinc levels in urine, faeces, testis, liver and kidney were assessed. Relative testis weight was decreased (data on absolute weight is neither shown nor mentioned) and histological evaluation of testis showed atrophy of seminiferous tubules with loss of spermatocytes and spermatids. Urinary excretion of zinc-65 was increased. Zinc content in testis was decreased whereas the content in liver and kidney was increased. The animals showing testicular atrophy also had increased urinary excretion of zinc and a putative link between low zinc levels in testis and testicular atrophy was established.

Study quality and assessment: Although no CAS-number or information on housing conditions is provided in this early study, other important information is given, such as number and weight of animals used, dose level and method and duration of dosing. Therefore the study is assessed to be of moderate quality. The data from the present study provide strong evidence of testicular adverse effects of DPP. Moreover, the study provides some evidence of a mode of action of DPP involving zinc, but it is not clear if the adverse effects are due to an endocrine disrupting mode of action. Therefore the study is assessed to provide weak evidence of an endocrine-related mode of action of DPP.

4.10.3.4 Summary of the plausible link between adverse effects and endocrine mode of action

Several *in vivo* studies investigating the endocrine mode of action of DPP showed an anti-androgenic mode of action of DPP (Table 1). Many studies showed decreased testosterone levels in foetuses either in plasma, testis or *ex vivo* testicular testosterone production (Gray et al. 2016; Beverly et al. 2014; Hannas et al. 2011; Howdeshell et al. 2008). Genes involved in steroid synthesis, regulation or transport have also been shown to be downregulated by DPP exposure (Hannas et al. 2012; Hannas et al. 2011; Liu et al. 2005). Finally, microsomal enzymes involved in steroid synthesis were affected by DPP exposure *in vivo* and *in vitro* (Yuan et al. 2012; Foster et al. 1983). Other *in vitro* studies investigating the endocrine mode of action of DPP and the monoester mono-n-pentyl phthalate (MPP) showed weak signs of anti-androgenic activity of DPP and no effects of MPP on testosterone production in Leydig cells (Christen et al. 2010; Jones et al. 1993). Some studies investigating zinc excretion and deposition in testis showed that low zinc levels may be involved in DPP related testis damage (Foster et al. 1982; Foster et al. 1980). Altogether, several rodent studies have demonstrated strong evidence of an anti-androgenic mode of action of DPP *in vivo*.

DPP is classified as a reproductive toxicant and adverse effects on the reproductive system in rodents that may be related to an endocrine disrupting mode of action have been shown in a large number of *in vivo* studies (Table 2). Effects on the male reproductive system that are known to be able to be induced via endocrine disrupting modes of action have been reported, e.g. decreased male AGD, increased nipple retention in males, decreased number of spermatocytes (decreased sperm count), malformations of male reproductive organs (hypospadias, malformations of vas deferens, epididymis and gubernacular cord), decreased weight of male reproductive organs in adulthood, decreased foetal testis weight and delayed descent of testis. Other changes in the male reproductive system were also seen although it is not clear if the adverse effects are due to an endocrine mode of action. The changes

include degenerative changes of testicular Sertoli cells and degeneration or loss of spermatocytes (Hild et al. 2007; Hild et al. 2001; Jones et al. 1993; Granholm et al. 1992; Creasy et al. 1988; Creasy et al. 1987; Creasy et al. 1983; Foster et al. 1982; Foster et al. 1980).

Effects of DPP were also seen on female fertility without effects on weights of female reproductive organs (Heindel et al. 1989), but the modes of action are unclear. All in all the evidence for adverse effects of DPP in males is strong but the evidence for adverse effects in the female reproductive system is moderate.

Summary and conclusions

Overall, several rodent studies have shown adverse effects on the male reproductive system and male reproductive development. Many of the effects observed are generally known to be androgen dependent and changes such as decreased AGD and increased nipple retention in males is associated with an anti-androgenic mode of action and low androgen levels. Studies on the endocrine-related modes of action of DPP showed effects on steroid production at several levels including downregulation of steroid-related genes, reduced activity of enzymes involved in steroid synthesis and decreased levels of testosterone in plasma and testis. The adverse effects observed for DPP can be attributed to an anti-androgenic mode of action, and it is highly biologically plausible, that there is a link between the decreased testosterone synthesis found for DPP and the adverse effects observed on male reproductive organs and male reproductive development. The evidence for an endocrine mode of action of DPP is strong, the evidence for adverse effects on male reproductive system is strong and there is strong evidence for a causal link. In conclusion, DPP meets the WHO definition of an endocrine disruptor.

Additional literature not included in the evaluation

Reviews on DPP have been used to check for additional literature not found in our literature search (Gangolli et al. 1982; Gray et al. 1982; NTP 1985).

Table 1. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of di-n-pentylphthalate (DPP)

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Gray et al. 2016		Decreased testosterone levels in plasma, testis and <i>ex vivo</i> testicular testosterone production.	High	Strong
Beverly et al. 2014		Decreased <i>ex vivo</i> testicular testosterone production.	Medium	Strong
Hannas et al. 2012		Downregulation of several genes in GD18 testis involved in steroid synthesis, steroid regulation and steroid transport.	High	Moderate
Yuan et al. 2012	Reduced activity of microsomal enzymes related to testicular testosterone synthesis.		High	Moderate
Hannas et al. 2011		Decreased <i>ex vivo</i> testicular testosterone production in a dose-related manner in testis GD 17 and 18 and downregulation of gene expression levels of androgen synthesis genes StAR and cyp11a and of insl3 in testis GD 18.	High	Strong
Christen et al. 2010	Androgen receptor reporter gene assay: Partial inhibition curves (anti-androgenic activity) were observed for DPP. No notable androgenic activity was seen.		High	Moderate
Howdeshell et al. 2008		Reduced testosterone production in <i>ex vivo</i> foetal testis exposed to DPP during foetal development (GD 8-18).	High	Strong
Liu et al. 2005		Gene expression levels in GD 19 foetal testes was changed for genes involved in regulating steroidogenesis and insulin signaling. DPP appeared to target Sertoli cells and gonocytes and the interaction between the two.	High	Moderate
Jones et al. 1993	Secretion of testosterone from cultured Leydig cells was not affected by MPP.		Medium	Moderate

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Creasy et al. 1988	An <i>in vitro</i> study on effects of MPP on Sertoli cells showed changes in Sertoli cell shape, development of microprocesses between Sertoli cells and germ cells and reorganization of actin filaments.		Low	Weak
Foster et al. 1983		Single doses of DPP decreased microsomal enzyme activity involved in testosterone synthesis in testes. Repeated dosing of DPP for 2 or 4 days increased the activity the enzymes. Cytochrome P450 in testes was also decreased by DPP.	Low	Moderate
Foster et al. 1982		Analysis of zinc deposits in testicular cells indicated that the testicular damage may be associated to zinc depletion.	Medium	Weak
Foster et al. 1980		Animals showing testicular atrophy had increased urinary excretion of zinc and decreased zinc levels in testis.	Medium	Weak

MPP: Mono-n-pentyl phthalate, the monoester of DPP, gestation day (GD)

Table 2. Overview of potential endocrine-related adverse effects of di-n-pentylphthalate (DPP)

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Gray Jr et al 2016	Rats, n=5 litters/group	Decreased male AGD PND2, malformations of male reproductive organs in adult offspring (hypospadias, malformations of vas deferens, epididymis and gubernacular cord), decreased weight of reproductive organs in adult offspring. Decreased foetal testis weight and delayed testis descent GD 18.	Medium	Strong
Hannas et al. 2011a	Rats, n=5 dams/group	Decreased male AGD PND2 and increased male NR PND13.	High	Strong
Hild et al. 2007	Rats, n=4/group	Degenerative changes in Sertoli cells, spermatocytes and spermatids were seen after DPP exposure. Spermatogonia appeared unaffected.	High	Strong
Liu et al 2005	Rats; n=10 control dams and n=5 exposed dams	AGD in GD 19 foetuses was decreased after DPP exposure GD12-19.	High	Strong
Hild et al. 2001	Rats, n= 3 adults/group and 10 prepubertal / group	An increased % of seminiferous tubules with a higher number of apoptotic germ cells per tubule compared to controls was seen. Morphologically, vacuolization of Sertoli cells and detachment of germ cells was observed. Serum inhibin B was decreased. In the second study (in prepubertal males), STF secretion (by Sertoli cells) was suppressed and serum inhibin B levels and epididymal ABP were decreased.	Medium	Strong
Jones et al. 1993	Rats; n=3/group	Rarefaction and vacuolization of Sertoli cells, necrosis of germ cells and shedding of spermatocytes and spermatids after DPP exposure. No changes were seen in Leydig cell morphology or ultrastructure after DPP exposure.	Medium	Strong
Granholtm et al. 1992	Rats, n=not described.	No changes were seen in rat testis weight but histological examination showed slight morphological changes in Sertoli cells with rarefaction of the basal cytoplasm.	Low	Weak
Heindel et al. 1989	Mice; study A: n=40 control and 20 treated	Fewer litters/pair, fewer live pups per litter and fewer pups born alive were seen in the 0.5% treatment group. Breeding pairs exposed to higher levels of DPP (1.25 or 2.5%) were infertile. In the cross-over breeding study, both male and female mice were infertile after treatment with 2.5%	High	Strong for males, moderate for females

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
	breeding pairs/group; study B: n=20 cross-over breeding pairs/group;	DPP. Decreased body weight was seen for male and female mice and decreased absolute and relative organ weights were found for testis, epididymis and seminal vesicles. Epididymal sperm count was decreased to a non-detectable level. Prostate weight was comparable to controls and liver weight was increased (absolute and relative). Histopathological changes were seen in testis, where degeneration of seminiferous tubules and interstitial cell hyperplasia was seen. In epididymis, accumulation of fluid and degenerated cells was seen. No effects were seen on estrous cycle length and no histopathological changes were found in female reproductive organs.		
Creasy et al. 1988	Rats; n=3/group	Changes in Sertoli cell shape and rarefaction of the cytoplasm was seen after DPP exposure.	Low	Moderate
Lindström et al. 1988	Rats, n=10/group, n=20 in fertility study, n=5 in recovery study	Study on reproductive organs: lower body weight in the two highest dose-groups (1 and 2 g/kg) was seen during the first week after dosing. Testicular weight and epididymal weight were lower than controls in the 1 and 2g/kg dose-groups and remained below controls for all 10 weeks they were studied. Sperm density was lower than controls in all dose-groups. Sperm morphology was abnormal in the highest dose-group. Histological assessment of testis showed effects on germ cells and intertubular spaces. Liver weights were increased 2 days after dosing but returned to control levels after 2 weeks. Prostate and seminal vesicle weights were never different from controls. Fertility study: less fertile males were found in the high-dose group compared to controls – they had less ability to impregnate female rats, there was an increased number of preimplantation losses after mating and an increased number of dead pups. Recovery study: no signs of recovery on germinal epithelium were seen 30 weeks after dosing.	Low-medium	Strong
Creasy et al. 1987	Rats, n= 2 control and 3 exposed	Stage-specific changes in the testicular tubules and degeneration of Sertoli cells. The changes appeared to be reversible.	Medium	Strong

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Gray and Gangolli 1986	Rats, n=5/group unless otherwise mentioned	Testicular tubular atrophy (in 15 weeks old males) 24 hours after exposure to 2200 mg/kg/day DPP, but was less severe and developed slower than in immature rats. A single dose of 2200 mg/kg DPP in immature 4-5 weeks old rats showed a complete or close to complete depression of ABP and fluid in rete testis, respectively. 440 mg/kg DPP decreased fluid and ABP. In rats 10 weeks of age, the highest dose (2200 mg/kg) also decreased fluid and ABP, but not in the same dramatic extent as in the younger rats. Repeated dosing of 220 mg/kg for 3 days did not change fluid or ABP production significantly. Mono-n-pentyl phthalate (MPP) was shown to cross the blood-testis barrier in very little amounts (n=3 controls and 2 dosed with MPP). MPP administered i.v. for 4 days showed histological testicular changes characteristic of phthalate changes at 50 mg/kg/day but not at 25 mg/kg/day	Low	Weak
Creasy et al. 1983	Rats, n=1 control and 3 exposed/time-point	Degenerative vacuolization of Sertoli cells 3 to 6 hours after oral dosing. Degeneration of spermatocytes and spermatids as well as depletion of spermatogonia and germinal cells was seen with repeated dosing.	Medium	Moderate
Foster et al. 1982	Rats, n=3/group	Vacuolization of Sertoli cells and necrosis of spermatids and spermatocytes was found.	Medium	Strong
Foster et al. 1980	Rats, n=12/group	Relative testis weight was decreased and histological evaluation of testis showed atrophy of seminiferous tubules with loss of spermatocytes and spermatids.	Medium	Strong

ABP: androgen binding protein. AGD: ano-genital distance. GD: gestation day. PND: postnatal day. STF: Seminiferous tubule fluid, MPP: Mono-n-pentyl phthalate, the monoester of DPP.

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Bisphenol AF (BPAF), CAS no.1478-61-1

Synonyms: 4,4'-(Hexafluoroisopropylidene)diphenol

BPAF is a fluorinated organic diphenol and a structural analog to Bisphenol A in which the two methyl groups are replaced with trifluoromethyl groups (Figure 1). BPAF has broad applications and is for example used as cross-linking reagent in the production of fluoropolymers and fluoroelastomers and as a monomer in production of many polymers. Since no registration dossiers on BPAF are available, it is assumed that it is produced and/or imported to EU in tonnages less than 100 tpa. BPAF has been detected in different environmental samples and in samples from humans. At present there is a lack of toxicological information on BPAF and is therefore currently undergoing extensive evaluations for *in vivo* toxicity by the National Toxicology Program, USA.

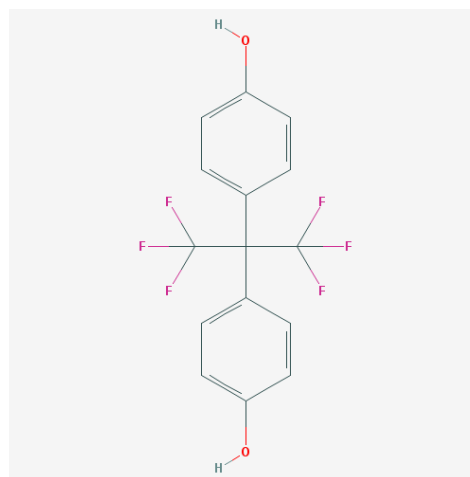


Figure 1. 2D structure from PubChem

4. Human health hazard assessment

4.10.3 Endocrine disruption

4.10.3.1 General approach – human health

4.10.3.2 *In vitro* information indicative of endocrine activity

Lei et al. (2017)

Summary: In this study, the effects of BPAF (>98% purity, CAS no. 1478-61-1) (and other bisphenols) on cell viability, DNA and plasma membrane damage, intracellular reactive oxygen species (ROS) formation, and Ca(2+) levels of MCF-7 cells were evaluated. Cell viability of MCF-7 cells was measured using the cell counting kit-8 (CCK-8) assay with BPAF concentrations ranging from 0.01-100 μ M for 24 h. Lactate Dehydrogenase (LDH) release was assessed to indicate the plasma membrane damage in MCF-7 cells, and for this BPAF was tested at concentrations from 1-100 μ M for 24 h. DNA damage was measured by alkaline single cell gel electrophoresis, i.e. the Comet assay, and BPAF was tested in concentrations from 1-50 μ M for 24 h. The production of ROS was measured after cells were exposed for 24 h to concentrations of BPAF from 0.01-50 μ M. Calcium levels were measured after 24 h exposure to 0.00001–10 μ M BPAF. At the same time, the estrogenic and thyroidal hormone receptor-mediated potentials (i.e., ER α and TR α activity) of BPAF were evaluated using the two-hybrid yeast bioassay with 10-12 dilutions (in triplicate).

The results showed that BPAF significantly increased cell viability in MCF-7 cells at low concentrations (0.01-1 μ M), but caused a significant decrease in cell viability at higher exposure concentrations (25-100 μ M). BPAF increased LDH activity (25, 50 and 100 μ M, i.e. at cytotoxicity levels), and caused increased DNA-damage (10, 25 and 50 μ M) in MCF-7 cells. BPAF also resulted in a concentration-dependent increase in both ROS production (0.01-25 μ M, i.e. below cytotoxicity) and intracellular Ca(2+) levels (0.0001-10 μ M). The two-hybrid yeast bioassay showed that BPAF is an estrogen receptor α (ER α) (EC50 = 5.3 μ M) and thyroidal hormone receptor α (TR α) (EC50 = 1.4

μM) agonist. Overall the results from the present study showed that BPAF exert cell biological effects, estrogenic and thyroidal effect with greater potency than BPA.

Study quality and assessment: In general, the study is well-described although the material and methods section does not specify the exact tested concentrations. This can instead be read from the graphs, and a large concentration range from 0.0001-100μM of BPAF was tested. The quality of the study is assessed to be strong. The study provides strong evidence for endocrine MoAs, i.e. ERα and TRα agonist, of BPAF as well as other biological/toxicological effects on MCF-7 cells.

Liang et al. (2017)

Summary: This study developed and validated an automated multi-parametric high-content analysis (HCA) using the mouse C18-4 spermatogonial cell line as a model. The validated HCA, including nuclear morphology, DNA content, cell cycle progression, DNA synthesis, cytoskeleton integrity, and DNA damage responses, was applied to characterize and compare the potential testicular toxicities of various concentrations of BPA and 3 analogous including BPAF (98% purity, CAS no. not reported) BPAF was tested at 0, 0.1, 1, 5, 10, 25 (≤ cytotoxicity level) μM (read from the relevant figures:) with exposure time of 24, 48 and 72 h (3 replicates in 3 separate experiments). Cell viability was determined by measuring the capacity of cells to take up neutral red (BPAF doses: 0, 25, 50, 75 and 100 μM, time: 24-72 h, 5 replicates in 2 separate experiments) The results revealed that BPAF exhibited higher cytotoxicity on spermatogonial cells compared with BPA and BPS at 25, 50, 75 and 100 μM, both after 24 and 48 h exposure. BPAF significantly reduced cell viability in a time and dose-dependent manner, and cytotoxicity started at the lowest tested concentration of 25 μM for 24 h. Moreover, BPAF induced dose- (5-25 μM) and time-dependent (24-72 h) alterations in nuclear morphology, cell cycle, DNA damage responses, and perturbation of the cytoskeleton of the spermatogonial cells.

Study quality and assessment: The study is well-described and is assessed to be of high quality. This study provides strong evidence for BPAF spermatogonial toxicity *in vitro*, however the MoAs for the alterations in spermatogonial cell morphology and potential testicular toxicity are not studied and a link to endocrine MoAs can therefore not be made.

Conley et al. (2016)

Summary: In this study, the T47D-KBluc estrogen receptor transcriptional assay (ERTA) was used to evaluate the estrogen receptor α (ERα)-mediated activity *in vitro* of BPAF (97% purity, CAS. no. 1478-61-1) and other bisphenols, as well as and 17α-ethinyl estradiol (EE2) 17β-estradiol (E2). BPAF was run on individual plates over a range of doses separated at half-log intervals for 24 h. Each plate included 4 technical replicates per dose group and each chemical was tested across 6 biological replicates. Cell viability was assessed before the cells were lysed and luciferase activity measured.

It was found that BPAF activates ERα but at a relatively low potency when compared to the reference compounds EE2 and E2. The main aims of the study were to evaluate how information from this *in vitro* assay can be used to predict results in the *in vivo* Uterotrophic assay and to explore the challenges and limitations of such extrapolations. The *in vivo* Uterotrophic assay and results on BPAF from this study is summarized in section 4.10.3.3.

Study quality and assessment: The study is well-designed and -described. The exact test concentrations of BPAF in ERTA are not specified but according to Figure 2 in the paper the tested concentrations range from $1 \cdot 10^{-10}$ to $1 \cdot 10^{-10}$ M (i.e., ~ 0.1 - $100 \mu\text{M}$). Also, the cytotoxicity results are not reported. Overall, the study is assessed to be of moderate quality. This study provides a strong evidence for an estrogenic MoA of BPAF *in vitro*.

Feng et al. (2016)

Summary: In the present study, the H295R cell line was used as a model to compare the cell toxicity and effects on steroid hormone synthesis by four bisphenols: BPA, BPS, BPF, and BPAF (99% purity, CAS no. is reported as B0945, which is instead a product number). First, the viability of H295R cells exposed to 10-500 μM BPAF for 24 h, 48 h, and 72 h was measured using the cell counting kit-8 (CCK-8) assay. Next, BPAF was tested at sub-cytotoxic concentrations (0, 0.1, 1, 10, 30, and 50 μM , 3 replicates of each exposure) for 48 h followed by quantification of hormone levels and gene expression.

The LC50 values at 72 h exposure indicated that the rank order of cytotoxicity of the tested chemicals was BPAF > BPA > BPS > BPF. The results demonstrated that all the tested BPA analogues were capable of altering steroidogenesis in H295R cells. More specifically, BPAF showed significant increase in progesterone (10, 30 and 50 μM) and significant reductions in testosterone and aldosterone (1, 10, 30 and 50 μM) and cortisol (10, 30 and 50 μM) (Figure 2).

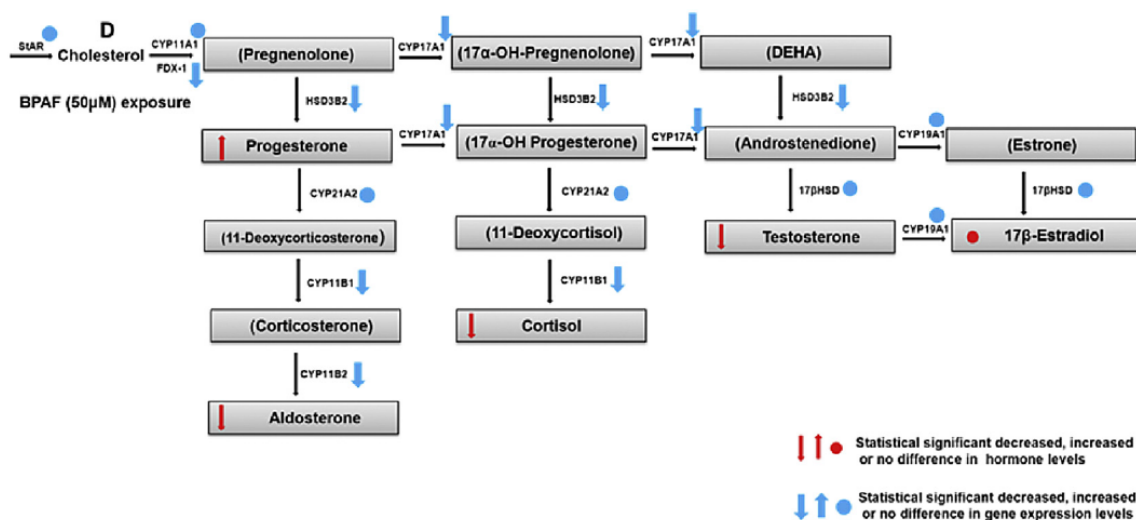


Figure 2. Overview of the steroidogenesis and the effect of 50 μM BPAF exposure (from Feng et al 2016)

Gene expression was significantly reduced for FDX1 (50 μM), CYP17A1 (1, 10, 30 and 50 μM), CYP11B2 (10, 30 and 50 μM), CYP11B1 and HSD3B2 (30 and 50 μM) in H295R cells. The inhibitory effects of BPAF on hormone production were probably mediated through the down-regulation of these steroidogenic genes.

Study quality and assessment: The study is well-described and investigates effects of BPAF on both hormone levels and enzyme expression. The OECD TG 456 was not applied or mentioned in the paper. Overall, the study is assessed to be of high quality. The links between altered expression of steroidogenic enzymes and hormone levels after BPAF exposure are discussed and can generally explain the lower testosterone level in the cells. Overall, the study provides strong evidence of endocrine MoA of BPAF on steroidogenesis.

Nakano et al. (2016)

Summary: The aim of this study was to analyse the effects of BPA and BPAF (purity and CAS no. not reported) exposure on oocyte maturation *in vitro*. Mice oocytes were cultured in the presence of different concentrations of BPA or BPAF (0, 2, 20, 50 and 100 µg/ml, 3 replicates/dose group) for 18 h (i.e., the time required to complete an oocyte maturation in mice) to determine the concentration-dependent effects on nucleus maturation rate. Next, oocytes were exposed to 2 µg/ml BPA or BPAF and cultured for 6, 9, 12, 15 or 18 h (3 replicates/group) in order to study the effects on the cell cycle delay during maturation. To clarify if BPAF and BPA affect oocyte progression into metaphase II, oocytes were exposed to 50 µg/ml BPA or BPAF for 18 or 21 h or for 12 h followed by 9 h in control medium (dimethyl sulphoxide (DMSO)) (3 replicates/group). Finally, oocytes cultured in the presence of 50 µg/ml BPA or BPAF for 21 h (3 replicates/group) were tested for the localization of α -tubulin and MAD2, a spindle assembly checkpoint (SAC) protein, using immunofluorescence to study effects on the spindle and SAC.

The first study showed that both BPA and BPAF at concentrations of 50 and 100 µg/ml significantly inhibited oocyte maturation, with BPAF treatment causing the most potent decrease in the number of oocytes reaching maturity. Oocyte exposure to 2 µg/ml BPA or BPAF did not suppress meiotic progression at 18 h, but when analysing effects of this exposure level at different exposure times, i.e. 6, 9, 12, 15 and 18 h, it was found that oocyte maturation rate was significantly delayed for BPAF at both 12 and 15 h. To determine whether the meiotic inhibition seen at the 50 and 100 µg/ml exposure to BPAF and BPA was due to arrest or delay, the oocytes were exposed for 21 h, and it was seen that BPAF arrested maturation while BPA caused a delay. After 12 h of culture in 50 µg/ml BPAF or BPA, oocytes were transferred to control medium for 9 h to explore if the effects on maturation were reversible, and only 63.3% oocytes treated with BPAF progressed to metaphase II (MII). Exposure to BPAF (50 µg/ml) resulted in spindle abnormalities but not in MAD2 re-localisation.

Study quality and assessment: Overall, the study is thorough; however, the study has some minor limitations in its description such as not reporting the chemical's purity, a cytotoxicity evaluation and it is not always clear how and why the specific work was performed. The study is therefore assessed to be of moderate quality. This study provides moderate evidence for effects on oocyte maturation *in vitro* after BPAF exposure.

Ruan et al. (2015)

Summary: In this study, 8 bisphenols (BPs) were identified in sewage sludge collected from wastewater treatment plants in 15 cities in China. The estrogenic potencies of the 8 BPs, including BPAF (98% purity, CAS no. not reported) and BPA, and the estrogenic activities of sludge samples were evaluated using a bioluminescence yeast estrogen screen (BLYES) assay. Cytotoxicity was determined by the LIVE/DEAD® Yeast Viability Kit. BPAF was tested in doses from approximately 50 nM to $4 \cdot 10^5$ nM (read from Figure 2b) with 4 replicates per dose group.

All 8 BPs exhibited estrogenic activity in the BLYES assay, and BPAF had the highest activity and showed significant bioluminescence induction when the concentration was between 100 and $5 \cdot 10^3$ nM. Furthermore, all 15 sludge samples elicited considerable estrogenic activity in the BLYES assay.

Study quality and assessment: The study is well-described although the number of experimental replicates and tested doses are not specifically given in the material and methods section but can be found in e.g. figures. Cytotoxicity was measured but only results for TBBPA and the sludge extracts are given, and from this it seems that the chemicals were only tested for estrogenic activity at

concentrations below their cytotoxic levels. Due to these few shortcomings, the study is assessed to be of moderate quality. The study provides moderate evidence for an endocrine MoA, i.e. estrogenic activity, of BPs, and reports that BPAF had a higher estrogenic potency than the remainder tested BPs including BPA.

Li et al. (2014)

Summary: In the present study, the effect and mechanism of BPAF-induced endogenous transcription was investigated in human breast cancer cells. After 24h *in vitro* BPAF (purity and CAS no. not reported) exposure to estrogen receptor α (ER α)-positive T47D and MCF7 cells (0, 1 nM, 10 nM, 100 nM, 1 μ M and 10 μ M), and ER α -negative MDA-MB-231 cells (0, 100 nM, 1 μ M and 10 μ M) (3 replicates per dose group), real-time PCR was performed to evaluate the dose-dependent effect of BPAF on endogenous transcription of 3 estrogenic responsive genes: trefoil factor 1 (TFF1/pS2), growth regulation by estrogen in breast cancer 1 (GREB1) and cathepsin D (CTSD). Time-dependent effects (1, 2, 4, 8, 24, 48 and 72 h exposure duration) of 1 μ M BPAF on estrogenic responsive gene transcription in T47D and MCF-7 cells were also performed. To explain the mechanism of BPAF-induced endogenous transcription (1 μ M for 24 h), gene-silencing with small interfering RNA (siRNA) against ER α , ER β and G protein-coupled estrogen receptor 1 (GPER) was used in T47D and MCF7 cells. In addition, the inhibition of BPAF-induced (1 μ M for 24h) ER activity was studied using ICI 182780 (1 μ M, ER α and ER β antagonist) in the ER α -positive T47D and MCF7 cells. The ER α -negative MDA-MB-231 cells were transfected with an adenovirus overexpressing ER α , exposed to BPAF and followed by gene expression quantification of TFF1, GREB1 and CTSD.

In both the T47D and MCF7 cells, BPAF (100 nM –10 μ M) significantly induced transcription of the 3 oestrogen responsive genes TFF1, GREB1 and CTSD in a dose-dependent manner. BPAF did not induce TFF1, GREB1 and CTSD mRNA expression in the ER α -negative MDA-MB-231 cells. A BPAF dose of 1 μ M over a time course of 1-72h, showed altered gene expression of TFF1, GREB1 and CTSD in both T47D and MCF-7 cells after 4h of exposure. Gene-silencing of ER α , ER β and GPER by siRNA revealed that BPAF-induced endogenous transcription was dependent on ER α and GPER, implying that both genomic and non-genomic estrogenic pathways might be involved in the endogenous transcription induced by BPAF. ER α -mediated gene transcription was confirmed by inhibition of ER activity using ICI 182780 in the T47D and MCF7 cells as well as in MDA-MB-231 cells overexpressing ER α . Moreover, the Src tyrosine kinase inhibitor, PP2, and the two MEK inhibitors, PD98059 and U0126, were used to elucidate the rapid non-genomic activation of Src/MEK/ERK1/2 cascade on endogenous transcription. It was shown that BPAF-induced transcription could be significantly blocked by PP2, PD98059, and U0126, suggesting activation of ERK1/2 was also required to regulate endogenous transcription. Taken together, these results indicate that BPAF-induced endogenous transcription of oestrogen responsive genes is mediated through both genomic and non-genomic pathways involving the ER α and ERK1/2 activation in human breast cancer cells.

Study quality and assessment: Overall the study is thorough, but the material and method section does not report the doses of BPAF exposure used in the study or the purity and CAS no. of BPAF. The doses can, however, be read from Figure 1. Furthermore, no results from cytotoxicity studies are given. Due to these shortcomings the study is assessed to be of moderate quality. The study provides strong evidence for two estrogenic MoAs of BPAF, i.e. activation of ER α and GPER.

Li et al. (2013)

Summary: The study examines the estrogenic receptor (ER) α and ER β agonistic activity of 12 EDCs, including BPAF (purity and CAS no. not reported). First, HepG2 and HeLa cells were used to determine the estrogen responsive element (ERE)-mediated estrogenic transcriptional activity of ER α and ER β via the luciferase reporter assay using the 2 luciferase reporters, 3xERE and pS2ERE. Next, Ishikawa cells stably expressing ER α were used to determine changes in selected endogenous ER target gene expression by real time PCR. All experiments were repeated at least 3 times, and BPAF was tested at 100 nM for 18 h in all experiments.

BPAF (100 nM for 18 h) strongly induced ER α 3xERE and pS2ERE in both HepG2 and HeLa cells, and in the HepG2 cells it also induced ER β 3xERE and pS2ERE responses.

BPAF (100 nM for 18 h) significantly induced endogenous ER α target genes (*PR*, *pS2*, *GREB1*, *SPUVE*, *WISP2*, and *SDF-1*) in the Ishikawa/ER α cells.

Study quality and assessment: The study is well-designed and thorough. Only one concentration (100 nM) of the EDCs, including BPAF, was tested but it was tested in multiple assays for similar effects and overall the results were in agreement. No cytotoxic levels are reported. Based on this the study is assessed to be of moderate quality. This study provides strong MoA evidence of BPAF as an ER α agonist and a potential ER β agonist.

Teng et al. (2013)

Summary: One aim of this study was to investigate the molecular mechanisms of how BPA, BPAF (purity not reported, CAS no. 1478-61-1) and BPS may affect estrogen receptor α (ER α) and androgen receptor (AR). For this transient transfection experiments with full-length ER α and or AR and their corresponding response elements were performed. In the AR functional assay, monkey kidney CV1 (AR negative) cells were bulk transfected with pSG5-AR, MMTV-luciferase (reporter gene to measure the transcriptional activity of AR) and Renilla-Luc. The assay was run in both agonist mode, i.e. 24h incubation with test compound with increasing concentrations (10^{-8} - 10^{-4} M), and antagonist mode, i.e. incubation with the AR agonist R1881 ($5 \cdot 10^{-10}$ M) and increasing concentrations of the test compound. For the ER α functional assay, CV-1 (ER α negative) or HepG2 cells were bulk transfected with pRST7-ER α , 3X-ERE-TATA-luc (reporter gene to measure the transcriptional activity of ER α), and Renilla -Luc. The assay were carried out as described for AR except E2 ($2 \cdot 10^{-10}$ M) was used in the antagonist mode assay. Each assay consisted of triplicate samples and the experiment was repeated 2–3 times.

The study confirmed that BPA and BPAF act both as ER α agonists and AR antagonists, and a competition experiment indicated that the AR antagonism was due to competitive antagonism.

Study quality and assessment: Overall the study is thorough and well-described, but the material and method section does not report the purity of the tested chemicals and the doses of BPAF exposure are not explicitly stated. Also, no information on cytotoxicity /cell viability is reported. The figures and figure text does not always report the results in the optimal format. Based on this the study is assessed to be of moderate quality. The study provides strong MoA evidence of BPAF being an ER α agonist and a competitive AR antagonist.

Delfosse et al. (2012)

Summary: The study examined how BPA, BPAF and bisphenol C (BPC) bind and activate estrogen receptor (ER) α and ER β and how this mechanistically differs from that of 17 β -estradiol (E2). First, the bisphenols ER α transcriptional activity and cell proliferation effect was examined using human breast cancer ER α -positive MCF-7 reporter cells (MELN cell line). Next, the effect of bisphenols on ERs transcriptional activity was tested by using HeLa reporter cells stably expressing human ER α and ER β (HELN ER α and ER β lines), allowing for a direct comparison of the effect of compounds on the two ERs in similar cellular context. A competitive binding assays with [³H]E2 was also performed to confirm observations from the previous experiments. The relative contributions of the ER transcriptional activation factors (AF-1 and AF-2) responsible for the ER activities were examined using HELN cells stably transfected with ERs deleted of their N-terminal AF-1 region. Next, it was examined if an altered cellular response could be observed due to AF-1 deletion by using the HELN ER cell line whose proliferation is known to decrease in response to E2 treatment. Each chemical was tested in HELN cells transfected with full ER α or AF-1 deleted ER α . The capacity of bisphenols to induce co-activator recruitment to ER α AF-2 was studied next by measuring the interaction of fluorescein-labelled SRC-1 (co-activator) with the ER α -LBD (ligand binding domain, containing AF-2). The effect of bisphenol on H12 (helix 12) active conformation stability was also studied, with and without addition of the co-activator SRC-1. Finally, to gain a better structural insight to the binding mode of bisphenols to ERs, the Y537S mutant ER α -LBD was crystallized in complex with each compound.

In the MELN assay, BPAF (and BPA and BPC) exerted a partial potency on luciferase reporter activity (~80% compared to E2) but was a full ER α agonist on cell proliferation. In the HELN assays, BPAF (and BPA and BPC) was activating ER α with a 60-70% potency of E2. BPAF also exerted ~60% potency on ER β . This indicates that BPAF is an ER agonist and a partial ER antagonist in the presence of E2. The competitive binding assays confirmed that BPAF binds to both ERs. The deletion of AF-1 strongly reduced the bisphenol-induced transcriptional activity of both ER α and ER β while no effects was seen on E2 ER activity. The cell response was also in agreement with this, as all bisphenols inhibited proliferation of HELN ER cells, but showed only very weak proliferative inhibition in the HELN AF-1 deleted cells. Overall, this indicates that the ER activity of bisphenols relies mainly on the AF-1 while the ER activity of E2 is independent of the AF-1 region. While E2 enhanced the binding affinity between the co-activator SRC-1 and the ER α -LBD and the ER antagonist 4-hydroxytamoxifen decreased the affinity, the bisphenols showed a weaker effect on the co-activator binding. The mobility of H12 was higher in the presence of bisphenols compared to E2 strongly stabilized H12 in the active conformation. Addition of SRC-1 resulted in a dose-dependent increase in H12 stability with BPA and BPAF treatment. When crystallized with BPA or BPAF the ER α -LBD displayed an active conformation with H12 capping the ligand binding pocket (LBP) and the SRC-1 bound to the AF-2 surface. BPAF displayed two orientations in the two subunits of the ER dimer; and agonist and antagonist positioning, indicating a regulatory crosstalk between the subunits.

Together, these experiments show that bisphenols can be considered as selective ER modulators (SERMs) that relies mainly on the AF-1 and are dependent on the cellular contents such as available amounts of co-activators.

Study quality and assessment: The study is very thorough and well-described although the doses in the different studies are not clear from the text but only appears in the figures. Overall, the study is assessed to be of high quality. It provides a strong MoA evidence for BPAFs interaction with ERs and detailed information on the molecular mechanisms behind this interaction.

Li et al. (2012)

Summary: In this study three human cell lines (Ishikawa, HeLa, and HepG2) representing three cell types were used to evaluate the dose- and cell-specific mechanistic actions of BPA, BPAF, and Zearaleone (Zea) on estrogen receptor (ER) α and ER β . To evaluate estrogen response element (ERE)-mediated transcriptional activity of ER α and ER β , the promoter activation in Ishikawa, HeLa, and HepG2 cells was examined using a luciferase reporter assay system at BPAF doses of 0, 1, 10, 100, or 1,000 nM. Similarly the antagonistic effects of BPAF were examined by treating the cells with 1 or 10 nM BPAF, with or without 10 nM E₂ co-treatment. The specific effects of BPAF (100 nM) on ER α functionality was analysed using wild-type (WT) ER α and specific ER α mutations in the following domains: H1 (ERE-mediated activation, no tethered-mediated activation), AA (tethered AA-mediated activation, no ERE-mediated activation), E1 (activation factor-1 (AF-1) inactive), and AF-2 (activation factor-2 (AF-2) inactive). Next, the role of co-activators on BPAF (100 nM) on ERE-mediated ER α activation was studied by co-treating the cells with the co-activators SRC2 or p300. To examine the phosphorylation events in the rapid ER action responses to BPAF, Ishiwaka cells transfected with a vector control or wild type (WT)-ER α were used. First the expression of ER α and the ER ERE-mediated activation was confirmed in the Ishiwaka/ER α cells. Then western blot was performed to identify phosphor-p44/42 MAPK and other kinases in the vector and ER α cells, respectively. Then the effect of 2 kinase inhibitors, PD 98059 (MAPK inhibitor) and PP2 (*src* family tyrosine kinase inhibitor), on BPAF-mediated expression of the ER target gene Progesterone receptor (PR) in Ishiwaka/ER α cells was examined. Finally, ER α -dependent responses to BPAF was examined by detecting endogenous gene expression of PR, and 3 other ER target genes in Ishiwaka/ER α cells using real time PCR.

For ER α , BPAF showed weak ERE-mediated estrogenic activity at low concentrations (≤ 10 nM) and strong activation at the higher concentration (1,000 nM) in the Ishikawa cells. The HeLa cells were less sensitive, while the HepG2 were highly responsive. For ER β , BPAF resulted in only a weak response in the Ishikawa and HepG2 cells, while the responses in the HeLa cells to 100 and 1000 nM BPAF were similar to E₂. In the antagonistic mode, BPAF did not antagonize E₂ ER α activation in any of the cell lines, but ER β activation by E₂ was reduced by BPAF (1 and 10 nM) in HeLa cells only. The functionality studies demonstrate that BPAF can activate ERE-mediated transcription via AF-2 in Ishiwaka cells. No activity was measured in the AA- or AF-2-ER α mutants, indicating that BPAF do not mediate tethered or AF-1 activation of ER α . It was also found that both co-activators, SRC-2 and p300, resulted in an enhanced BPAF-induced ER α activity. Regarding the effect on the rapid ER action response, it was found that BPAF could activate both the p44/42 MAPK pathway and the tyrosine kinase *src* pathways. BPAF also induced gene expression of the ER α target genes PR and GREB1. Together, these results indicate that BPAF can function as an ER agonist and antagonist, and activates ER α by ERE-mediated activation via the AF-2 function or via a non-genomic rapid-action response. The estrogenic activity of BPAF depended on the cell type and content.

Study quality and assessment: The study is thorough and well-described. No information on cytotoxicity of the tested compounds in the individual cell lines is reported. Based on this missing information, the study is assessed to be of moderate quality. The study provides strong evidence for BPAFs estrogenic MoA as well as detailed information on the specific functionalities on ER activation.

Sui et al. (2012)

Summary: In the study it was investigated whether BPA and selected analogues, including BPAF, bind to and activate human PXR (hPXR) and mouse PXR (mPXR). HepG2 cells were transfected with full-length hPXR together with CYP3A4-luc reporter or full-length mPXR together with (CYP3A2)₃-luc reporter and CMX-β-galactosidase control plasmid and treated with BPAF (0, 5, 10 and 20 μM for 24 h, read from Figure 4). Structural requirements of BPA analogues that activate hPXR were deduced by docking studies to tethered PXR, linker, SRC-1 (steroid receptor coactivator 1). BPAF was found to be an hPXR agonist (< BPA) and caused a dose-dependent activation, but it did not affect mPXR activity. The replacement of the CH₃ by CF₃ in BPAF helped BPAF retain a partial agonist activity relative to BPA.

Study quality and assessment: BPAF was only included in part of the studies reported and the cytotoxicity of BPAF in HepG2 is not reported. Overall the quality of the study is assessed to be moderate, and the study provides moderate evidence for a PXR agonist MoA of BPAF. PXR agonism may alter the expression of enzymes and transporters relevant for endogenous hormones and thereby indirectly result in ED.

Bermudez et al. (2010)

Summary: The present study characterizes the individual dose-response curves of estradiol-17β (E2), BPA, TBBPA, and BPAF (CAS no. 1478-61-1, 100% purity) on oestrogen-dependent luciferase expression in T47D-KBluc cells and determine how binary and ternary mixtures of E2 with BPAF and/or BPA interact with ERs. T47D-KBluc cells that naturally express ERα and ERβ were transfected with a triplet estrogen-response element (ERE) promoter-luciferase reporter gene construct, and exposed to E2, BPA or BPAF at multiple concentrations (1pM to 1 μM, read from Figure 1). BPAF was also tested in a binary mixture with E2 and a ternary mixture with E2 and BPA. BPAF caused a dose-dependent increase in oestrogen activity in the T47D-KBluc cells and was significantly more potent than BPA. Both the binary mixtures of E2 with BPAF and the ternary mixture of E2, BPA, and BPAF behaved in an additive manner experimentally, and at a concentration of 10 pM E2 the system reached saturation and additional BPAF did not produce an increase in activity.

Study quality and assessment: The study is well-described, although cytotoxicity results for TBBPA are only given. From this it seems that all the chemicals were only tested for estrogenic activity at concentrations below their cytotoxic levels. Overall, the study is assessed to be of moderate quality, and it provides moderate evidence for an estrogenic MoA of BPAF.

Matsushima et al. (2010)

Summary: The aim of the study was to determine the relative preference of BPAF (CAS no. 1478-61-1, 99% purity) for estrogen receptor (ER)α and ERβ and the BPA target receptor estrogen related receptor γ (ERRγ), and to clarify structural characteristics of receptors that influence BPAF binding. First the receptor-binding activities of BPAF (doses: 10⁻¹² to 10⁻⁵ M, read from Figure 2) relative to radio-labelled 17β-estradiol (E2) (5 nM) to ERα or ERβ ligand binding domain (LBD), or radio-labelled BPA (5 nM) to ERRγ LBD were measured with each assay run in duplicate and repeated at least 5 times. Next, functional luciferase reporter gene assays were performed to assess BPAF (doses: 10⁻¹² to 10⁻⁵ M, read from Figure 3) receptor activation in HeLa cells transfected with the receptor and a reporter gene. To measure the antagonistic activity of BPAF on ERβ, 4 concentrations (0.01, 0.1, 1, 1,

and 10 μ M) of BPAF was tested against 10^{-12} to 10^{-5} M E2. Similarly, 2 concentrations of E2 (10 or 100 nM) were tested against increasing levels of BPAF (10^{-12} to 10^{-5} M)

BPAF strongly (>BPA) and selectively binds to ERs over ERR γ . Furthermore, BPAF receptor-binding activity was 3 times stronger for ER β than for ER α . When examined using the functional luciferase reporter gene assays, BPAF was a full agonist for ER α in a dose-dependent manner at concentrations of 10^{-10} to 10^{-5} M. In contrast, it was almost completely inactive in stimulating the basal constitutive activity of ER β . When tested in ER β antagonist mode, BPAF acted as a distinct and strong antagonist against the ER β activity of E2.

Study quality and assessment: The study is thorough and well-described although the exact doses tested are not explicitly given in the material and methods and no cytotoxicity studies in the applied HeLa cells are reported. Due to such shortcomings, the study is assessed to be of moderate quality. The study provides strong evidence for an estrogenic MoA with ER α agonism and ER β antagonism.

Kitamura et al. (2005)

Summary: The potential endocrine-disrupting activities of BPA and 19 related compounds, including BPAF (CAS no. and purity not reported) were comparatively examined by means of different *in vitro* and *in vivo* reporter assays. First BPAF was tested for estrogen receptor (ER) activity in the ERE-luciferase reporter assay in MCF-7 cells (doses tested: 10^{-4} – 10^{-9} M) as well as for ER antagonism of 17 β -estradiol (E2) activity. Similarly BPAF was tested for AR agonism and antagonism (i.e. inhibition of dihydrotestosterone (DHT) activity) in transfected NIH3T3 cells (mouse fibroblast cell line). The induction of growth hormone production in GH3 cells after BPAF exposure was also examined as was the inhibition of triiodothyronine (T3) induced hormone production the cells.

BPAF exhibited strong estrogenic activity from 10^{-7} to 10^{-4} M in the MCF-7 cells. No androgenic activity was observed for BPAF, but BPAF was instead showing anti-androgenic activity on DHT in the NIH3T3 cells. BPAF did not induce or inhibit the thyroid hormone-dependent production of growth hormone by GH3 cells.

Study quality and assessment: The study is well-described and although cytotoxicity is not directly reported for each cell line, renilla luciferase activity in the transfected cells was used to control for cytotoxic effects. Overall, the study is assessed to be of moderate quality and provides strong evidence for estrogenic and anti-androgenic MoAs of BPAF.

Hasimoto et al. (2001)

Summary: In this study, the estrogenic activities of 13 BPA analogues, including BPAF (here called BP3, CAS no. not reported, 97% purity), in three *in vitro* bioassays are examined. BPAF was tested in concentrations from 10^{-7} to 10^{-3} M in both the yeast two-hybrid system (YES) and in a competitive ER-binding fluorescent polarization assay, and 10^{-9} to 10^{-4} M in MCF-7 cells (including proliferation called the E-screen), respectively. BPAF showed cytotoxicity at 10^{-4} M in the E-screen (according to the discussion section).

In the YES assay, BPAF showed a dose-dependent increase in estrogenic activity in the absence of S9, and the activity was enhanced with the addition of S9. BPAF could effectively displace the fluorescent non-steroid probe from the ER-FES complex from a concentration of 10^{-6} M and above. In

the E-screen, BPAF significantly increased the cell proliferation from concentrations of 10^{-7} M and above, except at the highest concentration 10^{-4} (i.e. cytotoxic dose). Overall, the estrogenic activity of BPAF/BP3 was stronger than that of BPA.

Study quality and assessment: In general the material and methods description is very short and inadequately described and cytotoxicity studies are not explicitly reported. The study can confirm the results in three independent, orthogonal assays, and also includes the effect on activity of metabolism (rat liver S9) in the YES assay. The number of biological and technical replicates are not given, and no p-values or other statistics are reported in text or figures. Overall, the study quality is assessed to be moderate. The study provides moderate evidence of BPAF having an estrogenic MoA.

Perez et al. (1998)

Summary: In this study BPAF (here called MM7, CAS no. not reported, $\geq 97\%$ purity) and other bisphenols were tested in the E-screen, which measures proliferation of MCF-7 cells, at doses from 10^{-8} to 10^{-5} M. The synthesis and secretion of 2 cell type-specific, oestrogen-responsive proteins, progesterone receptor (PgR) and pS2, were also measured in exposed MCF-7 cells. Finally, the relative binding affinity of BPAF at multiple doses (10^{-12} to 10^{-4} M) was measured in a competitive binding assay using cytosol from immature rat uteri incubated with radio-labelled 17β -estradiol (E2).

BPAF resulted in an increased MCF-7 cell proliferation as well as increased PgR and pS2 levels. BPAF showed high relative binding affinity to ER in the competitive binding assay.

Study quality and assessment: The study does not report the CAS no. of the tested chemicals or any statistical significant results in the related figures. Also, cytotoxicity levels for the MCF-7 cells are not reported. Overall, the study quality is assessed to be of moderate quality, and the study provides moderate evidence of an estrogenic MoA of BPAF *in vitro*.

The following four additional *in vitro* studies on BPAF were found when looking into the included literature: Kanai et al. (2001), Rivas et al. (2002), Yamasaki et al. (2003b), and Laws et al. (2006). These were not further evaluated as the abstracts indicate that all four studies support an *in vitro* oestrogenic MoA already adequately covered by the included studies, i.e. they do not provide new information.

4.10.3.3 In vivo effects with regard to an endocrine mode of action

Foster et al. (2017) (poster abstract and poster)

Summary: The poster describes a NTP modified one-generation study (MOG) of BPAF. Four groups of 20 time-mated dams were exposed to 0, 338, 1125 or 3750 ppm BPAF in the diet from gestation day (GD) 6 until weaning.

Significant delay in male puberty and advancement in female puberty was observed. Ten males at 3750 ppm did not achieve puberty assessed as Balano-Preputial Separation by PND 98. Two cohorts of offspring were mated and litter size was assessed on GD 21 or after birth. Both cohorts failed to produce any offspring at the highest dose of 3750 ppm in the diet and litters size was significantly decreased at 1125 ppm.

Study quality and assessment: Only very limited information is available in the abstract, but more information are available in form of a poster print. Optimally, the evaluation should be based on a study report or a paper. However, the NTP MOG study design is very similar to OECD TG 443, the group performing the BPAF study are very experienced and relevant results regarding the effects and dose levels are shown in the poster. Thus, the study is assessed to be of high quality and provides strong evidence of adverse effect on female and male reproduction. The effects observed are consistent with an oestrogenic mode of action.

Conley et al (2016)

Summary: This study evaluated the effect of oral exposure to BPAF (CAS no. 1478-61-1, 97% purity) (and other oestrogens) in the Uterotrophic *in vivo* assay, which is largely estrogen receptor (ER)- α -mediated. BPAF was given daily by oral gavage to adult ovariectomised female rats (age ~81 days) (n=3-6/group, 3-8 dose groups: 3-300 mg/kg/d (read from figure 2)) for 4 consecutive days. The rats were assessed for overt toxicity and euthanized 3 h after the final dose. Trunk blood was collected and uterine tissue excised and weighted with luminal fluid (wet), and after being drained for fluid (blotted). The fluid weight was estimated as the mass difference between wet and blotted. No general toxicity was observed at the tested BPAF concentrations, but BPAF caused a dose-related increase in uterus weight (wet, blotted and fluid) as well as histopathological changes (increased epithelial and glandular height). These results were used to evaluate the *in vitro* T47D-KBluc oestrogen receptor transactivation reporter assay's potential to accurately predict *in vivo* effects. T47D-KBluc is also mainly ER α -mediated, and as described in section 4.10.3.2, the same study found that BPA is an ER- α agonist *in vitro*. Extrapolation of the *in vitro* results on BPAF could predict the EC50 of *in vivo* results within the 95% confidence interval of the observed EC50.

Study quality and assessment: The study is well-designed and described and is assessed to be of high quality. Together with the results from the *in vitro* ER α activation study, this study provides strong evidence of an estrogenic MoA of BPAF.

Li et al. (2016)

Summary: Sixty male and 60 female 8-week old Sprague-Dawley rats were acclimated 1 week before pairing. The females were exposed to 100 (n=30) or 0 (control group: n=30) mg BPAF/kg/day (CAS no. 1478-61-1, 97% purity) by oral gavage from gestational day (GD) 3-19. On the day of birth (i.e., postnatal day (PD) 0), cross-fostering took place between treated and control litters, and cross-fostered mother rats were given 0 or 100 mg BPAF/kg/day, respectively, from PD 3-19. This resulted in four test groups: unexposed control (CC), pups exposed prenatally (TC), pups exposed postnatally (CT), and pups exposed both prenatally and postnatally (TT) (n=6/group according to text in figures). All animals were euthanized at PD 23, where blood was collected, and testes and epididymis excised and weighed. BPAF and hormones were measured in the blood and testes, and gene and protein expression was done in the testes.

The absolute and relative weights of the testis and the absolute weights of the epididymis of the exposed male pups (TT, TC and CT) on PD 23 showed no differences compared with the CC group. HPLC-MS/MS analysis showed that BPAF was transferred via cord blood and breast milk and bio-accumulated in the testes of the offspring. Pups exposed to BPAF both pre- and postnatally (TT) showed a significant increase in serum and testis testosterone levels compared with that of the control pups (CC), while all pups exposed to BPAF (prenatally and/or postnatally: TT, CT and TC) showed a

significant decrease in serum and testis inhibin B levels compared to unexposed offspring (CC). Compared with the CC group, RNA-sequencing revealed that 279 genes were significantly differentially expressed in the testes of pups exposed to BPAF both pre- and postnatally (TT). Specifically, mRNA levels of steroidogenic acute regulatory protein (StAR), estrogen receptor (ER) α , and androgen receptor (AR) in the TT group were increased compared to the CC group. The testes protein levels P450_{scc} and StAR, both involved in steroidogenesis, were increased in the TC and TT groups, and the testes protein levels of AR were significantly increased in the TT and CT groups. Together the decreased inhibin B and increased testosterone levels in the testes indicated that Sertoli and Leydig cell functions were both disturbed by gestational and lactational BPAF exposure, and these results were in agreement with the altered levels of genes and proteins involved in testosterone synthesis. The transcriptomic analyses also showed that BPAF alters the expression of genes involved in progression of meiosis and DNA recombination, indicating that BPAF has deleterious effects on meiotic progression and germ cell development during critical stages of differentiation.

Study quality and assessment: In general, the study is well-described and thorough, although more information on the housing conditions would have been preferred. Overall, the study quality is assessed to be high. The study provides strong evidence of a steroidogenic ED MoA of BPAF, and the link between the mechanistic information from the RNA seq/protein level analysis and the effects on testosterone level is strong.

Feng et al. (2012)

Summary: The aim of this study was to determine whether BPAF exposure produces adverse effects on testosterone production and to further elucidate the mechanism of BPAF toxicity in testes. Seven week old Sprague-Dawley (SD) male rats were acclimated for 1 week before exposure by oral gavage to BPAF (CAS no. is reported as B0945, which is instead a product number, 99% purity) for 14 days at doses of: 0, 2, 10, 50 and 200 mg/kg/d (n=6/group). Rats were weighed daily during the exposure and euthanized 24 h after the last exposure. Total cholesterol, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone were measured in serum. BPAF concentration and the gene expression of SR-B1, StAR, P450_{scc}, 3 β -HSD, CYP17 α , 17-beta-hydroxysteroid dehydrogenase (17 β -HSD), luteinizing hormone receptor (LHR), androgen receptor (AR), estrogen receptor (ER)- α , ER- β , HMG CoA reductase (HMGR), sterol regulatory element binding protein 1c (SREBP-1c), inhibin B, and Mullerian inhibiting substance MIS in the testes were quantified. Protein content in the testes was also determined. The body weights of the 50 and 200 mg/kg/d groups were decreased, the absolute testis weight was unchanged in the BPAF treatment groups compared to the control group. An increased relative testis weight was observed in the 200 mg/kg group. The BPAF concentration in the testes was found to increase with increasing doses of BPAF. Total cholesterol levels in serum were decreased in rats given a dose of 50 and 200 mg/kg/d. A significantly reduced serum testosterone level was observed in the 200 mg/kg group only, while increases in LH was seen in the 50 and 200 mg/kg groups, and FSH increased in the 10, 50 and 200 mg/kg groups. The 200 mg/kg BPAF exposure resulted in significant declines in the expression of genes involved in cholesterol biosynthesis (SREBP-1c), transport (SR-B1, StAR), and steroid biosynthesis (P450_{scc}, 17 β -HSD). Similarly, the testicular mRNA levels of inhibin B, ER α and LHR also decreased in rats given a dosage of 200 mg/kg/d BPAF. Protein expression of SR-B1, StAR, and P450_{scc} in testes was also significantly reduced in the 200 mg/kg/d BPAF group. Together, these data demonstrate that BPAF has the potential to impair the pituitary–gonadal function at different levels by increasing LH and FSH concentrations and decreasing testosterone levels in serum, and that the BPAF-induced inhibition of

testosterone production primarily resulted from the alteration of genes and proteins in the testosterone biosynthesis pathway.

Study quality and assessment: The study is well-described and the sample size of n=6 is large enough, at least to show effects at the highest doses. Therefore, the study is assessed to be high quality. The study provides strong evidence of a steroidogenic ED MoA of BPAF, and the link between the mechanistic information from the RNA seq/protein level analysis and the effects on testosterone level is strong.

Akahori et al. (2008)

Summary: The overall aim of the study was to explore the relationship between *in vitro* estrogen receptor (ER) α binding and the *in vivo* Uterotrophic assays for 65 chemicals spanning a variety of chemicals classes. BPAF (CAS no. 1478-61-1, >95% purity) (and 64 other chemicals) was tested in a recombinant human ER- α -LBD binding assay and in the immature rat Uterotrophic *in vivo* assay using 20 day old immature female Cr:CD (SD) IGS rats. Three doses were given (exact doses not specified) by s.c. injections for 3 consecutive days, alone or in co-administration with ethinyl estradiol (0.6 μ g/kg/day) for anti-estrogenic assay mode. BPAF was a hER α -LBD binder *in vitro*, and showed both estrogenic and anti-estrogenic activity *in vivo*.

Study quality and assessment: The description of the study has multiple shortcomings for examples are the exact doses not reported just as the results for the individual chemicals, including BPAF, are not given. The study is based on this assessed to be of low quality. The study provides moderate evidence of both an estrogenic and anti-estrogenic MoA of BPAF. The study does discuss the effects of ADME on extrapolation and since the chemicals are administered s.c. the effect of first-pass metabolism is minimal.

Yamasaki et al. (2003)

Summary: In this study 18 chemicals, including BPAF (CAS no. 1478-61-1, 98.8% purity), were tested in the immature rat Uterotrophic assay and Hershberger assay (under GLP guidelines) to assess the relationship between the results of the two assays. The selected chemicals were all positive in the reporter gene assay for estrogen receptor (ER) α activity and on the list of suspected endocrine disruptors published by the EU. In the immature rat Uterotrophic assay, 19 day old immature Crj:CD (SD) female rats were s.c. injected with BPAF at doses of 0, 8, 40 and 100 mg/kg/day (read from table 4) (n=6/group) for 3 consecutive days. To assess potential anti-estrogenic effects, some rats were co-administered with subcutaneous administration of ethinyl estradiol (EE, dose: 0.6 μ g/kg/day). Uteri were weighted with (wet) and without (blotted) intraluminal fluid. In the Hershberger assay, 56 postnatal day old castrated male Brl Han: WIST rats were administered with BPAF by oral gavage at doses of 0, 50, 200 and 400 mg/kg/day (read from table 5) (n=6/ group) for 10 consecutive days. Again to assess the potential anti-androgenic effects, some rats were co-administered with testosterone propionate (TP, dose: 0.2 mg/kg/day) by subcutaneous injection. Ventral prostate, seminal vesicle, bulbocavernosus/levator ani muscle (BC/LA), glans penis and Cowper's gland were weighed, and general toxic signs were registered.

In the Uterotrophic assay, BPAF alone caused a significant, dose-dependent increase in uterine weight at all doses, and significantly decreased the uterine weight in the group given EE + 40 mg/kg/day BPAF when compared to the vehicle + EE group. In the Hershberger assay, the 600 mg/kg/day dose caused toxic signs, including death, and the maximum dose was therefore reduced to 400 mg/kg/day. Decreased spontaneous locomotor activity was decreased in the 200 and 600 mg/kg group with and without testosterone propionate (TP). The BC/LA weight was decreased and the glans penis weight increased in the 400 mg/kg/day group. In the groups co-exposed to TP, increases in seminal vesicle (50 and 400 mg/kg/day), glans penis (50 and 400 mg/kg/day) and Cowper's gland (400 mg/kg/day) weights were seen.

Study quality and assessment: The study is well-described and assessed to be of high quality. The study provides moderate evidence of both an estrogenic and anti-estrogenic MoA of BPAF. The effects in the Hershberger assay were less consequent.

4.10.3.4 Summary of the plausible link between adverse effects and endocrine mode of action

There is strong evidence from *in vitro* and *in vivo* studies for both an estrogenic and anti-estrogenic MoA of BPAF (Table 1). The *in vitro* studies have shown that BPAF is an ER α agonist, an ER β antagonist and G-protein coupled ER (GPER) agonist, and that these effects depend on the cell type and doses. In Uterotrophic assays using both intact immature female rats and adult ovariectomised rats, BPAF caused an increase in urine weight when it was run in agonist mode, while the uterine weight declined when BPAF was tested in the antagonistic mode with co-administration of EE. The effects on uterus weight in this assay are known to be primarily ER α -mediated, and although the alterations in uterine weights are not regarded as an adverse effect as such but a MoA, the results provide weak evidence for adverse female reproductive effects. A single study has also reported delayed oocyte maturation in exposed rats but the underlying MoAs were not explored and therefore no conclusion regarding a link to an ED MoA can be made.

Studies have shown an anti-androgenic activity of BPAF *in vitro* and weak effects in the *in vivo* Hershberger assay, which uses alterations in accessory organ weights as endpoints for (anti)androgen effects. In addition, effects on steroidogenic enzymes *in vitro* and *in vivo* (in testis at both gene and protein level) have been identified, and this may explain the altered serum testosterone levels found in the BPAF exposed male rats. The effects on serum testosterone depended on the life stage with increased testosterone levels in foetal and prepubertal male rats exposed via cord blood or milk, and decreased testosterone levels in adult male rats. The effect on testosterone levels and antiandrogenic effects in the Hershberger assay in male rats provides weak evidence for adverse male reproductive effects. A single study found that BPAF caused spermatogonial toxicity *in vitro* but they did not study this effect's link to an ED MoA.

A new *in vivo* study using the NTP modified one-generation study (MOG) has shown marked delay in male puberty, advancement in female puberty and clear effects on fertility of offspring exposed to BPAF during development (Table 2). The study results are not published in a study report or paper yet, but are available as poster print and poster abstract. The NTP MOG study design is very similar to OECD TG 443, the group performing the BPAF study are very experienced, and relevant results regarding the effects and dose levels are shown in the poster. Thus, the study is assessed to be of high

quality and provides strong evidence of adverse effect on female and male reproduction. The effects observed are consistent with an oestrogenic MoA.

In addition to its oestrogenic, anti-androgenic and steroidogenic MoA, BPAF has also been shown to activate TR α and act as a human PXR agonist *in vitro*. BPAF may through PXR activation alter the turnover of multiple hormones.

The total evidence for adverse effects of BPAF is strong (Table 2), the evidence for an estrogenic MoA of BPAF is strong (Table 1) and the evidence for a plausible link between the MoA and adverse effects is also strong.

In conclusion, BPAF meet the WHO definition of an endocrine disruptor.

Additional literature not included in the evaluation

ANSES report (2013): the report on BPA and 8 structural analogous including BPAF concluded that at the present time, i.e. 2013, there were not enough toxicological data available on BPAF to make a comprehensive toxicological evaluation. More toxicological studies on BPAF have been published since the ANSES report was published in 2013 (see above). Both the ANSES report and previous studies have shown that most BPA analogous including BPAF share common mechanistic properties such as estrogenic activity.

NTP NIEHS (2008): This document is a chemical information profile for BPAF and summarises results from other studies on BPAF that have been included in the above.

Kemikalieinspektionen (2017): This is a report in Swedish that summarises the available information on 39 bisphenols including BPAF to assess them in a risk perspective, and includes many of the references included here.

Table 1. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of BPAF.

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Lei et al. (2017)	ER α and TR α activity as well as other biological/toxicological effects in MCF-7 cells		High	Strong
Liang et al. (2017)	Spermatogonial toxicity in the mouse C18-4 spermatogonial cell line, but no link to any ED MoAs studied		High	Weak
Conley et al. (2016)	ER α -mediated activity in the T47D-KBluc estrogen receptor transcriptional assay	Uterotrophic study in adult ovariectomised female rats (n=3-6/dose group), oral: dose-dependent \uparrow uterus weight and histological changes	Medium-high	Strong
Feng et al. (2016)	Effects on steroidogenesis in the H295R cell line: \uparrow progesterone, \downarrow testosterone, aldosterone and cortisol at sub-cytotoxic concentrations		High	Strong
Li et al. (2016)		Cross-sectional study in immature male rats (n=6/group): serum and testes testosterone \uparrow and inhibin B levels \downarrow in male infants exposed pre- and/or postnatally plus effects on testes gene and protein expression (where n=3-4/group), including increased expression of AR, ER α and StAR, P450 _{scc} . Together indicating effects on steroidogenesis.	High	Strong
Nakona et al. (2016)	Delayed mice oocyte maturation, but no link to any ED MoAs studied		Medium	Weak
Ruan et al. (2015)	Estrogenic activity in the bioluminescence yeast estrogen screen (BLYES) assay		Medium	Moderate
Li et al. (2014)	Estrogenic through activation of ER α and GPER in human breast cancer T47D and MCF7 cells		Medium	Strong
Li et al. (2013)	ER α agonism and potential ER β agonism in HepG2, HeLa and Ishikawa/ER α cells		Medium	Strong
Teng et al. (2013)	ER α agonism and a competitive AR antagonism in transfected monkey kidney CV1 cells		Medium	Strong

Reference	MoA	Quality of study	Evidence for ED	
Delfosse et al. (2012)	ER α and ER β activity on MCF7 and HeLa cells, and mechanism studies on the interaction with ER α	High	Strong	
Feng et al. (2012)		Study in adult male rat (n=6/group): \uparrow testis/BW ratio, serum total cholesterol and testosterone \downarrow , and LH and FSH \uparrow , plus testis gene expression of StAR, P450 _{scc} , 17 β -HSD, SREBP-1c, ER α , LHR, Inhibin B and SR-B1 \downarrow . Testes protein levels of StAR, P450 _{scc} and SR-B1 \downarrow . Together indicating effects on steroidogenesis.	High	Strong
Li et al. (2012)	Estrogenic activity in Ishikawa, HeLa, and HepG2 cells, and functionality studies of the ER activation	Medium	Strong	
Sui et al. (2012)	Human PXR agonism in transfected HepG2 cells, an indirect potential ED MoA	Medium	Moderate	
Bermudez et al. (2010)	Oestrogen activity in T47D-KBluc cells, and caused estrogenic additivity in a binary (with E2) and a ternary (with E2 and BPA) mixture until system saturation	Medium	Moderate	
Matsushima et al. (2010)	ER-LBD binding in a cell-free assay as well as ER α agonism and ER β antagonism in transfected HeLa cells	Medium	Strong	
Akahora et al. (2008)	Active for hER α -LBD binding	Uterotrophic study in immature female rats (n=6/dose group), s.c.: \uparrow uterus weight in estrogenic mode and \downarrow uterine weight in the EE co-administered anti-estrogenic mode	Low	Moderate
Kitamura et al. (2005)	Estrogenic potential in MCF-7 cells and anti-androgenic activity in NIH3T3 cells	Medium	Strong	
Yamasaki et al. (2003)		Uterotrophic study in immature female rats (n=6/dose group), s.c.: \uparrow uterus weight in estrogenic mode and \downarrow uterine weight in the EE co-administered anti-estrogenic mode	High	Moderate
		Hershberger study in adult castrated male rat (n=6/dose group), oral: \downarrow BC/LA weight in androgenic mode and \uparrow seminal vesicle, glans penis and Cowper's gland weights in the TP co-administered anti-androgenic mode	High	Weak

Reference	MoA	Quality of study	Evidence for ED
Hasimoto et al. (2001)	Estrogenic activity in the YES assay with and without S9, as well as in a displacement assay and the E-screen (MCF-7 cell proliferation assay)	Medium	Moderate
Perez et al. (1998)	Estrogenic in the E-screen and increases levels of the estrogen-responsive proteins, progesterone receptor and pS2, in the MCF-7 cells. The reported relative binding affinity to ER is high.	Medium	Moderate

estrogen receptor α (ER α), thyroidal hormone receptor α (TR α), steroidogenic acute regulatory protein (StAR), androgen receptor (AR), bioluminescence yeast estrogen screen (BLYES), G protein-coupled estrogen receptor 1 (GPER), ligand binding domain (LBD), ethynyl estradiol (EE), follicle-stimulating hormone (FSH), luteinizing hormone (LH), luteinizing hormone receptor (LHR), sterol regulatory element binding protein 1c (SREBP-1c), bulbocavernosus/levator ani muscle (BC/LA), testosterone propionate (TP), yeast two-hybrid system (YES)

Table 2. Overview of potential endocrine-related adverse effects of BPAF.

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Foster et al. (2017)	Rats, ~20/ group	<i>In vivo</i> study using the NTP modified one-generation study: marked delay in male puberty, advancement in female puberty and clear effects on fertility of offspring exposed to BPAF during development. The effects observed are consistent with an oestrogenic MoA.	High	Strong

National toxicology program (NTP),

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5. Environmental hazard assessment

5.6.2 Endocrine Disruption

5.6.2.1 General approach – environment

The peer-reviewed literature was investigated by use of Web of Science including all databases. The search terms were: bpaf + endocrin*, bisphenol af + endocrin*, bisphenol-af + endocrin*, (Hexafluoroisopropylidene)diphenol + endocrin*, 1478-61-1 OR 1478-611 OR 147861-1 OR 1478611 + endocrin*, bisphenol af + endocrin*, bisphenol-af + endocrin, bpaf + endocrin* A google search with the search terms bpaf + endocrin*, bisphenol-af + endocrin*, bisphenol af + endocrin* was also performed, but without relevant hits. Literature describing mammalian studies and *in vitro* studies based solely on mammalian cell lines was excluded because it is covered in the toxicological hazard assessment.

5.6.2.2 *In vitro* information indicative of endocrine activity

The results from *in vitro* studies are mentioned under *in vivo* studies because the *in vitro* studies were always performed in combination with *in vivo* studies, except for one study (Fic *et al.* 2014).

Fic et al. (2014)

Summary: The (anti)estrogenic and (anti)androgenic properties of BPA and BPA analogs including BPAF (CAS no. 1478-61-1, 97% purity) were investigated in the XenoScreen XL YES and YAS assays, which are commercial and modified versions of the original YES and YAS assays.¹

BPAF and its metabolite BPAF-M were tested first in eight concentrations (BPAF: 300 µM to 30 pM and BPAF-M: 30 µM to 30 pM) and thereafter in a narrower concentration range to determine EC50 (at least two independent additional experiments in duplicate). BPAF demonstrated agonistic estrogenic activity with an EC50 of 0.39 µM. BPAF is less potent than E2 (EC50: 0.0002 µM) but more potent than BPA (EC50: 3.60 µM). An anti-androgenic activity of BPAF was also demonstrated by inhibition of the AR in co-treatment with the anti-androgen flutamide. BPAF-M did not demonstrate any observable activity in any of the four assays.

Study quality and assessment: The paper is detailed and well written, and the results are in agreement with previous studies using the original YES/YAS assay. The study is assessed to be of high quality. It is shown that BPAF has both estrogenic (agonistic ER-binding) and anti-androgenic properties (antagonistic AR-binding) and thereby the MoA for BPAF involves receptor binding, but the MoA is not linked to any physiological effects. The evidence for an ED MoA is considered as strong.

¹ Yeast cells transfected with the human ERα or ARα and estrogen responsive elements (EREs) linked to a reporter gene (lacZ).

5.6.2.3 *In vivo* effects with regard to an endocrine mode of action

Moreman et al. (2017)

Summary: The toxicity and teratogenic effects of the bisphenols BPA, BPS, BPF and BPAF (>97% purity, CAS no. 1478-61-1) in zebrafish embryo/larvae were investigated, and also their estrogenic mechanisms were assessed in estrogen-responsive transgenic zebrafish larvae (ERE-TG). The toxicity and developmental deformities were determined after 96 hours (0-96 hpf) and the following BPAF concentrations were tested: 0.5, 0.75, 1.0 and 2.0 mg/L (n = 20 larvae in three replicates per treatment). Assessment of the estrogenic response by GFP induction was determined at 120 hpf (0-120 hpf) and at the following concentrations: 0.001, 0.01 and 0.1 mg/L (n = 20 larvae in three replicates per treatment). Co-exposure with the estrogen receptor antagonist ICI 182,780 was performed to investigate a possible ER-mediated MoA. The exposures were semi-static and ethanol (0.01%) was used as a solvent. The experimental setups used to determine toxicity/morphological abnormalities followed the OECD guideline for Fish Embryo Acute Toxicity Test (TG 236). The chemical concentrations were measured by LC-MS.

BPAF was the most potent bisphenol for toxic and developmental effects (BPAF was 6-7 times more potent than BPA for hatching rate and mortality); the LC50 (96 hpf) for BPAF was 1.6 mg/L, the EC50 for hatching success (72 hpf) was 0.92 mg/L, and cardiac edema occurred at 1 mg/L. Generally for all bisphenols, toxic and developmental effects occurred at concentrations several orders of magnitude higher than concentrations measured in the environment (the environmental concentrations of BPAF are generally in the range of ng/L).

BPAF was also the most potent bisphenol in the ERE-TG zebrafish larvae: GFP induction was observed in the heart at 0.01 mg/L and in the liver and tail region at 0.1 mg/L. By exposure of each single bisphenol chemical in combination with the estrogen receptor antagonist ICI 182,780, the induction of GFP expression in all tissues was completely removed, which clearly demonstrates that the responses are mediated by an ER-pathway. BPAF is more estrogenic than the other investigated BPs including BPA.

Study quality and assessment: The paper is very well written, and the experimental setup, including group size, replicates and quantification of exposure concentrations, is solid and well described. Therefore, the study is assessed to be of high quality. The receptor binding in the transgenic larvae is observed at concentrations below the LC50 and EC50s of developmental effects. The MoA was investigated by an ER-mediated GFP response, and the evidence for ED MoA is considered to be strong.

Cano-Nicolau et al. (2016)

Summary: The effects of BPA and BPA analogs including BPAF (CAS no. not provided but purchased from Sigma-Aldrich, 98% purity) were investigated both *in vivo* in zebrafish larvae (4-7 dpf) and *in vitro*. The effects on the (xeno)estrogen-sensitive marker *cyp19a1b* gene (Aromatase B), which is mainly expressed in the radial glial cells of the brain, were investigated *in vivo* by: 1) *cyp19a1b* induction levels by RT-qPCR, 2) distribution of *cyp19a1b* transcripts by *in situ* hybridization, and 3) *cyp19a1b* promoter activity in transgenic *cyp19a1b*-GFP larvae. The binding capacity of the test compounds to estrogen nuclear receptors (ER α , ER β 1, and ER β 2) was

investigated *in vitro* in 1) a transfected human glial cell culture and 2) a zebrafish estrogen receptor competitive binding assay.

Neither BPA nor the BPA analogs (1 μ M) affected the survival rate and motility and teratogenicity was not observed. Exposure of zebrafish larvae between 1-7 dpf to BPAF (1 μ M) caused significant up-regulation of *cyp19a1b* expression at 7 dpf compared with the solvent control (n=70 pooled heads and the exposure was repeated six times). This was confirmed by significant induction of *cyp19a1b* promoter activity in the brain of *cyp19a1b*-GFP transgenic larvae exposed to the same BPAF concentration from 2 hpf to 4 dpf (n=20 larvae per treatment). By *in situ* hybridization it was shown, that *cyp19a1b* transcripts of larvae exposed to BPAF (1-7 dpf) were detected in the same brain regions as in larvae exposed to EE2 and BPA (n=10 larvae per treatment), and thereby demonstrating a similar MoA for EE2, BPA and the BPAF. Human U251MG cells (an ER-negative human glial cell line) transfected with zfER α , zfER β 1, or zfER β 2, and the zebrafish *cyp19a1b* promoter upstream of luciferase as the reporter gene, were used to investigate which of the three estrogen nuclear receptors mediated the up-regulation of the *cyp19a1b* gene. BPA and BPAF significantly stimulated *cyp19a1b* promoter activity in ER α -containing cells but not in cells transfected with ER β 1 or ER β 2, which demonstrates that *cyp19a1b* activation is mediated by the zfER α . Further, simultaneous treatment with the estrogen nuclear receptor antagonist ICI 182 780 completely abolished the BPA and BPAF stimulations observed in ER α -containing cells. Taken together, the reporter gene assay provides evidence that BPA, BPF, and BPAF are ER α agonists in brain glial cells. A competitive receptor-binding assay was used to examine the receptor-binding affinity of BPA and BPA analogs for the three ERs (zfER α , zfER β 1, and zfER β 2). In perfect agreement with the reporter gene assay, they found that BPA, BPF, and BPAF bind *in vitro* to ER α , and BPAF had the highest binding affinity - also higher than BPA. An up-regulation of *cyp19a1b* (brain aromatase) leads to increased enzyme activity and therefore, increased levels of endogenous estrogen in the brain are expected. Elevated estrogen levels are likely to affect brain development at a functional level e.g. mating behavior.

Study quality and assessment: Both *in vivo* and *in vitro* experiments are generally very well described and the experimental setups, including group sizes, replicates, and positive (1 nM EE2)/negative (DMSO v/v: 0.1%) controls are solid and well described. However, the exposure concentrations were not verified by chemical analysis. The study quality is assessed as high. It is clearly demonstrated that the MoA for BPAF involves the ER α , and that the estrogenic effect of BPAF on the developing brain involves induction of the *cyp19a1b* gene (Aromatase B) similar to EE2 and BPA, but other estrogen sensitive responses in the brain and other tissues could also be involved.

Kwon et al. (2016)

Summary: The effects of BPAF (CAS no. 1478-61-1, >97% purity) or a combination of BPAF and SMX (Sulfamethoxazole) on the thyroid endocrine system in adult male zebrafish (*Danio rerio*) were investigated. The exposure period was 21 days and with the following exposure concentrations: BPAF 24.7 μ g/L, SMX 5.6 μ g/L or BPAF 24.7 μ g/L + SMX 5.6 μ g/L (n=12; four males in three replicates).

Effects on thyroxine (T4) plasma levels were investigated (n=3 from each group). Changes in thyroid gene transcription of brain and thyroid tissue (homogenate of the gill region) were investigated by microarray analysis (n=6 for each treatment group)¹ The expression of 10 genes related to the hypothalamic-pituitary-thyroid (HPT) axis in brain or thyroid tissue was investigated by qPCR and the transcription of four genes in thyroid homogenate samples from the microarray (n=4 for each treatment group) was verified by qPCR.

Total plasma T4 levels were unaffected by exposure to BPAF and SMX alone but increased significantly in the combined BPAF/SMX exposure (n=3). BPAF altered the transcription of genes related to thyroid hormone production and receptor activity, thyroid gland development, and deiodinase activity. The expression of *trh*, *trhr1* and *tsh β* in brain tissue was increased by BPAF and in the thyroid tissue *dio2* and *tpo* expression increased and decreased, respectively.

Study quality and assessment: The study was designed to investigate if BPAF exposure affects the thyroid hormone system of male zebrafish. The experiment has several shortcomings: 1) Low number of n: Three replicates with four fish each, but n is 3 to 6 in the analyses of hormone levels and gene expression, and it is very difficult to figure out if samples are pooled or not 2) Only one exposure concentration, 3) Discrepancy in the concentration units: the text says " $\mu\text{g/L}$ " but Figure 1 says " mg/L " and 4) the exposure concentrations were determined by LC-MS/MS "24 h before and after exposure", but it is unclear how many times the concentrations were determined during the 21 d exposure period (daily renewal of water). Based on these issues the overall quality of the study is assessed as low. The present study indicated that BPAF exposure alters the transcription of genes associated with the thyroid endocrine system, but BPAF alone has no effect on T4 levels. A clear link to the MoA related to the increased T4 levels in the combined BPAF/SMX exposure is not provided and the evidence for an ED MoA is weak.

¹ There is a mismatch in the information about sample size: they state that n=6/treatment but later they write that the samples were pooled three and three.

Tišler et al. (2016)

Summary: In this study, lethal and sublethal effects of BPF and BPAF (CAS no. not provided but purchased from Sigma-Aldrich, 99% purity) on bacteria (*Vibrio fischeri*), algae (*Desmodesmus subspicatus*), crustacea (*Daphnia magna*) and zebrafish embryos (*Danio rerio*) were investigated. Also, the effects of BPA (0.63-10.0 mg/L), BPF (0.84-13.4 mg/L), and BPAF (0.11, 0.23, 0.45, 0.90 and 1.8 mg/L)¹ on the reproduction of *D. Magna* were investigated after 21 days of exposure (n=10 individuals per treatment, repeated at least three times). BPAF was the most toxic compound to *D. magna*, *D. rerio* and *D. subspicatus*. The hatching success of zebrafish embryos was the most sensitive parameter in all the investigated species (The EC50 value for hatching of zebrafish embryos was 2.2 mg/L). A concentration of 0.45 mg BPAF/L reduced several reproductive parameters in *D. magna* significantly e.g. number of young/female, brood size, days to first brood and number of broods/female. The mortality was not increased at 0.45 mg/L but the body length of females was reduced, which could indicate general toxicity.

Study quality and assessment: The experimental setups are adequately described and the exposure concentrations were determined by chemical analysis. However, the test concentrations are very high and most endpoints are related to acute toxicity. The study is assessed to be of moderate quality. A possible ED MoA linked to the reproductive parameters was not investigated.

¹ The solubility of BPAF in water is 0.84 mg/L but no solvent was used (US EPA Estimation program interface (EPI), referred in Choi & Lee, 2017)

Yang et al. (2016)

Summary: The endocrine-disrupting effects of BPAF (CAS no. not provided, 98% purity) were studied by exposing 2-month-old zebrafish to 0, 0.05, 0.25, or 1 mg/L BPAF (n=4 tanks/group, 9 males or 9 females per tank (sexes held separately), each group in duplicate) for 28 days and evaluating the effect on growth, histopathology (liver, gonads, gills, and intestine), enzyme activity (SOD and MDA)¹, hormone levels (testosterone, estradiol and free T3), and gene expression (vitellogenin). Semi-static exposure with 50% water renewal every day and a solvent control group (0.1% DMSO) but no control group.

Estradiol levels increased in a concentration-dependent manner in males but only the highest concentration (1 mg/L) was significantly different from the control. BPAF (1 mg/L) increased free T3 levels of females significantly, but no effect was observed at the lower concentrations and also no effects on male T3 level. Male testosterone levels tended to decrease in a monotonic concentration-dependent manner, whereas in females an increase was observed at the lowest two concentrations (0.05 and 0.25 mg/L) and a decrease at the highest concentration (1 mg/L), but none of these changes in testosterone levels were significant. Also, no significant difference in T/E2 ratios between the groups was observed. Hormone levels were measured in whole-body homogenate of males or females (n=6 males or females per treatment from duplicate tanks).

Vitellogenin gene expression was increased significantly in male livers at 1 mg/L and in females at 0.25 mg/L (n=6 males or 6 females per treatment).

The authors state that "the overall fitness of the fish was not significantly affected" but male body length was significantly reduced at the two highest test concentrations, and exposure to 1 mg/L BPAF caused liver damage but only in male fish, which could indicate better detoxification in females. The liver damage was characterized by hepatocellular swelling and vacuolation and indicates liver toxicity. Histological examination of the male gonads indicated that exposure to 1 mg BPAF/L lead to acellular areas in the seminiferous tubules of the testes, which was in contrast to control males where the seminiferous tubules were filled with spermatids. Histological examination of the female gonads revealed significant inhibition of oocyte maturation as an increase in stage I oocytes and a decrease in stage IV oocytes was observed after exposure to 0.25 and 1 mg BPAF/L. It is not clear how many fish they used for the histological examination of the gills, intestine, liver, and gonads.

Study quality and assessment: The experiment is adequately described but n is either small (n=6 males or 6 females from two replicates) or not defined and the results are sometimes over-interpreted. The abstract describes effects on hormone levels but according to the figures most of these effects are not significant. The study is assessed to be of medium quality.

The histological effects observed in the gonads could be related to general toxicity. It is unclear if the effects on hormone levels are related to the observed effects on oocyte maturation and acellular areas in the seminiferous tubules. The increase in the expression of male vitellogenin at the highest concentration indicates an ER-mediated MoA, and the evidence for ED MoA is considered weak-moderate because n is low and the increase is only significant at the highest concentration.

¹ SOD: Superoxide dismutase, marker of antioxidant enzyme activity.

MDA: Malondialdehyde, marker of oxidative stress.

Shi et al. (2015)

Summary: In this study, zebrafish were exposed to BPAF (Product no B0945 - corresponds to CAS no 1478-61-1, 99% purity) at 5, 25 and 125 µg/L (n=50 per tank, three replicates), from 4 hour-post-fertilization (hpf) to 120 day-post-fertilization (dpf). The plasma levels of 17β-estradiol (E2) and testosterone (T) were measured, and the expression of several genes in the hypothalamus-pituitary-gonad (HPG) axis was examined in liver (*vtg1*), brain (*ERα* and *β*, *cyp19b*, *lhβ*, *fshβ*, and *gnrh2*) and gonad tissue (*fshr*, *lhr*, *star*, *cyp11a1*, *cyp17*, *cyp19a*, *hsd3β*, *hsd17β*, *hmgra*, and *hmbrb*). The exposure was semi-static with complete water renewal every 24 hours until 14 dpf and thereafter 50% renewal every day, and with a solvent control group (0,01% ethanol) but no control group. Hormone measurements: n=3 (for each sex) per exposure but 10 fish of same sex are pooled. Gene expression: n=3 (gonads) or 6 (liver and brain) organs from fish of the same sex are pooled from each tank.

In males, the concentration of E2 increased and the concentration of T decreased both in a monotonic concentration-response relationship and with significant differences in the two highest test concentrations compared with the controls. The female E2 concentration was significantly increased in the highest exposure group. The E2/T ratio increased significantly in males (25 and 125 µg/L) and females (125 µg/L). Liver *vtg1* increased significantly in males (25 and 125 µg/L) but not in females. Several genes involved in steroid synthesis were significantly increased or reduced in males, and *star* expression was down regulated in both sexes. The concomitant changes in hormone levels and mRNA expression levels of genes in the HPG axis demonstrated that the steroid hormonal balances of zebrafish were at least partly modulated through alteration of steroidogenesis. Effects on survival and malformation rate in the F1 generation were observed at the highest test concentration, and also reduced fertilisation rate in the highest exposure group, which could suggest sperm deterioration in males. The higher occurrence of malformations and lower survival rate in the offspring from the exposure groups suggested a possibility of maternal transfer of BPAF, which could be responsible for the increased prevalence of malformations in the offspring.

Study quality and assessment: Overall, the study is well described and the samples sizes are acceptable however, the tissue and plasma samples are pooled for hormone (n=3; 10 males or females are pooled) and mRNA measurements (n=3-6; 3 (gonads) or 6 (liver and brain) samples from males or females are pooled). By pooling the samples the individual variation is lost, and the study is assessed to be of medium-high quality. Male E2 level increase and T levels decrease. The link between changes in gene expression and hormone alterations are well argued for, and indicative of an ED MoA involving effects on the expression of vitellogenin and steroidogenic enzymes e.g. *star* and the *cyp* enzymes.

Tang et al. (2015)

Summary: The purpose of this study was to elucidate the disruptive effects of BPAF (0, 5, 50 and 500 µg/L) on thyroid function and expression of the representative genes along the HPT axis in zebrafish embryos. The embryos were exposed BPAF (CAS no. 1478-61-1, >99,5% purity) dissolved in DMSO from 2 hpf to 168 hpf (n=300 with 3 replicates). The exposure was semi-static with 50% water renewal every 12 hours and a solvent control group (0.1% DMSO) but no control group. BPAF exposure did not affect survival, body length or weight, but hatchability at 72 hpf was significantly increased at the two highest concentrations.

Total 3,3',5'-triiodothyronine (TT3), total 3,5,3',5'-tetraiodothyronine (TT4), free 3,3',5'-triiodothyronine (FT3) and free 3,5,3',5'-tetraiodothyronine (FT4) levels were measured by ELISA

(commercial kits) in whole-body homogenate (homogenate of 200 of the 300 larvae). The remaining 100 larvae were homogenized and used for mRNA analyses (RT-qPCR) of the following genes: *β-actin* (reference gene), *tsh-β*, *dio1*, *dio2*, *slc5a5*, *tg*, *ttr*, *tr-α*, and *tr-β*.

Generally, BPAF reduced the levels of the investigated thyroid hormones. Whole-body total T3 (TT3) and total T4 (TT4) decreased significantly at the intermediate and high exposure concentration. Whole-body free T3 (FT3) and free T4 (FT4) levels decreased in a monotonic concentration response-relationship: FT3 was decreased significantly in the intermediate and high concentration but FT4 levels were decreased significantly in all three exposure concentrations.

The exposure to BPAF affected several genes in the HPT axis e.g. the expression of *ttr* and *dio1* was significantly increased and the expression of *tra* and *slc5a5*¹ decreased in all or the two highest exposure groups.

Study quality and assessment: The experiments are well described and follow the OECD TG 236. The variation in the gene expression results is generally high and the responses are non-monotonic. The quality of the study is assessed to be medium-high. Overall, this study demonstrates that BPAF affects the whole-body concentrations of thyroid hormones and the transcription of genes involved in the HPT axis in zebrafish larvae. Decreased expression of *slc5a5* can lead to a decreased iodide transport and the thyroid cannot accumulate iodide, which might reduce the production of T4. The HPT gene expression results provide a possible underlying MoA of the decreased T3/T4 levels found in zebrafish larvae.

¹ *slc5a5* encodes a sodium/iodide transporter in the thyroid. The transporter plays a role in the iodine uptake from the blood into the thyroid where iodide is incorporated in T3 and T4.

Yamaguchi et al. (2015)

Summary: The objective of this study was to evaluate the potential estrogenic effects of BPA and BPA analogs including BPAF (CAS no. not provided, >97% purity) in Japanese medaka (*Oryzias latipes*) using *in vivo* assays and *in silico* docking simulation analysis. Male medaka were exposed for 8 hours to BPAF (0.05, 0.5, 5 and 50 μM, n=3 males per treatment, repeated at least twice) and mRNA expression levels of estrogen-responsive genes (*Vtg1*, *Vtg2*, *ChgH*, *ChgL*, and *ERα*)¹ in the livers were determined. Males died of abdominal swelling when exposed to 50 μM BPAF. Both negative control (0.01% DMSO) and positive control (E2: 3.7 nM) groups were included.

The expression of *Vtg1* increased significantly in a monotonic concentration-response relationship in all three exposure groups and *Vtg2* expression increased significantly in the highest exposure group (5 μM). Expression levels of *ChgH*, *ChgL* and *ERα* increased significantly in the two highest exposure groups. The interaction potential of BPA and BPA analogs with medaka *ERα* was investigated *in silico* in a three-dimensional model of the *ERα* ligand-binding domain (LBD) and docking simulations were performed. The docking simulations showed that BPA and BPAF are agonists of *ERα* LBDs in both the medaka and the common carp (*Cyprinus carpio*) but the medaka *ERα* is more sensitive to binding of BPs. BPA and BPA analogs including BPAF probably induce the expression levels of *Vtgs* and *Chgs* by activation of the *ERα*; at least *ERα* is partially involved in the regulation of liver *Vtg* and *Chg* genes in male medaka.

Study quality and assessment: The experimental setup and data are well described but the group size is small (n=3 males per treatment; repeated twice) and the chemical concentrations are not determined. The study quality is assessed as medium-high.

¹ ChgH and ChgL encode the female specific proteins choriogenin H and L. The choriogenins are precursor proteins for proteins in the inner egg envelope and they are synthesized in response to E2.

Song et al. (2014)

Summary: The toxicity of bisphenol A (BPA), tetrabromobisphenol A (TBBPA), tetrachlorobisphenol A (TCBPA), and BPAF (CAS no. not provided, 98% purity) was investigated in zebrafish embryos/larvae and the endocrine related endpoints were investigated in adult male zebrafish. Two-month-old male zebrafish were exposed to BPAF concentrations of 0.5, 1.0 and 1.5 mg/L (n= 10/tank, 3 replicates) for 21 days in a semi-static exposure (100% water renewal every day) with control and solvent control groups (0.5% DMSO). Plasma vitellogenin levels increased significantly in a monotonic concentration-response relationship (n=10) and reached the same level as the positive control (5 µg/L E2) in the highest exposure group (1.5 mg/L). The *in vitro* estrogenic activity of BPAF (0.5, 1, 5 and 10 µM) was investigated in the MVLN assay¹. The estrogenic activity increased with increasing exposure concentration, but the increase was only significant at the highest test concentration. Both the *in vivo* and *in vitro* assay showed a stronger estrogenic activity of BPAF compared with BPA.

Study quality and assessment: The material and methods section is inadequate e.g. lack of information about exposure concentrations, and the number of experimental animals in this section does not comply with information in the figure legends. Overall, the study is assessed to be of medium quality. Both BPA and BPAF induce a significant monotonic concentration-dependent increase in vtg levels *in vivo* and clearly illustrate an ER-mediated MoA, and the *in vitro* assay shows a clear ER activity of BPA and BPAF. The link is assessed to be moderate to strong. Both the *in vivo* and *in vitro* assay showed a stronger estrogenic activity of BPAF compared with BPA.

¹ A human reporter gene assay: MCF-7 cells with endogenous estrogen receptors and transfected with an estrogen-responsive reporter gene)

5.6.2.4 Summary of the plausible link between adverse effects and endocrine mode of action

Studies investigating the link between endocrine effects of BPAF and population relevant endpoints in non-mammalian vertebrates like phenotypic sex ratio and reproduction are unfortunately not available. Therefore, it is not possible to link the very well documented estrogenic mechanistic effects of BPAF to population relevant adverse effects non-mammalian vertebrates.

Adverse effects like skewed sex ratio have been documented in fish species after BPA exposure (e.g. Drastichova *et al.* 2005, OhSooNa *et al.* 2002) and since several studies report similar ER-mediated MoA for BPA and BPAF a read-across approach could be relevant. Especially because several of the studies demonstrate a stronger ER-binding affinity of BPAF compared with BPA.

With the current lack of information on population relevant endpoints in non-mammalian vertebrates (Table 3) BPAF is not an endocrine disrupting compound according to the IPCS/WHO definition. However, clear ER-mediated MoA is established in *in vitro* and *in vivo* studies of BPAF. In mammalian species there is strong evidence for marked effects on fertility and these are evaluated as population relevant (see 4.10.3.4 above). Therefore BPAF meet the WHO definition of an endocrine disruptor.

5.6.2.5 Environmental relevance

BPAF has been detected in environmental samples but in lower concentrations than BPA, but because BPA is substituted with BPA analogs there is an increasing concern that environmental concentrations of BPA analogs might increase in the future. BPAF is normally detected in sediment and water samples in the low ng/g and ng/L range, but concentrations several orders of magnitude higher have been detected in sediment and water samples in China e.g. close to a BPAF manufacturing plant (Liao *et al.* 2012, Song *et al.* 2012, Yang *et al.* 2014). The environmental BPAF concentrations are generally lower than the effect concentrations in the studies described above, but because BPA and BPA analogs have similar ER-mediated MoA a combined environmental exposure could cause concern.

Table 3. Overview of *in vitro* and *in vivo* endocrine disruptive (ED) mode(s) of action (MoA(s)) of BPAF.

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Moreman et al. (2017)		BPAF (0.01 mg/L) induced GFP in the estrogen-responsive transgenic larvae (0-120 hpf). It is clearly demonstrated that the MoA is ER-mediated.	High	Strong
Cano-Nicolau et al. (2016)	The binding capacity of BPAF to zfERs was investigated in human glial cell culture and a zebrafish estrogen receptor competitive binding assay, and it was shown that BPA and BPAF are ER α agonists <i>in vitro</i> . BPAF has a higher binding affinity compared with BPA.	The induction of the <i>cyp19a1b</i> gene (aromatase B) by BPAF exposure was investigated by RT-qPCR, in situ hybridization and a <i>cyp19a1b</i> -GFP zebrafish, and showed that exposure to BPAF induced the <i>cyp19a1b</i> gene (aromatase B) in the brain in a pattern similar to EE2 and BPA.	High	Strong
Kwon et al. (2016)		Several genes related to the thyroid system are affected but only significantly increased T4 levels in males after combined BPAF and SMX exposure	Low	Weak
Tišler et al. (2016)		Several reproductive parameters in <i>D. magna</i> significantly but MoA was not investigated	Medium	Weak
Yang et al. (2016)		Increased female T3 levels and increased male E2 levels and <i>vtg</i> gene expression at the highest test concentration. Damage of male liver cells at the highest concentration. Effects on spermatid concentration and oocyte development. The MoA is weak-moderate.	Medium	Weak-moderate
Shi et al. (2015)		Concentration dependent increase/decrease in E2/T plasma levels of males. Increased <i>vtg1</i> expression in male liver tissue at the two highest concentrations. The expression of several genes in the steroidogenic pathway was affected.	Medium-high	Moderate

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Tang et al. 2015		Monotonic concentration-response decrease in TT3, TT4, FT3 and FT4 in whole-body homogenate of zebrafish larvae. The expression of several genes related to the thyroid system was affected.	Medium-high	Moderate
Yamaguchi et al (2015)	<i>In silico</i> : Docking simulations revealed that BPA and BPAF are agonists of ER α LBDs in both the medaka and the common carp but the medaka ER α is more sensitive to binding of BPs.	Short-term exposure (8 hours) of adult male medaka to BPAF (0.05-5 μ M) significantly increased the expression levels of liver Vtgs, Chgs, and ER α .	Medium-high	Weak-moderate
Song et al. (2014)	Activation of ER by BPAF in the MVLN assay (significant at the highest test concentration)	Plasma vtg increase in a monotonic concentration-response relationship in male zebrafish. BPAF has a stronger estrogenic activity compared with BPA.	Medium	Moderate
Fic et al. (2014)	BPAF has both estrogenic (agonistic ER-binding) and anti-androgenic properties (antagonistic AR-binding) in modified YES/YAS assays. Clear ER-mediated MoA but no link of MoA to physiological effects.		High	Strong

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Isobutyl paraben, CAS no. 4247-02-3

Synonyms: Isobutyl 4-hydroxybenzoate, isobutyl-p-hydroxybenzoate, isobutyl parahydroxybenzoate, 2-methylpropyl 4-hydroxybenzoate, IBP

Isobutyl paraben (C₁₁H₁₄O₃) (Figure 1) is a nonlinear paraben (Figure 1). Parabens are a group of alkyl esters of p-hydroxybenzoic acid, and many different parabens exist, e.g. methylparaben, ethylparaben and butylparaben. Isobutyl parabens is used as preservatives in foods, pharmaceuticals and cosmetics and is used in 0-10 tonnes per annum. Isobutyl paraben has been classified in ECHA as a skin sensitizer 1B (H317: May cause an allergic skin reaction). Moreover, it has been classified as a skin (H315: causes skin irritation) and eye irritant (H318: causes serious eye damage and H319: causes serious eye irritation)

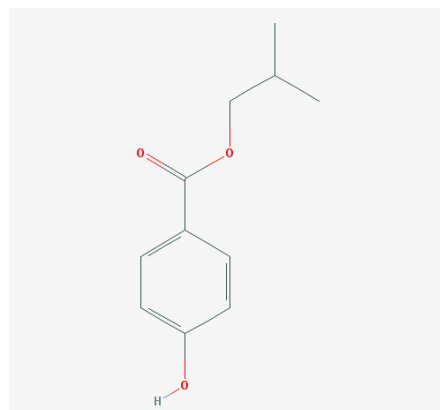


Figure 1: 2D structure from PubChem

4. Human health hazard assessment

4.10.3 Endocrine disruption

4.10.3.1 General approach – human health

4.10.3.2 *In vitro* information indicative of endocrine activity

Kim et al. (2012)

Summary: The aim of the study was to investigate the additive, synergistic or antagonistic effects on estrogenic activity of isobutyl paraben and octylphenol using GH3 rat pituitary cells. Isobutyl paraben was tested at 0, 0.1, 1 or 10 μ M and gene expression and protein expression of Calbindin-D_{9k} (CaBP-9k) and progesterone receptor (PR) and estrogen receptors (ER) reporter gene expression was assessed in the cells. Expression of the ER reporter gene and expression of CaBP-9k gene and protein was upregulated at all tested doses of isobutyl paraben. The PR gene was also upregulated in all the tested doses of isobutylparaben but only the highest dose (10 μ M) upregulated expression of PR protein.

Study quality and assessment: The study is well-described and thorough. The methods section describes the concentrations of isobutyl paraben tested and the incubation conditions and they used positive and negative controls and performed duplicate or triplicate samples. Information on cytotoxicity was apparently not tested and the CAS-number of the chemicals used is not provided. Based on this, the study is assessed to be of medium quality. The study provides moderate evidence of an estrogenic mode of action of isobutyl paraben involving estrogen receptors.

Kim et al. (2011)

Summary: The objective of this study was to validate the estrogenic activity of a range of chemicals, including parabens, using the stably transfected transcriptional activation assay. Isobutyl paraben was tested in a hER α -HeLa-9903 cell line transfected with a human ER α gene with a firefly luciferase gene as a reporter gene. The study showed (22,000 fold) lower estrogenic activity of isobutyl paraben compared to 17 β -estradiol. Compared to other parabens tested, isobutyl and butyl paraben were the two parabens with highest estrogenic activity.

Study quality and assessment: The study is well-described and well-structured and it provides much information on the cell assay performed such as positive and negative controls, incubation conditions, dilutions etc. However, information on cytotoxicity or triplicates is not described and no CAS number or purity of isobutyl paraben is given. The study is therefore assessed to be of medium quality. The present study investigated the activity of isobutyl paraben in a single assay and the resulting information on the estrogenic mode of action of isobutyl paraben is limited. The data provide weak evidence of an estrogenic mode of action of isobutyl paraben

Kim et al. (2010)

Summary: In this study, the aim was to determine the relative androgen receptor (AR) binding affinity of a range of chemicals, e.g. parabens, phthalates and testosterone. Isobutyl paraben showed higher competitive affinity to AR than other parabens tested. The binding affinity relative to dihydrotestosterone was almost 17000 times lower for isobutyl paraben.

Study quality and assessment: The description of the methods and the interpretation of the findings are poor. The CAS-numbers of chemicals or of the radioligand are not given and controls are not used. The concentrations tested can be read with some difficulty from a figure of low graphical quality. Technical notes for the user in the laboratory are included in the description of the methods giving the impression that the materials and methods section is not written with the reader of the article in mind. However, other details are included such as a description of the AR used, a description of the preparation of the assay buffer and the experiment was performed in triplicates. The study is assessed to be of medium quality. This study investigated the relative binding affinity to AR but does not investigate other antiandrogenic mechanisms of action and the knowledge provided on the antiandrogenic properties of isobutyl paraben are limited. The data provide weak evidence of an antiandrogenic mode of action of isobutyl paraben.

Vo et al. (2010)

Summary: In this study a competitive ligand binding assay was performed in addition to a female pubertal assay to assess the estrogenic effects of several parabens *in vitro* and in female rats. The binding affinity of isobutyl paraben to ER α and ER β was investigated. Isobutyl paraben showed affinity to both receptors and no preference to any of the receptors could be determined.

Study quality and assessment: The *in vitro* study is described very briefly and it is not mentioned where the receptors used in the assay originate from (e.g. rat or human) or whether triplicates were performed. The quality of the *in vitro* part of the study is assessed to be of low quality. The study provides weak evidence of an estrogenic mode of action of isobutyl paraben.

Dabre et al. (2002)

Summary: This study investigated the estrogenic activity of isobutyl paraben and n-butyl paraben in an array of *in vivo* and *in vitro* assays. *In vitro* studies were performed in three different human breast cancer cell lines. A competitive ER α binding assay and the ability to regulate gene expression of oestrogen responsive genes were performed in MCF-7 cells. Oestrogen dependent growth was assessed in MCF-7 and ZR-75-1 cell lines and MDA-MB-231 cells were used as a negative control.

Isobutyl paraben increased expression of oestrogen regulated genes, stimulated oestrogen dependent growth of cell lines and inhibited binding of oestradiol to ER α . These *in vitro* data support each other and altogether they indicate an estrogenic activity of isobutyl paraben at receptor, gene and at a cellular physiologic response level.

Study quality and assessment: The study is well-described and much information is included for the *in vitro* part of the study, such as source of cell lines, use of negative and positive controls and clear descriptions of the interpretation and objective of each study. Although the CAS number and purity of isobutyl paraben is not reported, the *in vitro* study is assessed to be of high quality. The *in vitro* studies investigated the estrogenic activity of isobutyl paraben at several levels, including at receptor, at gene and at a cellular physiologic response level. The *in vitro* part of the study provides strong evidence of an estrogenic mode of action of isobutyl paraben.

Okubo et al. (2001)

Summary: In the present study the estrogenic activity of parabens was investigated *in vitro*. MCF-7 cells were used to study estrogenic induced cellular proliferation, gene expression levels of ER α and PR and the protein expression of ER α . A competitive receptor binding assay was performed to investigate the affinity to human ER α and ER β .

Proliferation of MCF-7 cells was increased by isobutyl paraben. Compared to the positive control, 17 β -estradiol, the effect of isobutyl paraben was 170,000 times lower. Suppression of the proliferative effect by the antiestrogen ICI 182,780 interacting with ER, suggested an estrogenic mode of action of isobutyl paraben on proliferation of MCF-7 cells. Gene expression of ER α was decreased and PR was increased by isobutyl paraben. Although the effects were lower than the effects of 17 β -estradiol, gene expression of ER α and PR were changed in the same directions as for 17 β -estradiol. Protein expression of ER α was decreased by isobutyl paraben although not in the same extent as for 17 β -estradiol. This is in good accordance with the findings on gene expression level of ER α . Isobutyl paraben had 1000-fold lower affinity to human ER α and ER β compared to the positive control DES, indicating that the paraben does not have specific preference for ER α or ER β .

Study quality and assessment: The study is well-described and thorough. Although Okubo and co-workers did not mention whether they assessed cytotoxicity, this does not affect the reliability of the study as the results indirectly imply that there was no cytotoxicity at the relevant doses. Proliferation was observed and proliferation levels were not suppressed below control levels at dilutions of ICI 182,780 below 10⁻⁸. Although the CAS-number and purity of isobutyl paraben is not described, the study is assessed to be of high quality. Based on the data on estrogenic activity of isobutyl paraben at the gene, protein and cellular proliferation levels together with the study on affinity to human oestrogen receptors, the study provides strong evidence of an estrogenic mode of action of isobutyl paraben.

4.10.3.3 *In vivo* effects with regard to an endocrine mode of action

Yang et al. (2016)

Summary: The aim was to investigate reproductive effects in male rats of a low dose of a mixture of bisphenol A and isobutyl paraben after developmental exposure. The two compounds were tested separately and in a mixture. Pregnant rat dams (n=3/group) were dosed by oral gavage with 2.5 mg/kg/day isobutyl paraben from gestation day 6 to post-natal day 12. At delivery, birth weight, number of pups and the sex ratio were recorded. Male pups were assessed for anogenital distance (AGD), nipple retention, preputial separation, testis descent, body weight, pinna detachment, incisor eruption and eye opening. The litters were adjusted to 8 male pups per group from PND4. Male pups were killed on PND70 and blood was sampled for hormone analysis (LH, FSH, testosterone and 17 β -estradiol), testis and epididymis were weighed and stored for histopathological analysis and epididymal sperm count and motility (n=5/group) was assessed. The hormone level of 17 β -estradiol was decreased by isobutyl paraben in adult male pups. Sperm count and motility was reduced compared to controls in adult male offspring in the isobutyl paraben group. No other endpoints investigated were affected by isobutyl paraben.

Study quality and assessment: The study is well described and although the chow was not tested for phytoestrogen content, oestrogen contamination was minimized from other sources (stainless steel cage, glass water bottles and wood bedding). Only 3 pregnant dams were used for this developmental study and data from littermates were analysed but the litter effect was not included in the statistical analysis. The study is assessed to be of low to medium quality. This study showed adverse effects on sperm motility and sperm numbers, but the underlying mode of action is not clear. Effects on sperm count have been seen for several endocrine disrupters after developmental exposure suggesting an endocrine mode of action of isobutyl paraben. Moreover, the study showed effects on the intrinsic oestrogen levels indicating an endocrine mode of action of isobutyl paraben *in vivo*. The study provides weak evidence of an endocrine mode of action and adverse effects of isobutyl paraben.

Kim et al. (2015)

Summary: In this study, a 28-day repeated dose toxicity study with dermal exposure to isobutyl paraben, isopropyl paraben or a mixture of the two was performed. The test followed the OECD test guideline 410. Male and female rats (5 weeks old) were dermally exposed to 0, 100, 300 or 600 mg/kg isobutyl paraben (10 rats per group) for 28 days (5 days per week) by topical application to the skin in a shaved dorsal area of the trunk. Body weight and food and water consumption was measured. At necropsy, blood was sampled (for haematological parameters, biochemical parameters and hormone analysis of T3, FSH, estradiol, testosterone and insulin) and livers, kidneys, hearts, brains, testes, prostates, ovaries and vaginas were weighed. Skin, liver, kidney brain and heart were assessed histopathologically. Histopathological evaluation of the skin showed changes in the females. Epidermal hyperplasia with hyperkeratosis (1 out of 3 examined in the 100mg/kg dose-group and 3/3 in the 300 and 600 mg/kg groups) and pustules (1/3 in the 100 and 300 mg/kg dose-groups and 3/3 in the 600 mg/kg dose-group) were seen. Biochemical parameters measured in the blood showed increased levels of Na⁺ in a dose-dependent manner in males from 50mg/kg and higher and increased levels of Cl⁻ in females from the 600 mg/kg group compared to controls; however these changes were within the normal range. The lack of changes in organ weights and haematological parameters indicated no signs of hematotoxic, hepatotoxic or nephrotoxic effects of isobutyl paraben in the tested doses.

Study quality and assessment: The study is well-described and thorough and follows the OECD TG 410 for repeated dose dermal toxicity. It is unclear how many male and female rats were used in the study (5 or 10 of each sex per group), and a little more details on the housing conditions would have been preferred. The study is assessed to be of medium quality. Due to the lack of effects on endocrine-related organs and hormonal parameters, the study provides no evidence of adverse effects with an endocrine mode of action.

Kawaguchi et al. (2010)

Summary: In this study the effects of perinatal exposure to isobutyl paraben on social behaviour was analysed. Pregnant rat dams were continuously exposed to isobutyl paraben sc. through a Silastic capsule implanted 3 weeks before mating. Female offspring (n=5-6) were tested for social recognition at 16 weeks of age.

Treated rats showed impaired social recognition. After repeated presentation to a rat, females exposed to isobutyl paraben spent the same time interacting with the rat compared to the first presentation, whereas control females spent less time interacting with the rat on the 4th trial.

Study quality and assessment: The study is well-described and relevant information is given. The study is assessed to be of high quality. The study provides evidence of effects on social behaviour, but it is not clear if the adverse effects are due to an endocrine mode of action. This study provides strong evidence of adverse effects.

Vo et al. (2010)

Summary: In this study a female pubertal assay was performed to investigate the effects of parabens (methyl-, ethyl-, propyl-, isopropyl- butyl- and isobutyl paraben) in female rats and an affinity ligand binding assay was performed to assess the estrogenic activity *in vitro*. Female prepubertal rats (10/group) were dosed PND21-40 with 0, 62.5, 250 or 1000 mg/kg/day isobutyl paraben by oral gavage. 17 α -ethynylestradiol was used as an estrogenic positive control. Oestrous cycle was assessed during the exposure period. At necropsy on PND41 blood was sampled for hormone analysis (estradiol, prolactin, tetra-iodothyronine (T4) and TSH) and organs (uterus, ovary, liver, kidneys, adrenal glands and thyroid glands) were weighed and stored for histopathology.

At all doses of isobutyl paraben myometrial hyperplasia was seen in uteri. It is less clear what changes were observed histologically in ovaries, but a decreased number of corpora lutea, increased number of cystic follicles and thinning of the follicular epithelium was apparently seen. Serum levels of T4 were decreased in the low-dose isobutyl paraben group but not at higher doses.

Study quality and assessment: The present study has some limitations and missing information. Although animals were fed a soy-free diet to limit contamination of phytoestrogens through the feed, other sources of oestrogen contamination such as cage material or drinking bottles were not avoided. The rats were bred in-house but it is not described whether some of the 200 female prepubertal rats used in the study were littermates. The description of histopathological findings in ovaries is confusing and it is unclear what kind of changes they found in this organ. Vaginal opening was assessed, but is not described in the materials and methods section. Finally, the relevance of assessing the regularity of the oestrous cycle in vaginal smears from PND21 is not clear as the female rats are not sexually mature at this age and the mean day of vaginal opening in some of the exposure groups

was above 36 meaning that they had less than 4 days to assess vaginal smears in some of the animals in those groups. As a consequence, the study is assessed to be of low quality. The histopathological changes observed in female reproductive organs may be related to estrogenic effects of isobutyl paraben, but the underlying mechanism of action is not clear. The role of isobutyl paraben on thyroid hormones is unclear and the changes in T4 levels may be a chance finding. The study provides weak evidence of adverse effects in female reproductive system with an estrogenic mode of action of isobutyl paraben. Also, a weak evidence of a thyroid disrupting mode of action of isobutyl paraben is provided based on the hormonal effects seen at the lowest dose.

Kawaguchi et al. (2009a)

Summary: In this study, the effects of isobutyl paraben on emotional behaviour and learning performance were analysed in rats. From 3 weeks before mating, mated dams were exposed continuously to isobutyl paraben through an implanted Silastic capsule. Offspring were tested for neurobehavioral function. In the open field test, 5 weeks old offspring (one male and one female per litter from 7 treated and 8 control litters) were tested. The same animals were tested in the elevated plus maze at 6 weeks of age. In the passive avoidance test, females were ovariectomised (ovx) at 7 weeks of age and treated with or without oestrogen and 11 weeks old males and ovx females with or without oestrogen from 8 treated and 8 control dams (one of each per litter) were tested. The same animals were used in the Morris water maze. The amount of isobutyl paraben released from the Silastic capsule was estimated by incubation of capsules in saline at 37°C for 42 days.

Approximately 4.36 mg/L/day of isobutyl paraben was released from the capsule *in vitro*. Behavioural testing showed sex-specific effects. Male offspring exposed to isobutyl paraben perinatally spent shorter time in the open arms of the elevated plus maze and showed decreased performance in the passive avoidance test. No effects of isobutyl paraben on offspring behaviour were seen in the open field or the Morris water maze. Developmental exposure to isobutyl paraben affected emotionality but not activity levels. Long-lasting effects on behaviour appeared to be present in male rats after developmental exposure to isobutyl paraben and the anxiogenic effects observed in male offspring may be comparable to behavioural effects seen for gonadal hormones or compounds that mimic oestrogen. The results indicate that the estrogenic action of isobutyl paraben shown in the literature may feminise the male brain.

Study quality and assessment: This study is well-described and the relevant information is given in the text. Although no CAS-number of the compound is reported, the study is assessed to be of high quality. The adverse effects on male behaviour are in good accordance with the estrogenic activity of isobutyl paraben reported by others and this study provides strong evidence of adverse effects linked to an estrogenic mode of action of isobutyl paraben.

Kawaguchi et al. (2009b)

Summary: The aim was to clarify the estrogenic effects of gestational and through lactation exposure to isobutyl paraben on the endocrine system of pregnant dams and offspring. This study was the same as for Kawaguchi et al. (2009a), but with assessments of other effects. Pregnant rat dams were continuously exposed to isobutyl paraben sc. through a Silastic capsule. Dams were killed after weaning and blood was collected and pituitary glands, adrenal glands and uterus were weighed. AGD was measured in 7 days old offspring (4 males and 4 females per litter) and vaginal opening and oestrous cycle from 7 weeks of age were assessed in female offspring (1 female per litter). Offspring

were killed at 3 weeks of age (1 of each sex per litter) and at 12 weeks of age (1 male per litter). At necropsy of offspring blood was collected and pituitary glands, adrenal glands, testis and uterus were weighed. One testis from 12 weeks old male offspring was used for sperm count. The 12 weeks old males were stressed before necropsy and adrenal glands were collected. One 7 weeks old female per litter was ovariectomised and implanted with Silastic capsules with or without estradiol. At 12 weeks of age (5 weeks after implantation of the capsules) female offspring treated with estradiol were killed and blood was collected and pituitary glands and uterus were weighed. Blood samples from ovariectomised females not treated with estradiol were collected at 13 and 17 weeks of age. Blood was analysed for hormone levels (LH, FSH, testosterone, estradiol, prolactin, ir-inhibin, TT3, TT4 and corticosterone).

No overt signs of toxicity were seen. There were no effects on litter size or ratio of male pups. Plasma corticosterone levels in dams were decreased after isobutyl paraben exposure and uterus weight was increased. No effects were found in the offspring.

Study quality and assessment: The study is well-described and all relevant information is given in the text except for the CAS number of the tested isobutyl paraben. The study is assessed to be of high quality. The data from the study show evidence of effects of isobutyl paraben on uterus weights of dams but not in female offspring. The study provides weak evidence of adverse effects related to an estrogenic mode of action and no evidence an antiandrogenic or a thyroid disrupting mode of action.

Koda et al. (2005)

Summary: The objective of the study was to test the oestrogenic activity of the agents in UV filters, including isobutyl paraben, using ethinyl estradiol and bisphenol A (BPA) as positive controls in a uterotrophic assay. Ovariectomised female rats (13-14 weeks old at study start, n=6) were dosed s.c. daily for 3 days with 0, 100, 250 or 625 mg/kg/day. Body weight, wet and blotted uterine weight was assessed. Wet and blotted uterine weights were increased with 250 mg/kg isobutyl paraben per day or more, indicating an estrogenic activity of the chemical *in vivo*.

Study quality and assessment: The study is well-described, the estrogenic activity of the feed was tested and aluminium cages and water supply without plastic were used to minimize contamination with BPA. The study is assessed to be of high quality. This study provides strong evidence of an estrogenic mode of action of butyl paraben *in vivo*.

Dabre et al. (2002)

Summary: This study investigates the estrogenic activity of isobutyl paraben and n-butyl paraben in an array of *in vivo* and *in vitro* assays. The immature rodent uterotrophic assay was used in the *in vivo* study. Immature (18 days of age) female CD1 mice (7 per group) were dosed s.c. with 0, 1.2 or 12 mg/kg/day for 3 days. Body weight and uterus wet weight were assessed at necropsy.

Isobutyl paraben increased uterus weight relative to body weight at 1.2 and 12 mg/kg/day. The results indicate an estrogenic activity of isobutyl paraben *in vivo*.

Study quality and assessment: In the *in vivo* part of the study, information on housing conditions is missing. In a study with focus on oestrogen activity of a compound like in the present study, some information on (phyto)oestrogen content in the feed, cage material or cage enrichment would be

preferred. However, in the present case with a very short exposure period (3 days), this information is less critical and the remaining work is very well described. Although CAS-number and purity of the compound is not reported, the quality of the *in vivo* study is assessed to be high. The *in vivo* data show an estrogenic mode of action of isobutyl paraben and provides strong evidence of an estrogenic mode of action of isobutyl paraben.

REACH registration dossier

The REACH dossier did not include data on reproductive toxicity studies or other *in vivo* or *in vitro* data. Acute toxicity and skin sensitisation and irritation were evaluated based on QSAR prediction using the Danish QSAR database. Genetic toxicity was assessed based on an *in vitro* study. No other information on toxicity testing is available in the REACH dossier.

4.10.3.4 Summary of the plausible link between adverse effects and endocrine mode of action

Several *in vitro* and *in vivo* studies were performed with focus on the endocrine mode of action of isobutyl paraben (Table 1). Most of the *in vitro* studies investigated the estrogenic mode of action of isobutyl paraben and showed estrogenic activity at different levels. The paraben has been shown to bind ER α and ER β , to upregulate oestrogen regulated genes and proteins and to induce oestrogen regulated proliferation of cells (Kim *et al.* 2012; Kim *et al.* 2011; Vo *et al.* 2010; Dabre *et al.* 2002; Okubo *et al.* 2001). *In vivo* studies confirmed the estrogenic mode of action of isobutyl paraben in uterotrophic assays with s.c. exposure (Koda *et al.* 2005; Dabre *et al.* 2002). At the hormonal level, there are some discrepancies between the studies. Yang *et al.* (2016) showed decreased 17 β -estradiol in adult male pups in a developmental study (Yang *et al.* 2016). In contrast, no effects on serum estradiol levels were seen in rat studies with adult exposure in males or females (Kim *et al.* 2015), peripubertal exposure in females (Vo *et al.* 2010) or developmental exposure in females (Kawaguchi *et al.* 2009b). Taken together, the data provide strong evidence of an estrogenic mode of action of isobutyl paraben.

The *in vivo* data on endocrine-related adverse effects of isobutyl paraben are scarce (Table 2). Two studies showed adverse effects that may be related to the estrogenic mode of action shown *in vitro* and *in vivo*. Histopathological changes in female reproductive organs were observed in a female pubertal assay and may be related to an estrogenic effect of the compound (Vo *et al.* 2010). However, the changes reported in the study are not well-described in an extent that makes it unclear what changes were found in the ovaries and the underlying mechanism of action can not readily be discussed. Another study showed adverse effects on male behaviour after perinatal exposure and according to Kawaguchi *et al.* (2009a) the sex-specific anxiogenic affects observed were comparable to behavioural effects seen for gonadal hormones or compounds that mimic oestrogen (Kawaguchi *et al.* 2009a). Another study on behaviour showed impaired social recognition in female offspring exposed perinatally to isobutyl paraben, but it is not clear if the adverse effects are due to an endocrine mode of action. (Kawaguchi *et al.* 2010). Adverse effects on sperm motility and sperm numbers in male pups (Yang *et al.* 2016) suggest an endocrine mode of action of isobutyl paraben that may be oestrogenic. Altogether, the published data provide moderate evidence of adverse effects linked to an estrogenic mode of action of isobutyl paraben.

The androgenic activity of isobutyl paraben has been investigated to a lesser extent. An *in vitro* androgen receptor binding assay showed activity of isobutyl paraben (Kim *et al.* 2010). On the other hand isobutyl paraben does not appear to affect testosterone levels in *in vivo* studies. No effects on testosterone levels were found in male offspring at weaning or as adults after developmental exposure (Yang *et al.* 2016; Kawaguchi *et al.* 2009b) and no effects on testosterone levels were seen in males in a 28-days repeated dose toxicity study with dermal exposure (Kim *et al.* 2015). Adverse effects on sperm motility and sperm numbers in male pups (Yang *et al.* 2016) suggest an endocrine mode of action of isobutyl paraben that may be anti-androgenic. All in all there is weak evidence of adverse effects linked to an anti-androgenic mode of action of isobutyl paraben.

Few studies investigated effects of isobutyl paraben on thyroid hormones *in vivo*. A single study showed effects on serum T4 at PND41 in a female pubertal assay (Vo *et al.* 2010), whereas other studies showed no effects on TT3, TT4 or T3 levels in a 28-days repeated dose toxicity study with dermal exposure and in young and adult pups from a developmental toxicity study (Kim *et al.* 2015; Kawaguchi *et al.* 2009b). The role of isobutyl paraben on the thyroid hormones is unclear and the changes in T4 levels found by Vo *et al.* (2010) may be a chance finding or due to a possible litter effect. Thyroid hormones are known to be important for brain development and the long-lasting effects on behaviour reported by Kawaguchi and co-workers (2010 and 2009a) showing impaired social recognition in female offspring (Kawaguchi *et al.* 2010) could be linked to thyroid disruption. The mechanisms underlying the behavioural changes were not investigated and the link between these effects and thyroid disruption is unclear.

In summary, data provide strong/moderate evidence of an estrogenic mode of action of isobutyl paraben. Adverse effects on sperm motility and sperm numbers in male pups and effects on sexual dimorphic behaviour suggest an endocrine mode of action of isobutyl paraben that may be oestrogenic. Thus, there is weak to moderate evidence for a causal link. Altogether, the published data provide moderate evidence of adverse effects linked to an endocrine (estrogenic) mode of action of isobutyl paraben.

In conclusion, isobutyl paraben does meet the WHO definition of an endocrine disruptor with an estrogenic mode of action and with a possible anti-androgenic and thyroid disrupting mode of action.

Table 1. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of isobutyl paraben.

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Yang et al. 2016		Decreased 17 β -estradiol in male pups after developmental exposure to isobutyl paraben.	Medium	Weak
Kim et al. 2012	Expression of the ER reporter gene and expression of CaBP-9k gene and protein was upregulated in all tested doses of isobutyl paraben. The PR gene was upregulated in all the tested doses of isobutyl paraben but only the highest dose (10 μ M) upregulated expression of PR protein in GH3 rat pituitary cells.		Medium	Moderate
Kim et al. 2011	22000 fold lower estrogenic activity of isobutyl paraben compared to 17 β -oestradiol in a stably transfected transcriptional activation assay in a hER α -HeLa-9903 cell line.		Medium	Weak
Kim et al. 2010	The AR binding affinity relative to dihydrotestosterone was almost 17000 times lower for isobutyl paraben in an androgen receptor binding assay.		Medium	Weak
Vo et al. 2010	Isobutyl paraben had affinity to ER α and ER β , but did not appear to have a preference to one of the receptors.	Serum levels of T4 were decreased in the low-dose group but not at higher doses.	Low	Weak-weak
Koda et al. 2005		Increased wet and blotted uterus weight in uterotrophic assay with s.c. exposure in ovariectomised rats (n=6/group)	High	Strong
Dabre et al. 2002	Estrogenic activity was seen on binding to ER α , upregulation of oestrogen regulated genes in MCF-7 cells and on oestrogen regulated proliferation in MCF-7 and ZR-75-1 cell lines.	Increased uterus weight (relative to body weight) in uterotrophic study in immature female rats (n=7/ group) with s.c. exposure.	High	Strong

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Okubo et al. 2001	Increased proliferation of MCF-7 cells, which was suppressed by the antioestrogen ICI 182,780 interacting with ER, suggesting an estrogenic mode of action of isobutyl paraben. Gene and protein expression of ER α was decreased and gene expression of PR was increased by isobutyl paraben. Isobutyl paraben had 1000-fold lower affinity to human ER α and ER β compared to the positive control DES.		High	Strong

ER: estrogen receptor; AR: androgen receptor; T4: tetra-iodothyronine; PR: progesterone receptor; s.c.: subcutaneous; DES: diethylstilbestrol

Table 2. Overview of potential endocrine-related adverse effects of isobutyl paraben.

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Yang et al. 2016	Rat, n=3 litters/group	Sperm count and motility was reduced by isobutyl paraben in adult male pups after developmental exposure to isobutyl paraben.	Low - Medium	Weak
Kim et al. 2015	Rat, n=10/group	No effects were seen on hormone levels or organ weights in adult rats exposed dermally for 28 days.	Medium	Weak
Kawaguchi et al. 2010	Rat, n=5-6	Female offspring exposed perinatally to isobutyl paraben showed impaired social recognition compared to controls.	High	Strong
Vo et al. 2010	Rat, n=10/group	At all doses myometrial hyperplasia was seen in uteri and in ovaries a decreased number of corpora lutea, increased number of cystic follicles and thinning of the follicular epithelium was apparently observed histologically.	Low	Weak
Kawaguchi et al. 2009a	Rat, n=8/group	Male offspring spent shorter time in the open arms of the elevated plus maze and showed decreased performance in the passive voidance test after developmental exposure to isobutyl paraben.	High	Strong
Kawaguchi et al. 2009b	Rat, n=7-8 litters	Plasma corticosterone levels in dams were decreased after isobutyl paraben exposure and uterus weight was increased.	High	Weak

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REACH registration dossier: <https://echa.europa.eu/da/registration-dossier/-/registered-dossier/17752>
(Link used in November 2017)

Hexachlorophene (HCP), CAS no. 70-30-4

Synonyms: HCP

The substance Hexachlorophene (Figure 1) is a white free-flowing odorless powder. The powder is insoluble in water and denser than water. It is an organochlorine compound used as a detergent cleanser, and it is an antibacterial emulsion for topical administration. It has bacteriostatic action against staphylococci and other gram-positive bacteria. Cumulative antibacterial action develops with repeated use. Cleansing with alcohol or soaps containing alcohol removes the antibacterial residue. Used as a surgical scrub and a bacteriostatic skin cleanser. It may also be used to control an outbreak of gram-positive infection where other infection control procedures have been unsuccessful. It is also used in agriculture as a soil fungicide, plant bactericide and acaricide. Since no registration dossiers on Hexachlorophene are available, it is assumed that it is produced and/or imported to EU in tonnages less than 100 tpa

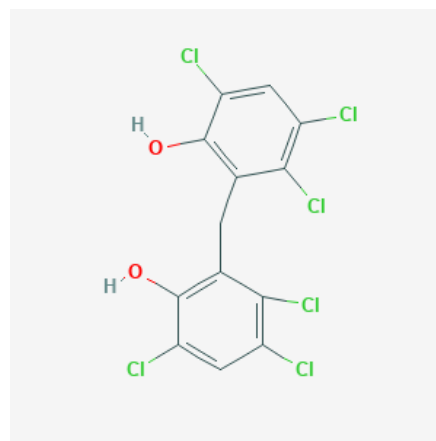


Figure 1: 2D structure from PubChem

4. Human health hazard assessment

4.10.3 Endocrine disruption

4.10.3.1 General approach – human health

4.10.3.2 *In vitro* information indicative of endocrine activity

Jung et al. (2004)

Summary: This study investigated the anti-estrogenic effect of 50 chemicals, including hexachlorophene (HCP), using 3 *in vitro* assays. First the yeast two-hybrid assay with the rat estrogenic receptor (ER) α and the coactivator TIF2 (transcriptional intermediary factor 2) was used to investigate the anti-estrogenic activity of HCP at concentration ranging from 10^{-3} to 10^{-9} M. Toxicity of HCP in the assay was assessed and found at concentrations of $>5 \mu\text{M}$. Next, the inhibitory effect of HCP on the transcriptional activity of 17β -estradiol was tested in a reporter gene assay system using ER-positive human breast cancer cells (MCF-7). Finally, the binding activity of HCP to human ER α was examined in a competitive binding assay. HCP showed a dose-dependent anti-estrogenic activity against rat ER α in the two-hybrid assay at concentrations below toxicity ($\text{IC}_{50}=1 \mu\text{M}$). This result could not be confirmed in the MCF-7 reporter gene assay at concentrations below toxicity. In the competitive binding assay, however, it was found that HCP can bind to human ER α .

Study quality and assessment: The study is well-described although purity and CAS number of the substance used could not be found. The number of experimental replicates and tested doses are not specifically given in the material and methods section but can be found in the figures. Overall, the study is assessed to be of high quality, and provides moderate evidence for an anti-estrogenic MoA of HCP in multiple *in vitro* screening systems.

4.10.3.3 *In vivo effects with regard to an endocrine mode of action*

ErDOS et al. (2013)

Summary: This study aims to evaluate the suitability and performance of circulating Inhibin B as a biomarker of seminiferous tubule damage in rats. In that context HCP was administered as one of eight testicular toxicants - only the data collected regarding HCP will be addressed in this summary. HCP (Sigma-Aldrich; CAS#70-30-4; purity by NaOH titration 99,1 %; lot#053K3603) was administered by oral gavage to male Wistar Han rats (n=15 / age 8-10 weeks) at doses of 0 or 25 mg/kg/day for 4 or 9 days. A higher dose group (n=4-9) received decreasing doses of HCP due to toxicity, starting with 50 mg/kg/day on day 1 and 2, 40 mg/kg/day on the 3rd day and 30 mg/kg/day on day 4-9. On day 5 or 10 plasma samples were collected for Inhibin B analysis and necropsy was conducted. Data was collected from terminal body weight, histology of testicular tissue (seminiferous tubule degeneration, degeneration/necrosis of the round and elongated spermatids, seminiferous tubule vacuolation and spermatogenesis) and ELISA was used to determine plasma concentrations of Inhibin B. The study result for HCP showed a statistically significant correlation ($p < 0.05$) between decreases in Inhibin B and seminiferous tubule toxicity but only observed at the early time points. In the animals from the HCP study exposed to 25 mg/kg/day brain lesions of white matter degeneration was observed in 5/5 in the postmortem examination and significant adverse physical signs was noted.

Study quality and assessment: Although the effect of HCP on testis was not the main focus, the study is well-described and thorough and is assessed to be of high quality. It is unsure if the observation regarding white matter degeneration and the adverse physical signs could have an unknown effect on inhibin B levels. On the basis of that, the study provides a weak evidence for an ED MoA of HCP.

James et al. (1980)

Summary: This study aimed to examine the spermatogenesis in Dawley rats (n=30) and Beagle dogs (n=4) after oral exposure to HCP. At the time of study no literature was available for a suitable HCP dose for dogs so a dose-range study was conducted on 4 sexually mature male beagle dogs. On the basis of that study a dose of 3 mg/kg/day was used in the primary study. For the canine part, HCP was administered by gelatin capsules at the level of 3 mg/kg/day for 9 weeks, no additional groups and no control group. Two dogs was killed after 9 weeks and 2 dogs were retained for additional 13 weeks after last dose and no HCP was administered. The rats were exposed to HCP by oral gavage at levels of 0 mg/kg/day (control), 5 mg/kg/day for 9 weeks. In each group 5 rats was killed after 4 and 9 weeks and 5 rats were retained for 13 weeks after last dose and no HCP was administered. For the canine study length and breadth of testis were measured and semen collected, starting pre-dose with one measurement then 2, 4 and 8 weeks of exposure and finally after 4, 8 and 12 weeks in the withdrawal period. Ejaculates were examined for Vol., motility, density, sperm concentration and morphology. The concentration of Na⁺ and K⁺ ions, alanine aminotransferase, aspartate aminotransferase and acid phosphatase was determined in each sample of seminal plasma. Canine luteinizing hormone (LH) was assayed using a heterologous ovine double antibody system. For the rat study sera were obtained after 1, 4 and 9 weeks of exposure and in week 13 of the withdrawal period. Serum testosterone concentrations, Follicle-stimulating hormone (FSH) and LH were assayed. For both species High-power light microscope was used for visual germ cell count of type B spermatogonia, pachytene spermatocytes, early or late spermatids and sertoli cells. In addition to macroscopic post mortem evaluation, the pituitary glands, testis and secondary sex organs were weighed and preserved for histological examination. The results detected no clinical change in pituitary-testicular relationship and the semen production of dogs was unaffected. In the counting of

germinal cells in the seminiferous tubules, some statistically significant differences ($P < 0,05$) were found for both dogs and rats. For dogs, decreased numbers of B spermatogonia were recorded after 9 weeks of exposure. For rats a decrease in the number of all germinal cell types counted was apparent at 4 weeks examination, but not at 9 weeks. After 13 weeks of HCP withdrawal, no effect on cell counts was apparent in dog or rat.

Study quality and assessment The description of this study has some shortcomings for example are there no control group for the dog study and even though it is mentioned in the text, that nothing was found, there is no presentation of the values measured regarding the concentration of Na^+ and K^+ ions, alanine aminotransferase, aspartate aminotransferase, acid phosphatase and FSH/LH assays. For the chemical used no report of the purity and CAS no. could be found. The study is assessed to be of medium quality and it provides a weak evidence for HCP's adverse effect on the spermatogenesis in rats and dogs

Gellert et al. (1978)

Summary: This study investigates effects on mating behavior and prostatic development in Sprague-Dawley rats after exposure to HCP (3 %) by topical application. The study was separated into a female and a male part. In both studies the rats were lathered for 10 minutes a day through day 1- 8 of their life. The wash was conducted with pHisoHex, 3% HCP (n= 25), pHisoDerm, 0% HCP (control, n=7) or they remained unwashed 0 % HCP (control, n=8). All pups were kept separated 1.5 h after exposure to minimize ingestion of HCP due to nuzzling and licking on the skin. In the female part following parameters were noted; Onset of puberty by vaginal opening, estrus cycle by vaginal smear, fertility and delivery of normal pups. Body weight and changes in pituitary, ovary and adrenals were noted post mortem. All female rats were sacrificed at the age of 6 month. In the male study the mating behavior and fertility was assessed at several ages. At 7 month of age each male were placed with two normal females (proven fertility) in a period of two weeks. Progeny was counted, sex determined at birth and mortality up to 5 days was noted. At 9,5th month of age each male were placed with two primed (100 µg estradiol benzoate, 48 h before testing) females for two consecutive overnight cohabitation periods. Conclusion of intromission and/or ejaculation was made if a vaginal plug, sperm or a bloody vaginal smear were registered. At 11 month of age, each male was placed with a primed female for a test-period of 35 min. Number and frequency of behaviors (mounting, intromission and ejaculation) was noted. Heart blood was taken under light ether-anesthesia and a radioimmunoassay for testosterone was carried out. At 12th month of age all rats were orchidectomized and a saline smear was made from the cut surface of the caudal epididymis to evaluate motile sperm. The weight of testis was noted. At 13th month of age all rats were sacrificed, weight of the adrenals was noted and selected portions of the internal genitalia were removed and fixed for histologic examination. During the exposure period, all pups washed with pHisoHex showed ataxia and tremor by the age of day 9 and increased mortality by day 14 . No clinical signs or mortality was seen in the control groups (pHisoDerm and unwashed). The result from the female study revealed no changes in onset of puberty, estrus cycle, fertility, pituitary, ovary, adrenals and body weight in any group. In the male study the two control groups were combined for statistical analysis of data, since no significant differences existed between them. Neonatal male rats exposure to pHisoHex, 3% HCP showed inhibition of reflex ejaculation, infertility and prostatic cysts and fibrosis. Spermatogenesis was not affected.

Study quality and assessment :

In general, the study is well-described, although information regarding the number of rats in the control group of the female study and housing condition would have been preferred. No report of the purity and CAS no. of the used chemical could be found. The study is assessed to be of medium quality and provides no evidence for adverse effect on female reproduction and a moderate evidence for effects on mating behavior and prostatic development in male rats.

Kennedy et al. (1976)

Summary: This study evaluated the effects on reproductive performance, perinatal and postnatal development of rats in two parts. In the first part, COBS random bred albino rats were used to investigate the effects on reproduction. HCP (G-11 brand) was administered by gavage as a 3% suspension in 1% aqueous methylcellulose to three groups (n=30) at levels of 0 mg/kg/day, 5 mg/kg/day, 10 mg/kg/day. For males the administration of HCP started 63 days prior to mating and females received the compound 14 days before mating and continued until weaning of the litter at day 21. Mating was done at the age of 100 days by rotation of males within each group. Parameters registered were fertility, mating behavior, pregnancy, length of gestation and lactation behavior. At day 14 of the gestation 10 females from each dose group were killed and examination of the ovaries, uteri and fetus were conducted. The remaining females continued until weaning at day 21. Registration of weight was obtained at 0, 1, 4 and 21 days after delivery, a complete examination for external defects was conducted. In the second study regarding peri- and postnatal development HCP was administered orally by gavage to three groups (n=20) at levels of 0 mg/kg/day (control), 15 mg/kg/day, 30 mg/kg/day. Exposure started at day 15 of gestation and continued throughout lactation. Parameters examined were maternal body weight, length of gestation, number of progeny, death, behavior and survival rate. Body weight was assessed on day 0, 1, 4 and 21 after birth. The result from the internal examination at day 14 of gestation showed an increase in resorption sites in females receiving HCP 10 mg/kg/day. In the same dose group, pups surviving the later stage of lactation were reduced. In the group exposed to HCP at levels of 30 mg/kg/day maternal toxicity (weight loss) was observed and a pharmacotoxic response of hindlimb weakness and severe hypo-activity with a following comatose stage was observed in two animals. In the same group the number of viable pups delivered was decreased and the number of stillborn pups increased.

Study quality and assessment. The study is well-described and thorough, although the study is old it provides information on housing, number and age of animals. The CAS no. and purity of the HCP compound was not reported. Overall, the study quality is assessed to be high and it provides moderate evidence of the adverse effect on the reproductive performance (resorption sites, viable and stillborn pups) and the peri- and postnatal development of rats.

Kennedy et al. (1975)

Summary: This study investigated the influence of HCP on reproduction in rats (albino CD strain) after exposure by feeding. HCP was administered to 4 groups (n=24/dose) in the daily feeding at levels of 0 ppm, 12.5 ppm, 25 ppm and 50 ppm. All dose-groups started on the experimental diet at the age of 21 days. Preparation of diet was as followed; HCP acetone solution was added to a commercial ration to reach the final concentration of 12.5 ppm, 25 ppm and 50 ppm. Diet was available *ad libitum* until sacrifice and prepared fresh on a weekly basis. At the age of 100 days the F₀ generation was

housed for breeding with two females per male. F_{1A} were retained for 21 days. The female rats of F₀ were allowed 10 day rest period before mating again. At weaning of the F_{1B}, 8 males and 16 females were randomly selected for breeding of F₂. This procedure continued for 3 successive two litter generations. Registrations were obtained for weight gain, gestation time, no. of viable and stillborn progeny and external physical abnormalities. At sacrifices, examination for gross pathological changes was done on all parental animals and tissue was retained for histologic examination. The result of the study revealed no change in any parameters measured.

Study quality and assessment: The study is generally well-described but information about the absolute exposure doses of HCP are not explicitly stated for the individual animal. The compound was administered as a component in the diet and all tested animals had *ad libitum* access to the feeding - body weight gain was registered to be normal but no information on food consumption is described. The material and method section does not report CAS no and purity of the chemical used or information on housing conditions. The study is assessed to be of low quality and since no change was measured in any parameter it provides no relevant evidence for the adverse effect of HCP on reproduction.

Kimmel et al. (1974)

Summary: This study investigates the teratogenicity of HCP in pregnant rats (Charles River strain). The study consist of two parts, the first part investigate the effect on the developing rat embryo after absorption of HCP from the vaginal mucosa, the second part covers the kinetics of HCP by comparing the uptake, distribution and excretion of HCP labeled with radioactive carbon after oral, dermal and intravaginal administration. The first part will be of concern to this summary. In the study mating of rats were conducted and day 0 of gestation was designated by vaginal presence of sperm. At the 7th day of gestation, 0.2 ml vehicle (control, n= 12) , 300 mg/kg (45%) HCP suspension (n=12), 80 mg/kg (12%) HCP suspension (n=5), 20 mg/kg (3%) HCP suspension (n=6) or commercial 3% HCP (n=6) was inserted into the vaginal lumen under ether anesthesia. A gaze plug was inserted into the vagina and the orifice was partially closed by suture. An additional 0.2 ml of HCP or vehicle was applied by tuberculin syringe on day 8, 9 and 10 of gestation and at the 11th day the plug was removed. All animals were killed at day 20 of gestation. Parameters registered for the maternal rats were implants and resorption sites, weight and gross abnormalities of fetus. Two thirds of the fetuses were fixed in alcohol and stained with alizarin red and parts (brain and eyes) were prepared by routine for histology. The result revealed an apparent dose-response relationship in the rate of offspring malformations (urogenital defects like chryptorchidism and missing uterine horn, hydrocephaly, anophthalmia, microphthalmia and wavy ribs). In rats exposed to 45 % suspension almost half the surviving fetuses were affected. The group exposed to 12 % showed less teratogenic effect and the 3 % group showed no effect. In the two groups of highest concentration, the pregnant rats developed a moderate to severe toxicity during treatment (weakness, weight loss and diarrhea).

Study quality and assessment: It is an old study but it is well described and thorough. The CAS no. and purity for the chemical used was not reported and information regarding housing conditions would have been preferred. Overall the study is assessed to be of medium quality and it provides a strong evidence for the teratogenic adverse effects of HCP. For some of the malformations produced, i.e. urogenital defects like chryptorchidism and missing uterine horn, there may be a plausible link to an ED MoA. Thus the study provides weak evidence for endocrine-related adverse effects.

Frederic R. Alleva (1973)

Summary: The aim of this study was to evaluate the effect of HCP on reproduction in hamsters (Lakeview strain) after exposure of a near lethal dose. HCP was dissolved in Mazola corn oil and the solution was injected subcutaneously (SC). A dose-range study on HCP lethality was conducted prior to the main study where male and female neonatal hamsters were exposed to HCP doses at 0, 1.56, 3.12, 6.25, 12.0, 12.5, 24, 25 mg/kg, adm was subcutaneous injection at the age of 0 or 2 days. It was concluded that the maximal non-lethal dose of HCP in neonatal hamsters were 6 mg/kg bw. In the main study male and female groups were exposed to a single dose at different age (0, 2, 4 and 12 days). The compound was administrated at three levels (0, 3 or 6 mg/kg) with two control groups (untreated or only oil); untreated (control, n=14 oil with 0 mg/kg/0 days of age (control, n=11), oil with 0 mg/kg/2 days of age (control, n=7), 3 mg/kg/0 days of age (n=31), 3 mg/kg/2 days of age (n=25), 3 mg/kg/4 days of age (n=34 3 mg/kg/12 days of age (n=5), 6 mg/kg/0days of age (n=12), 6 mg/kg/2 days of age (n=19), 6 mg/kg/4 days of age (n=20 and 6 mg/kg/12 days of age (n=2). Two additional groups received three SC injections of 3 mg/kg at the age of 0, 1 and 2 days (n=46 the dose was administrated as one injection each day. At the age of 20 days vaginal washing were obtained daily and puberty was assigned when a 4-day cycle of the vaginal discharge signaling ovulation was found. Mating was done at the age of 85-88 days between similarly treated partners and fertility was considered successful when delivery of pups was registered. The result showed no effect on fertility and body weight and for the female groups there was no effect on time of puberty and estrus cycle regularity.

Study quality and assessment: Only very limited written information was presented in the article and all details of the experiment were outlined in 3 tables. No purity or CAS no. for the chemical could be found and more information on housing condition would have been preferred. Overall the study is assessed to be of medium quality and since the study did not find any effect it provides no relevant evidence for adverse effects on reproductive after exposure to HCP.

Gaines et al. (1973)

Summary: This study evaluated the effect of HCP in five different setups. Three of them are of relevance to reproduction The aim of the three reproduction studies was to evaluate the toxicity after oral exposure regarding to the size of litter, survival to weaning, body wt., brain lesions and determination of HCP excreted in the milk of lactating rats. Study 1- a two- generation study, F₀: HCP was administrated to the groups (n=20) in the diet at levels of 0 (control, 20 and 100 ppm starting from the age of 4-5 weeks. All rats were euthanized after 258 days of exposure. Breeding of F_{1a} was conducted after 54 days and breeding of F_{1b} after 166 days (all mating was done among rats of same dietary level). F_{1b} continue with the feeding like F₀ and 72 animals from F_{1b} were used for breeding of F₂ at the age of 95 days. F₁ was euthanized after 145 days of exposure and F₂ after weaning at the age of 21 days. Number of litters born and weaned, average no. per litter, survival (%) and mean body weight and organ weight were registered and tissues from selected organs was preserved for microscopy. Study 2 – Administration of HCP by oral intubating to female rats at the age of 100 days, during the organogenesis, day 7-15 of pregnancy. Five groups (n=8-10) were given 0, 1, 5, 10 and 20 mg/kg/day. Measurements were obtained for numbers of litters born and weaned, average no. per litter, survival (%) and body wt. Study 3 – Determination of HCP in the milk of lactating rats. Female rats (n=2 in each group) were exposed to HCP for 241 days by diet at levels of 0 ppm 20 ppm and 100 ppm Concentrations level was evaluated from a whole milk sample (2 ml) taken from the stomach of each pup (age 3-4 days) in the litter. Results from the two-generation study revealed that abnormal findings by microscopic examination of the organs were limited to the brain in

the group fed 100 ppm. Findings included small vacuolated areas in the white matter and mild status spongiosus. Reproduction was not affected in rats exposed to 20 ppm but in the group fed 100 ppm the survival to weaning in F1b was significantly reduced to 62.5 %. Reproduction data of the study where HCP was administered on day 7-15 of gestation, no still births was observed and pups was evaluated to be normal. Analysis of stomach content revealed 0.007 and 0.33 ppm in the milk of rats fed 20 ppm and 100 ppm respectively.

Study quality and assessment: The material and method section does not report the purity and CAS no. of HCP but it is mentioned that all tests were conducted with the same batch of USP grade. A description of housing conditions would have been preferred and only limited statistically significant results are presented in the figures. The absolute dose of HCP administered to the individual rat is a point of concern in the two studies where rats were fed by diet because the mixing technique was done by grinding using a mechanical mixer. The question about even distribution of HCP in the diet remains unanswered but weight gain was normal during the study. Overall, the study quality is assessed to be medium and it provides weak evidence of the adverse effect on reproduction (decreased pup survival) after HCP exposure.

Thorpe et al. (1969)

Summary: In this study HCP (2,2 methylene bis [3,5,6-trichlorophenol]) was tested in 8 month old rams. The aim of the study was to examine the effect on the liver and the seminiferous epithelium (SE) in testis. HCP (5% solution in arachis oil) was administered by stomach tube in all cases. HCP was given in three groups (n=3-6) as a single oral dose at the levels of 0 mg/kg (control), 25 mg/kg and 50 mg/kg. In each group a unilateral orchiectomy was performed under regional local anesthesia at intervals of 2, 7 and 21 days after exposure. Atrophy of the SE was registered at both HCP doses of 25 mg/kg and 50 mg/kg. Depletion of the SE was less obvious at day 7 post-exposure in the low concentration group compared to the high concentration group but lesions were indistinguishable in 75 % of the samples by day 21. Liver biopsy revealed periportal fatty changes in the groups that received multiple doses of HCP. Enzyme histochemical results suggested that the hepatic lesions were a direct result of the toxic properties of HCP.

Study quality and assessment: In general, the study is well-described, although more information on the housing conditions would have been preferred and there is no report of the purity and CAS no. of the tested chemical. The study is assessed to be medium quality and provides a moderate evidence for a toxic adverse effect in the testis and liver of rams after exposure to HCP.

Thorpe et al. (1967)

Summary: This study examines the pathological effect of HCP in male wistar rat (albino strain) using 3 different experiments. In the first study HCP was administered to 4 groups (n=20-30/group) as a single oral dose given by catheter at 0 mg/kg (control) 25 mg/kg, 75 mg/kg, and 125 mg/kg. A minimum of 4 rats from each group were euthanized at 1, 2, 5, 12 and 21 days post-dose. The second study tested the effect of 5 doses where HCP was administered to 3 groups (n=12) orally by catheter on 5 consecutive days at levels of 0 mg/kg (control), 25 mg/kg and 75 mg/kg and all animals were euthanized on the 5th day. In both studies, blocks of testis, liver, kidney, small and large intestine were fixed for microscopically examination. In the 3rd study the teratogenic effect in female rats were investigated. The results from the first two studies showed atrophy of the seminiferous epithelium

after exposure to a single dose of 125 or 5 days of exposure to 75 mg/kg and these results were in concordance with findings of reduced sperm in epididymides. Lesions were detected at the 2nd day post-dosing but were found to be reversible since the epithelium was observed to be restored by day 21 post-dosing. The third study, looking at the structure of the major viscera and skeleton of fetuses the results revealed no interference with pregnancy or development.

Study quality and assessment: The material and method section is not explicit, about the report of the purity and CAS no. of the tested chemical and the age of the tested animals and more information on the housing conditions would have been preferred. The study is overall assessed to be of medium quality. The lesions in the testis were evaluated quantitatively by the method of Chalkley (1943) and the results provide strong evidence for adverse effects on the seminiferous epithelium after exposure to HCP.

4.10.3.4 Summary of the plausible link between adverse effects and endocrine mode of action

To our knowledge one *in vitro* study (Jung et al, 2004) (Table 1) addressing the anti-estrogenic effect of HCP is currently available. This study is of high quality and showed a dose-dependent anti-estrogenic activity against rat ER α in the two-hybrid assay at concentrations below toxicity (IC₅₀=1 μ M). The result could not be confirmed in the MCF-7 reporter gene assay at concentrations below toxicity but in the competitive binding assay, however, it was found that HCP can bind to human ER α . Thus, there is moderate evidence for ED MoA, i.e. anti-estrogenic activity.

The majority of research regarding HCP consists of older publications and encounters several *in vivo* studies looking at the adverse effect of HCP exposure (Table 2). None of the *in vivo* studies have included known sensitive endpoints for anti-oestrogenic effects in females, but the studies provide strong evidence for adverse effects on testis histology manifested as seminiferous toxicity. Treatment with anti-estrogens can lead to lower sperm numbers, abnormal sperm morphology and reduced fertility in male rodents, changes also seen for ER α knockout mice (reviewed by Hess et al. 2003). The effects on testis and epididymides reported for hexachlorophene may thus be related to an anti-estrogenic mode of action. However, ER α receptors have been reported to be absent in human testis and epididymides and the link between the ER α activity observed *in vitro* and the adverse effects observed in the reproductive organs *in vivo* for hexachlorophene is weak. It should be noted, that the data on the ED modes of action (*in vivo* and *in vitro*) of HCP are scarce, and other mechanisms besides of ER α activity or other modes of action of the substance that could explain the adverse effects observed cannot be excluded.

In conclusion, hexachlorophene meets the WHO definition of a potential endocrine disruptor. According to the DK-EPA suggested ED criteria, hexachlorophene is assessed as a suspected endocrine disruptor.

Table 1. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of Hexachlorophene

Reference	MoA		Quality of study	Evidence for ED MoA
	<i>In vitro</i>	<i>In vivo</i>		
Jung et al. 2004	HCP showed a dose-dependent anti-estrogenic activity against rat ER α in the two-hybrid assay at concentrations below toxicity (IC ₅₀ =1 μ M). This result could not be confirmed in the MCF-7 reporter gene assay at concentrations below toxicity. In the competitive binding assay, however, it was found that HCP can bind to human ER α .		High	Moderate
Erdoş et al. 2013		The study result for HCP showed a statistically significant correlation ($p < 0.05$) between decreases in Inhibin B and seminiferous tubule toxicity but only observed at the early time points.	High	weak

Hexachlorophene (HCP), estrogen receptor α (ER α)

Table 2. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of Hexachlorophene (HCP)

Reference	Species, N	Adverse effects	Quality of study	Evidence for adverse effects
Erdos et al. 2013	n=15	Postmortem findings showed white matter degeneration in the brain in 5/5 rats. Histomorphologic testicular findings included seminiferous tubular degeneration	High	Weak
James et al. 1980	Rats n=30 Dogs n=4	Semen production of dogs were unaffected. In the counting of germinal cells in the seminiferous tubules, some statistically significant differences ($P < 0,05$) were found of both dogs and rats. For dogs, decreased numbers of B spermatogonia were recorded after 9 weeks of exposure. For rats a decrease of all cell types counted was apparent at 4 weeks examination. After 13 weeks of HCP withdrawal, no effect on cell counts was apparent in dog or rat.	Medium	weak
Gellert et al. 1978	Rats Female, n= 10-30 Male, n= min.10	During the exposure period, all pups washed with pHisoHex showed ataxia and tremor by the age of day 9 and mortality by day 14 was male=25% and female=13%. No clinical signs was seen in the control groups (pHisoDerm and unwashed) and mortality by day 14 = 0%. The result of the female study revealed no changes in onset of puberty, estrus cycle, fertility, pituitary, ovary, adrenals and body weight in any group. In the male study the two control groups were combined for statistical analysis of data, since no significant differences existed between them. Neonatal male rats exposure to pHisoHex, 3% HCP showed inhibition of reflex ejaculation, infertility and prostatic cysts and fibrosis. Spermatogenesis was not affected.	Mediun	no evidence for the female study Moderate for the male study

Reference	Species, N	Adverse effects	Quality of study	Evidence for adverse effects
Kennedy et al. 1976	Rats Female,n=120 Malen=30	The result from the internal examination at day 14 of gestation showed an increase in resorption sites in females receiving HCP 10 mg/kg/day. In the same dose group, pups surviving the later stage of lactation were reduced. In the group exposed to HCP at levels of 30 mg/kg/day maternal toxicity (weight loss) was observed and a pharmacotoxic response of hindlimb weakness and severe hypo-activity with a following comatose stage was observed in two animals. In the same group the number of viable pups delivered was decreased and the number of stillborn pups increased.	High	Moderate
Kennedy et al. 1975	Rats n=32-64	The study revealed no change in any parameters measured. It should be noted that the diet containing HCP was administrated <i>ad libitum</i> with no record of individual intake.	Low	None
Kimmel et al. 1974	Rats n=41	The result from the first part revealed an apparent dose-response relationship in the rate of offspring malformations (hydrocephaly, anophthalmia and microphthalmia). Rats exposed to 45% suspension affected almost half the surviving fetuses. The group exposed to 12% showed less teratogenic effect and the 3% group showed no effect. In the two groups of highest concentration, the pregnant rats developed a moderate to severe toxicity during treatment (weakness, weight loss and diarrhea).	Medium	High
Alleva, frederic R. 1973	Rats n= 106 - 120	After a single subcutaneous injection, the result showed no effect on fertility and body weight and for the female groups there was no effect on time of puberty and estrus cycle regularity.	Medium	None
Gainé et al. 1973	Rats females, n= 76-86 Males n= 30	Results from the two-generation study revealed that abnormal findings by microscopic examination of the organs were limited to the brain in the group fed 100 ppm. Findings included small vacuolated areas in the white matter and mild status spongiosus. Reproduction was not affected in rats exposed to 20 ppm but in the group fed 100 ppm the survival to weaning in F1b was significantly reduced to 62,5 %. Reproduction data of the study where HCP was administrated on day 7-15 of gestation, no still births was observed and pups was evaluated to be normal. Analysis of stomach content revealed 0,007 and 0,33 ppm in the milk of rats fed 20 ppm and 100 ppm respectively.	Medium	Weak

Reference	Species, N	Adverse effects	Quality of study	Evidence for adverse effects
Thorpe et al. 1969	Sheeps n= 15	In the testis study atrophy of the SE was registered at HCP rates of 25 mg/kg and 50 mg/kg. Depletion of the SE was less obvious at day 7 post-exposure in the low concentration group compared to the high concentration group but lesions was indistinguishable in 75 % of the samples by day 21. Liver biopsy revealed periportal fatty changes in the groups that received multiple doses of HCP. Enzyme histochemical results suggested that the hepatic lesions were a direct result of the toxic properties of HCP.	Medium	Moderate
Thorpe et al. 1967	Rats Female, n= 84 Male, n=122	The results from the first two studies showed atrophy of the seminiferous epithelium after exposure to a single dose of 75 mg/kg. Lesions was detected at the 2 nd day post-dosing but were found to be reversible since the epithelium was observed to be restored by day 21 post-dosing. In the third study, looking at the structure of the major viscera and skeleton of fetuses the results revealed no interference with pregnancy or development.	Medium	Moderate

Hexachlorophene (HCP), seminiferous epithelium (SE)

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