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.

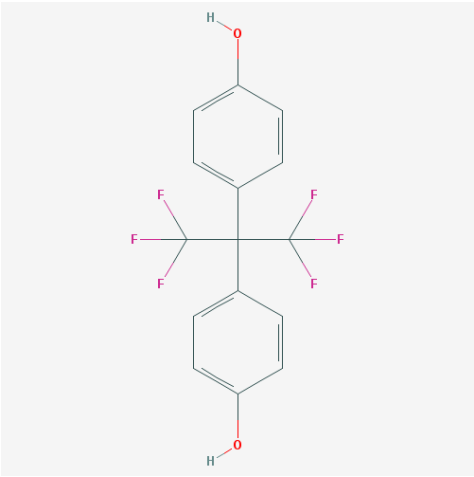

Figure 1. 2D structure from PubChem

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 µM 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 µM 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 µM for 24 h. The production of ROS was measured after cells were exposed for 24 h to concentrations of BPAF from 0.01-50 µM. Calcium levels were measured after 24 h exposure to 0.00001–10 µM BPAF. At the same time, the estrogenic and thyroidal hormone receptor-mediated potentials (i.e., ERα and TRα 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 µM), but caused a significant decrease in cell viability at higher exposure concentrations (25-100 µM). BPAF increased LDH activity (25, 50 and 100 µM, i.e. at cytotoxicity levels), and caused increased DNA-damage (10, 25 and 50 µM) in MCF-7 cells. BPAF also resulted in a concentration-dependent increase in both ROS production (0.01-25 µM, i.e. below cytotoxicity) and intracellular Ca(2+) levels (0.0001-10 µM). The two-hybrid yeast bioassay showed that BPAF is an estrogen receptor α (ERα) (EC50 = 5.3 µM) and thyroidal hormone receptor α (TRα) (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 at0, 0.1, 1, 5, 10, 25 (≤ cytotoxicity level) µM (read from the relevant figures:) with exposure time of24, 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 µM). 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).

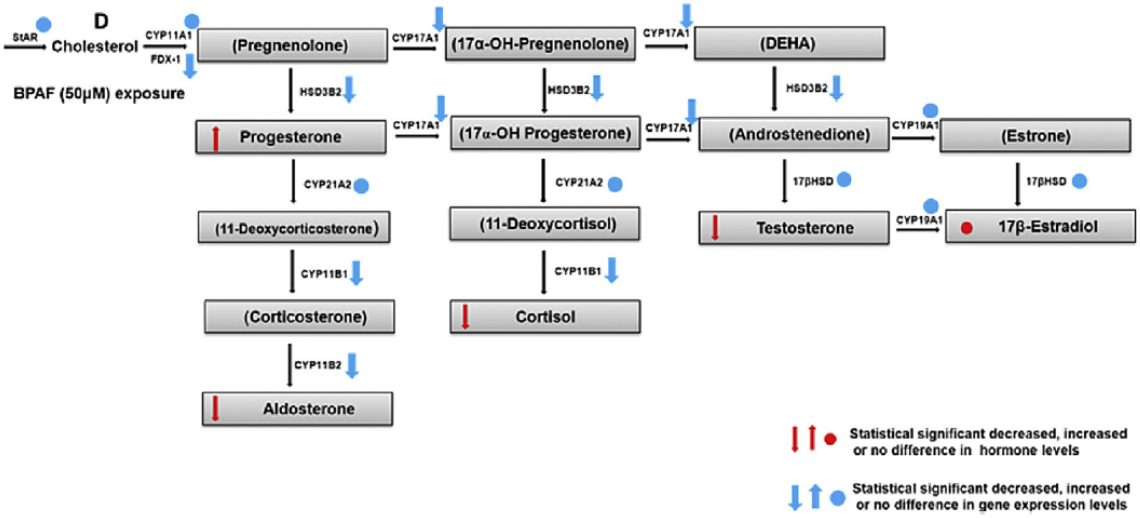

Figure 2. Overview of the steroidogenesis and the effect of 50 µM BPAF exposure (from Feng et al 2016)

Gene expression was significantly reduced for FDX1 (50 µM), CYP17A1 (1, 10, 30 and 50 µ), 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 (ERα)-positive T47D and MCF7 cells (0, 1 nM, 10 nM, 100 nM, 1 µM and 10 µM), and ERα-negative MDA-MB-231 cells (0, 100 nM, 1 µM and 10 µM) (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 µM 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 µM for 24 h), gene-silencing with small interfering RNA (siRNA) against ERα, ERβ and G protein-coupled estrogen receptor 1 (GPER) was used in T47D and MCF7 cells. In addition, the inhibition of BPAF-induced (1 µM for 24h) ER activity was studied using ICI 182780 (1 µM, ERα and ERβ antagonist) in the ERα-positive T47D and MCF7 cells. The ERα-negative MDA-MB-231 cells were transfected with an adenovirus overexpressing ERα, 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 µM) 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 ERα-negative MDA-MB-231 cells. A BPAF dose of 1 µM 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 ERα, ERβ and GPER by siRNA revealed that BPAF-induced endogenous transcription was dependent on ERα and GPER, implying that both genomic and non-genomic estrogenic pathways might be involved in the endogenous transcription induced by BPAF. ERα-mediated gene transcription was confirmed by inhibition of ER activity using ICI 182780 in theT47D and MCF7 cells as well as in MDA-MB-231 cells overexpressing ERα. 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 ERα 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 ERα and GPER.

Li et al. (2013)
Summary: The study examines the estrogenic receptor (ER) α and ERβ 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 ERα and ERβ via the luciferase reporter assay using the 2 luciferase reporters, 3xERE and pS2ERE. Next, Ishikawa cells stably expressing ERα 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 ERα 3xERE and pS2ERE in both HepG2 and Hela cells, and in the HepG2 cells it also induced ERβ 3xERE and pS2ERE responses.

BPAF (100 nM for 18 h) significantly induced endogenous ERα target genes (*PR, pS2, GREB1, SPUVE, WISP2, and SDF-1*) in the Ishikawa/ERα 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 ERα agonist and a potential ERβ 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 (ERα) and androgen receptor (AR). For this transient transfection experiments with full-length ERα 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 ERα functional assay, CV-1 (ERα negative) or HepG2 cells were bulk transfected with pRST7-ERa, 3X-ERE-TATA-luc (reporter gene to measure the transcriptional activity of ERα), 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 ERα 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 ERα 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 ERβ and how this mechanistically differs from that of 17β-estradiol (E2). First, the bisphenols ERα transcriptional activity and cell proliferation effect was examined using human breast cancer ERα-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 ERα and ERβ (HELN ERα and ERβ 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 ERα or AF-1 deleted ERα. The capacity of bisphenols to induce co-activator recruitment to ERα AF-2 was studied next by measuring the interaction of fluorescein-labelled SRC-1 (co-activator) with the ERα-LBD (ligand binding domain, containing AF-2). The effect of bisphenol on H12 (helix 12) active conformation stability was also studies, 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 ERα-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 ERα agonist on cell proliferation. In the HELN assays, BPAF (and BPA and BPC) was activating ERα with a 60-70% potency of E2. BPAF also exerted ~60% potency on ERβ. 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 ERα and ERβ 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 ERα-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 crystalized with BPA or BPAF the ERα-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 ERβ. To evaluate estrogen response element (ERE)-mediated transcriptional activity of ERα and ERβ, 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 E_2 co-treatment. The specific effects of BPAF (100 nM) on ERα functionality was analysed using wild-type (WT) ERα and specific ERα 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 ERα 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)-ERα were used. First the expression of ERα and the ER ERE-mediated activation was confirmed in the Ishiwaka/ERα cells. Then western blot was performed to identify phosphor-p44/42 MAPK and other kinases in the vector and ERα 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/ERα cells was examined. Finally, ERα-dependent responses to BPAF was examined by detecting endogenous gene expression of PR, and 3 other ER target genes in Ishiwaka/ERα cells using real time PCR.

For ERα, 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 ERβ, 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 ERα activation in any of the cell lines, but ERβ 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-ERα mutants, indicating that BPAF do not mediate tethered or AF-1 activation of ERα. It was also found that both co-activators, SRC-2 and p300, resulted in an enhanced BPAF-induced ERα 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 ERα target genes PR and GREB1. Together, these results indicate that BPAF can function as an ER agonist and antagonist, and activates ERα 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 CH_3 by CF_3 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 characterize 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 expresses 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,

and 10 µM) 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 ERRγ. Furthermore, BPAF receptor-binding activity was 3 times stronger for ERβ than for ERα. When examined using the functional luciferase reporter gene assays, BPAF was a full agonist for ERα 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 ERβ. When tested in ERβ antagonist mode, BPAF acted as a distinct and strong antagonist against the ERβ 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 ERα agonism and ERβ 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 17β-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 form 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, ≥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 pmm 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 ERα-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* ERα 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 P450scc 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, P450scc, 3β-HSD, CYP17α, 17-beta-hydroxysteroid dehydrogenase (17β-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 (P450scc, 17β-HSD). Similarly, the testicular mRNA levels of inhibin B, ERα and LHR also decreased in rats given a dosage of 200 mg/kg/d BPAF. Protein expression of SR-B1, StAR, and P450scc 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. Tree 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 µg/kg/day) for anti-estrogenic assay mode. BPAF was a hERα-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 ethynyl estradiol (EE, dose: 0.6 µg/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 Hersberger 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 ERα agonist, an ERß 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 PBAF was tested in the antagonistic mode with co-administration of EE. The effects on uterus weight in this assay are known to be primarily ERα-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 TRα 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
	In vitro	*In vivo*		
Lei et al. (2017)	ERα and TRα 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)	ERα-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 ↑ uterus weight and histological changes	Medium-high	Strong
Feng et al. (2016)	Effects on steroidogenesis in the H295R cell line: ↑ progesterone, ↓ 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 ↑ and inhibin B levels ↓ 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, ERα and StAR, P450scc. 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 ERα and GPER in human breast cancer T47D and MCF7 cells		Medium	Strong
Li et al. (2013)	ERα agonism and potential ERβ agonism in HepG2, HeLa and Ishikawa/ERα cells		Medium	Strong
Teng et al. (2013)	ERα 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)	ERα and ERβ activity on MCF7 and HeLa cells, and mechanism studies on the interaction with ERα		High	Strong
Feng et al. (2012)		Study in adult male rat (n=6/group): ↑testis/BW ratio, serum total cholesterol and testosterone ↓, and LH and FSH ↑, plus testis gene expression of StAR, P450scc, 17ß-HSD, SREBP-1c, ERα, LHR, Inhibin B and SR-B1 ↓. Testes protein levels of StAR, P450scc and SR-B1 ↓. 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 ERα agonism and ERβ antagonism in transfected HeLa cells		Medium	Strong
Akahora et al. (2008)	Active for hERα-LBD binding	Uterotrophic study in immature female rats (n=6/dose group), s.c.: ↑ uterus weight in estrogenic mode and ↓ 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.: ↑ uterus weight in estrogenic mode and ↓ uterine weight in the EE co-administered anti-estrogenic mode	High	Moderate
		Hershberger study in adult castrated male rat (n=6/dose group), oral: ↓ BC/LA weight in androgenic mode and ↑ 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 (ERα), thyroidal hormone receptor α (TRα), 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	*In vivo* 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 (ERα, ERβ1, and ERβ2) 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 µM) affected the survival rate and motility and teratogenicity was not observed. Exposure of zebrafish larvae between 1-7 dpf to BPAF (1 µM) 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 zfERα, zfERβ1, or zfERβ2, 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 ERβ1 or ERβ2, 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 ERα-containing cells. Taken together, the reporter gene assay provides evidence that BPA, BPF, and BPAF are ERα 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 (zfERα, zfERβ1, and zfERβ2). In perfect agreement with the reporter gene assay, they found that BPA, BPF, and BPAF bind *in vitro* to ERα, 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 ERα, 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 µg/L, SMX 5.6 µg/L or BPAF 24.7 µg/L + SMX 5.6 µg/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 *tshβ* 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 "µg/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*, *sclc5a5*, *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 *trα* 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 assay[1]. 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 adverse endocrine-mediated endpoints 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.

Adverse effects like skewed sex ratio have been documented in fish species after BPA exposure BPA (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 available information (Table 3) BPAF is not an endocrine disrupting compound according to the IPCS/WHO definition. Studies investigating population relevant endpoints in non-mammalian vertebrates are unavailable at present, but because a clear ER-mediated MoA is established in *in vitro* and *in vivo* studies BPAF should be considered as a suspected endocrine disrupting compound.

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**
	In vitro	*In vivo*		
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 *in vitro*. BPAF has a higher binding affinity compared with BPA.	The induction of the *cyp19a1b* gene (aromatase B) by BPAF exposure was investigated by RT-qPCR, in situ hybridization and a *cyp19a1b*-GFP zebrafish, and showed that exposure to BPAF induced the cyp19a1b 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 *D. magna* significantly but MoA was not investigated	Medium	Weak
Yang et al. (2016)		Increased female T3 levels and increased male E2 levels and vtg 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 *vtg1* expression in male liver tissue at the two highest concetrations. The expression of several genes in the steroidogenic pathway was affected.	Medium-high	Moderate

Reference	MoA		Quality of study	Evidence for ED MoA
	In vitro	*In vivo*		
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)	*In silico*: Docking simulations revealed that BPA and BPAF are agonists of ERα LBDs in both the medaka and the common carp but the medaka ERα is more sensitive to binding of BPs.	Short-term exposure (8 hours) of adult male medaka to BPAF (0.05-5 µM) significantly increased the expression levels of liver Vtgs, Chgs, and ERα.	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 monotic 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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