OPINION OF THE MEMBER STATE COMMITTEE

ON ENDOCRINE DISRUPTING PROPERTIES FOR THE ENVIRONMENT OF

4,4’-methylenediphenol (BPF)

EC number: 210-658-2

CAS number: 620-92-8

and

4,4'-[2,2,2-trifluoro-1-(trifluoromethyl)ethylidene]diphenol (BPAF)

EC number: 216-036-7

CAS number: 1478-61-1

and

eight BPAF salts

according to a MSC mandate[1]

Adopted on 13 December 2022

[1] Annex 1: Request to the Member State Committee for an opinion on the endocrine disrupting properties for environment of BPF and BPAF and for eight BPAF salts; I(2022)0077 of 5 May 2022

I ADOPTION OF THE OPINION OF THE MEMBER STATE COMMITTEE

Rapporteur, appointed by MSC: Asger Bolwig
Co-rapporteur, appointed by MSC: Johanna Barthelemy-Berneron
Dossier submitter: Germany
The MSC opinion was adopted on 13 December 2022.
The MSC opinion was adopted by consensus.

II OPINION OF THE MEMBER STATE COMMITTEE

MSC has formulated its opinion on:

a) whether the information provided in the Competent Authority of Germany (DE CA) evaluations of endocrine disrupting properties for the environment of BPF and BPAF and eight BPAF salts is sufficient and adequate to develop an opinion of a similar robustness to a substance of very high concern (SVHC) agreement.

b) whether the information provided, taking into account the comments received in the call for information and responses to them, shows that the substances meet the criteria for endocrine disruption according to the WHO/IPCS definition[2].

After examination of the information provided by the DE CA and the comments related to the endocrine disrupting properties to the environment of BPF and BPAF and eight BPAF salts raised during the call for evidence, MSC agreed that a scientifically robust conclusion can be drawn on the endocrine disrupting properties to the environment of the substances. The information included in the evaluation by DE CA shows that the substances meet the criteria for endocrine disruption as defined by the WHO/IPCS and as interpreted by the JRC Endocrine Disruptor Expert Advisory Group[3]. The additional data on BPAF identified by the Rapporteurs during the MSC opinion development and included in the MSC opinion do not change this conclusion, but further supports the conclusion.

[2] WHO/IPCS (2002): World Health Organisation, International Programme on Chemical Safety. Global assessment of the state-of-the-science of endocrine disruptors. WHO/PCS/EDC/02.2

[3] JRC (2013): Key Scientific issues relevant to the identification and characterisation of endocrine disrupting substances – Report of the Endocrine Disruptors Expert AdvisoryGroup (ED EAG). Comission E.

III SCIENTIFIC GROUNDS FOR THE OPINION

The reports submitted by DE CA are provided in Annexes 2 and 3, and the response to the comments received during the consultation of interested parties in Annexes 4 and 5. The scientific grounds for this opinion are detailed in Annex 6. Additional studies relevant for the assessment of the endocrine disrupting properties of BPAF were identified by the rapporteur after the public consultation and these are included under 'other supporting evidence'.

IV PROCESS FOR ADOPTION OF THE OPINION

In a letter of 5 May 2022, attached as Annex 1, ECHA's Acting Executive Director asked the Member State Committee (MSC) to draw up an opinion on whether BPF, BPAF and eight BPAF salts are endocrine disruptors for the environment. The opinion is based on:

- information prepared by the Member State Competent Authority of Germany (DE CA) and submitted to ECHA on 4 May 2022, and a revised version for BPF submitted on 28 July 2022 (Annexes 2 and 3);
- comments received in the 45-day consultation;
- responses to the comments as prepared by DE CA (Annexes 4 and 5);
- the scientific grounds provided in Annex 6 to this document, including the additional studies identified by the Rapporteurs as described in the section "Other supporting evidence".

The request from ECHA's Acting Executive Director is not a request for identification of these substances as substances of very high concern (SVHC) and therefore this opinion of MSC will not lead to the Candidate List listing of these substances.

MSC was requested to submit its opinion to the Risk Assessment Committee (RAC) and its Rapporteurs for RAC's deliberations on the Restriction proposal for bisphenol A and bisphenols exhibiting a similar concern for the environment from uses which lead to emissions to the environment.

Following the receipt of the ED's request, MSC agreed on the Terms of reference for the MSC rapporteur and on the indicative timeframe for the development of this opinion. Based on the mandate, MSC appointed Asger Bolwig and Johanna Barthelemy-Berneron as the rapporteur and the co-rapporteur, respectively, at MSC-78 on June 15 2022 to draft its opinion. The MSC discussed the draft opinion at MSC-79 and at MSC-80. The MSC opinion was adopted by consensus at MSC-80 on 13 December 2022.

ANNEXES:

Annex 1 – Request to the Member State Committee for an opinion on the endocrine disrupting properties for environment of BPF and BPAF and for eight BPAF salts; I(2022)0077 of 5 May 2022

Annex 2 – Evaluation by DE CA of endocrine disrupting properties for the environment of BPF. Provided by DE CA on 28 July 2022.

Annex 3 - Evaluation by DE CA of endocrine disrupting properties for the environment of BPAF and its salts. Provided by DE CA on 4 May 2022.

Annex 4 – Responses to comments received in the consultation of interested parties on Evaluation of BPF. Provided by DE CA on 8 July 2022.

Annex 5 – Responses to comments received in the consultation of interested parties on Evaluation of BPAF and its salts. Provided by DE CA on 8 July 2022.

Annex 6 – Scientific grounds for the opinion

Annex 6 – Scientific grounds for the opinion

Bisphenol F (BPF)

The dossier submitter's assessment of 4,4'-methylenediphenol (Bisphenol F, **BPF**) shows that the substance meets the criteria for endocrine disruption as defined by the WHO/IPCS (2002). The substance BPF acts as an endocrine disruptor (**ED**) in the environment based on available information from both *in vitro* and *in vivo* data in fish as described in the following sections:

Adverse effects relevant for ED identification:

Two *in vivo* studies (Klimisch 2) in *Danio rerio* show clear and consistent adverse and population relevant effects on reproduction and sexual development.

In the first study, six male and six female zebrafish in semi-static conditions were exposed to 1, 10, 100 and 1000 µg/L (nominal) BPF for 21 days following OECD TG 229 and OECD TG 230 (Yang *et al*., 2017). The reproductive performance, indicated by the number of eggs in F0, hatching rate and survival rate of F1 were all significantly reduced at 1000 µg/L of BPF. Histological alterations of testis in adult males, and ovaries in adult females were observed. In males, they were characterised by a decrease in the number of spermatogonia and spermatocytes, and an increased number of spermatids. In females, a higher proportion of pre-vitellogenic stage I oocytes but lower proportions of pre-vitellogenic stage II, vitellogenic stage III, and post-vitellogenic stage IV oocyte were observed in the exposed group. Accordingly, the gonadosomatic index (**GSI**) decreased significantly in both male and female groups exposed to 1000 µg/L BPF.

In the second study, Yang *et al*. (2018) investigated the effects of long term exposure of BPF on development and sexual differentiation of zebrafish. In this experiment, 500 *Danio rerio* eggs were maintained in semi-static condition and exposed to BPF solution (1, 10, 100 or 1000 µg/L, nominal), from fertilization until larvae were 60 days post-fertilization (**dpf**), covering the sex differentiation stages when zebrafish are sensitive to endocrine disrupting compounds (**EDC**). The hatching rates decreased significantly in the 100 and 1000 µg/L groups. Apical effects were observed, as indicated by a significant decrease at 60 dpf of the specific growth rates of body weight and body length in the higher exposure group (1000µg/L) compared to control. After 60 days of exposure, a significant trend for a female-biased sex ratio was observed (58% F, 32% M and 10% intersex in the 100 µg/L group and 64% F, 14% M and 22% intersex in the 1000 µg/L group). Histological examination revealed oocytes with a higher proportion of pre-vitellogenic stage I and a lack of other developmental stages in individual female fish with abnormal ovaries in the 100 and 1000 µg/L exposure groups, indicating a delay in the ovarian development. In males, exposure to 100 and 1000 µg/L BPF caused abnormal testicular development, characterized by different stages of spermatogonia together with primary oocytes. For both males and females, measurements of testosterone level in the full body homogenate at the end of the experiment showed a dose-dependent decrease (statistically significant from 10 µg/L). A corresponding dose-dependent increase of 17ß-estradiol was measured for both sexes (statistically significant from 10 µg/L).

No information regarding T-modality related to the adverse effect of BPF was included in the dossier submitter assessment of BPF.

Endocrine activity:

The available *in vitro* and *in vivo* mechanistic data clearly and consistently demonstrate

an estrogenic and anti-androgenic activity of BPF. All studies are rated as Klimisch 2, except the study performed in accordance with the OECD TG 455 and TG 458 (Park *et al*., 2020), which is rated Klimisch 1.

In Yang *et al*. (2017), gene expression alterations were measured in the hypothalamic-pituitary-gonadal (HPG) axis in gonads and brains of male and female adult zebrafish. Gonadotropin releasing hormones GnRH2, GnRH3, GnRHR1, and GnRHR2 were significantly upregulated in the brains of male fish in the BPF-treated groups (100 µg/L and 1000 µg/L), followed by significant upregulation of follicle stimulating hormone (**FSHβ**) and Luteinizing hormone (**LHβ**) expression (as low as 1 µg/L for FSHβ). Exposure to all concentrations of BPF caused upregulation of *cyp19a1b*. In females, significant downregulation of FSHβ gene expression was measured after exposure to 100 µg/L. *Cyp19a1b* gene expression was significantly upregulated but only at the concentration 100 µg/L. ERα expression was significantly upregulated in BPF-exposed brains of both males and females (≥100 µg/L). Stronger effects on gonadal gene expression alteration were reported in males. Exposure to BPF affected significantly the upregulation of follicle stimulating hormone receptor (**FSHR**) and Luteinizing hormone receptor (**LHR**) (≥100 µg/L) as well as *cyp11a* (1000µg/L), *cyp19a* (≥100 µg/L) gene expression, and the downregulation of the *cyp17*, 17β-HSD and StAR (1000 µg/L). Significant downregulation of 17β-HSD and StAR gene expression was observed in the ovaries in the 100 and 1000 µg/L exposed groups, whereas FSHR gene expression was upregulated (stronger effect at 100 µg/L then decrease at 1000 µg/L). Exposure to 1000 µg/L BPF caused significant upregulation of *cyp11a* expression and downregulated expression of LHR. Plasma vitellogenin (**VTG**) levels as endpoint for oestrogenic or anti-oestrogenic, and aromatase inhibition activity in males and females were not measured. However, the dose-dependent increasing expression of VTG1 gene in male liver homogenate (statistically significant from 10 µg/L) is correlated with the increasing 17β -estradiol (**E2**) level in full body homogenate (statistically significant from 100 µg/L), while testosterone level is decreasing (statistically significant from 10 µg/L) in the study.

In Yang *et al*. (2018), gene expression of *cyp19a1a* and VTG were also measured in zebrafish larvae. This study demonstrated that *cyp19a1a* expression was significantly upregulated at 20 dpf (10 and 1000 µg/L), 30 dpf (10, 100 and 1000 µg/L) and at 42 dpf in all exposure groups. No significant difference was found after 10 dpf. In the present study, VTG expression was detected during the early stages of sex differentiation. During the exposure period, VTG expression increased significantly at 20, 30 and 42 dpf in the 10, 100 and 1000 µg/L exposure groups, which coincided with the increased E2 levels after exposure to BPF.

The *in vitro* and *in vivo* study in zebrafish by Le Fol *et al.* (2017) evaluated the estrogenic activity of BPF using an *in vivo* zebrafish embryo assay (EASZY assay) based on the cyp19a1b-GFP transgenic line expressing the Green Fluorescent Protein (**GFP**) under the control of the ER-regulated *cyp19a1b* gene in the brain. These data also support the inducing effect of BPF on *cyp19a1b* transcript levels in the hypothalamus of zebrafish embryos. In BPF-exposed zebrafish embryos, a concentration-dependent induction of GFP was observed with a significant effect from 1 µM. At higher concentrations, the GFP signal reached a plateau at 5 µM (maintained up to 20 µM). The EC50 of BPF was 1.2 µM suggesting a higher *in vivo* estrogenic activity as compared to bisphenol A (EC50 5.7 µM). Additionally, the study confirmed the significant dose-dependent increase in the level of plasmatic VTG in adult male zebrafish after 7 days of exposure to 0.1 and 1 µM BPF.

Furthermore, the study by Qiu *et al*. (2019) showed that 60 days exposure to 0.1, 1, 10, 100 and 1000 µg/L BPF in juvenile (one month old) zebrafish leads to increased expression of reproductive neuroendocrine-related genes and increased levels of hormones in the zebrafish brain as well as increased levels of VTG in liver. The study however, did not evaluate the differences of tested parameters between male and female. The endocrine hormones LH and FSH were significantly increased in brain of zebrafish compared to

control (at 10 µg/L for FSH content levels, and 10 and 1000 µg/L regarding the LH content levels). GnRH and VTG content and VTG gene expression were increased after BPF exposure as compared to controls. Specifically, BPF concentration ≥10 µg/L induced GnRH content, all the treatment groups induced significantly VTG content and BPF concentration ≥10 µg/L induced VTG gene expression. As previously reported in other studies, the mRNA levels of *cyp19a* (10 µg/L), but also *cyp19b (*≥100 µg/L*),* have been significantly induced after BPF exposure. The mRNA levels of estrogen receptor (**ER**) were significantly increased in response to concentration ≥10 µg/L BPF exposure, while ER was significantly increased in response to 100 µg/L. The authors suggest that chronic BPF exposure affects the regulation of the reproductive neuroendocrine system through activation of the estrogen receptor.

Several *in vitro* reporter gene and transactivation assays show a clear agonistic activity of BPF on both ERα and ERβ subtypes of the receptor proteins (Kitamura *et al.*, 2005; Le Fol *et al*., 2017; Pelch *et al*., 2019; Park *et al*., 2020). Anti-estrogenic activity of BPF was observed in the studies by Pelch *et al.* (2019) and Park *et al.* (2020) in the transactivation assay set up and in an MCF-7 cell-based ERα protein expression level.

No androgen agonistic activity is reported in the available *in vitro* studies. Anti-androgenic activity was observed in several assays (Kitamura *et al.,* 2005; Punt *et al.*, 2019; Pelch *et al.*, 2019; Park *et al.,* 2020; Šauer *et al.,* 2021). Reported IC50 values and effect concentrations of BPF and BPA were similar or at least in the same order of magnitude.

Although the most prominent endocrine activity is related to estrogenic and androgenic modalities, two *in vitro* studies also show an interference with testosterone production in cellular assays (Eladak *et al*., 2015) and found that BPF and BPA comparably decreased basal testosterone secretion by human and mouse foetal testis in a culture assay set up. Growth hormone production was analysed in GH3 cells by Kitamura *et al.* (2005) and there were no effects of BPF and BPA.

The dossier submitter mentioned that there is also human health data available supporting the conclusion for EAS-modality activity of BPF. However, only the *in vitro* study from Eladak *et al.* (2015) is mentioned and the human health data were not further investigated in this assessment.

The estrogen agonistic and anti-androgenic activity of the substance is consistent across different cell lines and fish studies. The modalities are considered to be sufficiently investigated and BPF, based on the available *in vivo* and *in vitro* studies, shows significant endocrine activity.

Other supporting evidence:

In vitro and *in vivo* data from fish and amphibians point to an interference of BPF with the hypothalamic-pituitary-thyroid (HPT) axis. BPF showed binding to both subtypes of the thyroid receptor protein in a competitive binding assay rated as Klimisch 2 by Zhang *et al.* (2018). The same study found that bisphenol S (**BPS**) and BPF recruited coactivator to Thyroid hormone receptor (**TRβ**) but not to TRα in a coactivator recruitment assay but with weaker potencies compared to BPA. Additionally, Zhang *et al.* (2018) performed a TR-mediated reporter gene transcription assay and found agonistic actions of BPF in the absence or presence of Triiodothyronine (**T3**). The study further showed that BPA, BPF and BPS induced TH-dependent GH3 cell proliferation and that BPA and BPF inhibited T3 induction in the cells in presence of T3. Šauer *et al.* (2021), Klimisch 2 study, showed that BPF can bind to the Transthyretin (**TTR**) transport protein. TTR binding potency of BPF was comparable to that of BPAF and higher compared to BPA in this assay.

Additionally, *in vivo* mechanistic data from *Xenopus laevis* and *Pelophylax nigromaculatus* tadpoles, in three Klimisch 2 studies, show that BPF exerts some activity on the HPT axis. Zhu *et al.* (2018) found that in the absence of T3, BPF inhibited development of the tadpoles at metamorphic climax, but promoted pre- and pro-metamorphic development, displaying a developmental stage-dependence. The study performed by Zhang *et al.* (2018) on *Pelophylax nigromaculatus* tadpoles showed that BPA, BPF, BPS induced TH-response gene transcription in the tadpoles. In the presence of T3, altered T3-induced gene transcription was observed in a biphasic concentration-response manner. Niu *et al.* (2021) found effects of BPF on thyroid specific gene expression as well as antagonised T3-induced brain remodelling in *Xenopus laevis*.

The dossier submitter mentioned that there is also available human health data supporting the conclusion for EAS-modality activity of BPF. However, only the *in vitro* study from Eladak *et al.* (2015) is mentioned and the human health data were not further investigated in this assessment.

Plausible link between adverse effects and endocrine activity:

BPF may have multiple modes of endocrine action (estrogenic, anti-androgenic, thyroidal activity and interference with steroidogenesis) that might interact and are difficult to distinguish from one another. However, the estrogenic and/or anti-androgenic effects of BPF in fish are consistently observed in the available studies showing significant effects on egg production, hatching and survival of F1 larvae, sex ratio and gonadal development.

GnRH is the primary hormone regulating the synthesis and release of FSH and LH. In the Yang *et al*. (2017) study, GnRH2, GnRH3, GnRHR1, and GnRHR2 were significantly upregulated in the brains of male fishes in the BPF-treated groups. This result suggests that GnRH concentration can be modulated by BPF, which could subsequently affect the production of gonadotropic hormones. The expression of FSHβ and LHβ in males was indeed upregulated. On the contrary, significant downregulation of FSHβ gene expression in females after exposure to 1000 µg/L BPF suggested possible delays in oogenesis and maturation. This finding was also revealed in the histological examination of ovaries exposed to BPF.

Aromatase (Cyp19) is the key enzyme in estrogen biosynthesis from testosterone. The *in vivo* zebrafish studies demonstrated that *cyp19a* expression in males was significantly upregulated after exposure to BPF. VTG is a biomarker for estrogenic endocrine disruption in males and is usually produced in response to stimulation by estrogenic chemicals. Hence, the increase in VTG1 gene expression coincided with the increased E2 level after exposure to BPF.

Estrogenic and anti-androgenic modes of action are well known to be involved in the regulation of sexual development and reproduction. Considering the observed concomitant decrease in plasma testosterone levels and the increase in plasma estradiol levels, as well as the increase in VTG levels and gene expression in male fishes, the link between these endocrine activities and the observed adverse effects on fish is highly plausible.

Therefore, the observed effects fit with an estrogenic and/or anti-androgenic mode of action (**MoA**).

The link between the observed effects and the specific estrogenic and/or anti-androgenic activity of BPF is further supported by the analogy of BPF to BPA and bisphenol B (BPB). In *in vitro* studies (Kitamura *et al*., 2005; Le Fol *et al.,* 2017; Pelch *et al*.; 2019, Park *et al*., 2020), BPF showed similar EC50 values in the low µM range as BPA. Additionally, as reported in Punt *et al*. (2019), a previous yeast estrogen bioassay performed by van

Leeuwen *et al.* (2019) also shows estrogen agonistic activity of BPF and BPA, both at EC50 values of 20 µM. BPA is already identified as an ED in the environment based on estrogenic and or anti-androgenic effects. The data available for BPA and BPB, both of which share very similar chemical structures compared to BPF, show well defined adverse effects and modes of action that fit with an estrogenic MoA in fishes. Furthermore, BPF shows activity on the HPT axis, which also fits to the effects observed in the available in vitro data and effects reported for BPA. Based on similar data, BPA and BPB have already been identified as SVHC due to their endocrine disrupting properties for the environment.

Conclusion on ED properties of BPF:

Overall, BPF has estrogen agonistic properties and induces adverse effects in fish that are plausibly mediated by this endocrine activity.

Furthermore, *in vivo* and *in vitro* evidence shows that BPF has androgen antagonistic properties. This endocrine activity could also plausibly contribute to the observed adverse effects on reproduction and sexual development in fish.

The effects observed in fish (reduced number of viable eggs, reduced hatching and survival rates, increased malformation rate in F1 generation embryos and larvae and above all intersex individuals and altered sex ratio) are relevant for the environment because an effect on the reproductive function and the sexual development can have consequences at a population level.

Therefore, there is convincing scientific evidence to conclude that BPF fulfils the definition of an endocrine disruptor to the environment.

Bisphenol AF (BPAF) and eight BPAF salts

The dossier submitter's assessment of 4,4'-[2,2,2-trifluoro-1-(trifluoromethyl)ethylidene]diphenol (Bisphenol AF, **BPAF**) and its eight BPAF salts demonstrates that the substances meet the criteria for endocrine disruption as defined by the WHO/IPCS (2002). BPAF and its salts act as endocrine disruptors for the environment based on available information from both *in vitro* and *in vivo* data in fish as described in the following sections.

Adverse effects relevant for ED identification:

The dossier submitter included three *in vivo* studies in their assessment (Shi *et al.*, 2015; Song *et al*., 2014; Yang *et al*., 2016).

Shi *et al.* (2015) investigated the ED effects of BPAF in a fish full cycle toxicity test in a semi-static setup using 3 replicates with 120 embryos, 50 larvae and exposure concentrations of 5, 25 and 125 µg/L (nominal). In the F0 generation, no obvious difference on the hatch time, hatchability and survival rates between solvent control and exposure groups were observed during the 120 d exposure of BPAF. The fecundity of F0 females decreased but this was not statistically significant in the 125 µg/L group. However, a statistically significant reduction in fertilization success of spawn eggs was observed in this group.

In the F1 generation, malformation rates in the 125 µg/L-group was significantly higher than the control after 3 dpf (most malformed larvae died in first few days) while the survival rates were significantly lower after 6 dpf in the 125 µg/L-group.
In F0 males, significantly increased plasma E2 was observed in the 25 and 125 µg/L-groups and plasma thyroid (**T**) levels were significantly reduced in the 25 and 125 µg/L-groups.
In F0 females, significantly increased E2 levels were observed in the 125 µg/L-group while the T levels were unchanged.

In liver of F0 males, VTG1 was significantly increased in the 25 and 125 µg/L groups. VTG1 was unchanged in female fish. The brain expression of GnRH2, FSHb, LHb, *cyp19b* in males were significantly increased in the 125 µg/L-group. No changes were observed in the female brain. Gonadal expression of FSHR, *cyp19a*, *cyp11a1* was significantly increased and testis STAR with cyp17 was significantly decreased in the 125 µg/L-group. There was increase of FSHR and decrease of star expression by ovaries in the 125 µg/L-group. VTG concentrations were not determined.

The dossier submitter evaluated this study as Klimisch 1. The MSC disagrees on Klimisch 1 and consider this study to be Klimisch 2. The study does not follow OECD TG 234 although covering the exposure period from 0 to 60 days post hatching (**dph**) (even to 120 dph). The sex ratio was not reported and this is an important endpoint for adverse endocrine specific effects. Furthermore, there was no analytical confirmation of the test substance concentrations.

Song *et al*. (2014) conducted two toxicity tests; one test using embryos exposed for 6 days and another test using two-months old male fish exposed for 21 days in a semi-static setup using 3 replicates with 30 embryos OR with 10 males randomly selected. The test substance concentrations were 0.5, 1.0 and 1.5 mg/L (nominal) for test of hatching rate and hatching time of embryos and 0.5, 1.0, 1.5 and 2.0 mg/L (nominal) for test of mortality of zebrafish embryos/larvae exposed to BPAF.
In male adults, VTG induction was observed in all concentrations of BPAF (21d).

In the embryo test, 100% mortality at 2.0 mg/L after 144 hours post fertilization (**hpf**) was observed and statistically significant increase of pericardial oedema was observed at 1.5 mg/L. Slightly delayed hatching was also observed in the 1.0 and 1.5 mg/L-groups (not significant).
Body length, number of fish in swim-up stage at one or more time periods and behavioral abnormalities were not reported.
The dossier submitter evaluated this study to be Klimisch 2. VTG induction in adult male fish support estrogenic MoA.

Yang *et al.* (2016) tested BPAF in two months old zebrafish exposed for 28 days using three replicates with 9 fish in a semi-static setup with concentrations of 0.05, 0.25 and 1 mg/L (nominal).
Male fish hepatocytes were swollen and irregularly shaped in the 1 mg/L-group. Vacuolization in liver in the 1 mg/L-group was observed but no hepatic damage was seen in any female fish.
No obvious alterations were observed in the gills and intestines of both sexes. In males, germ cells were found in all stages of spermatogenesis. In females, in contrast to the control fish, the majority of cells from BPAF-treated females were in stage I, although a small number of cells were at stage IV. Exposure to 0.25 and 1 mg/L BPAF resulted in a significantly higher proportion of stage I cells and caused significantly lower proportion of stage IV cells relative to the control fish, suggesting that BPAF inhibits ovarian maturation.
Male fish had reduced T levels in whole-body homogenates in a dose-dependent manner as well as increased E2 levels with increasing BPAF concentration. Female fish had increased T levels in the 0.05 and 0.25 mg/L-groups and decreased T levels in the 1 mg/L group. An increase in E2 levels in the 0.05 and 1 mg/L-groups was also observed in female fish but slightly decreased in the 0.25 mg/L-group. VTG gene expression in the liver was significantly upregulated in males of the 1 mg/L-group
The dossier submitter evaluated this study to be Klimisch 1. The MSC disagrees and considers this study to be Klimisch 2 and notes that no analytical verification of the test concentrations were performed. VTG gene induction in male fish support estrogenic MoA as do increased E and decreased T concentrations.

The MSC agrees with the conclusions of the dossier submitter that the available data clearly and consistently show that BPAF has an estrogenic/anti-androgenic MoA.

Endocrine activity:

Three *in vitro* studies are included in the assessment by the dossier submitter (Li *et al.*, 2012; Li *et al.*, 2013; Matsushima *et al.*, 2010).
Li *et al.* (2012) used *in vitro* models for evaluation of effects of BPAF on ERα and ERβ using three human cell lines at concentrations of 1 nM, 10 nM, 100 nM and 1000 nM (Ishikawa, HeLa, and HepG2). They demonstrated estrogenic activity as agonist for ERα in a dose-dependent manner. At low concentrations (≤ 10 nM), BPAF had weak estrogenic activity compared with E2, but stronger activation was observed at higher concentrations (1,000 nM) via the p44/42 MAPK pathway for BPAF.
The reliability of this study is assessed to be Klimisch 2. The study is a well performed non-guideline study. BPAF induced ER mediated activity supporting an estrogenic MoA.

Li *et al.* (2013) used HepG2 and HeLa cells to determine the agonistic activity of BPAF on ERα and ERβ via the luciferase reporter assay at concentration of 100 nM. They found activation of ERα3xERE-mediated responses in HepG2 cells and significant induction of 3xERE and pS2ERE mediated activity. They also reported activation of ERβ3xERE and pS2ERE mediated activity and effects on ERα target genes (PR, pS2, GREB1, SPUVE, WISP2, and SDF-1) in Ishikawa/ERα stable cells, as well as significant induction of endogenous ERα target genes PR, pS2, GREB1, SPUVE, WISP2 and SDF-1. In contrast,

expression of target genes in the Ishikawa/vector stable cells did not change with bisphenol-AF treatments.
The reliability of this study is assessed to be Klimisch 2 as this is a well performed non-guideline study. BPAF induced ER mediated activity and ER target genes, supporting an estrogenic MoA.

Matsushima *et al.* (2010) investigated the receptor binding activity of BPAF to ERα and ERβ proteins in a competitive binding assay at concentrations of 0.01, 0.1, 1.0, and 10 μM. They demonstrated binding to ERs.
The authors found that receptor binding activity of BPAF was three times stronger for ERβ (IC50 = 18.9 nM) than for ERα. Furthermore, BPAF almost completely inactivated the basal constitutive activity of ERβ. BPAF acted as an antagonist against the activity of 17β-estradiol. Hence, the authors concluded that BPAF is a full agonist for ERα but a highly specific antagonist for ERβ. BPAF was almost completely inactive in stimulating the basal constitutive activity of ERβ.
The reliability of this study is assessed to be Klimisch 2. It is a well-planned non-guideline study. BPAF induced ER mediated activity with strongest binding to ERβ supporting an estrogenic MoA.

The MSC agrees with the dossier submitter in its conclusion that BPAF shows estrogenic activity in the presented *in vitro* studies in a dose-dependent manner.

Other supporting evidence:

Additional studies relevant for the assessment of the endocrine disrupting properties of BPAF were identified by the rapporteur during the assessment. These studies were not included in the Art. 77(3) report by the dossier submitter and have not been provided or discussed during the public consultation.

In vitro EAS-modality:
Feng *et al.* (2016) demonstrated that BPF, BPS and BPAF altered steroidogenesis in H295R cells. They tested bisphenol concentrations of 0, 10, 30, 50, 70, 100, 200, 300 and 500 μM. While BPF predominantly led to increased progesterone and estradiol levels, BPAF showed induction of progesterone and reduction of testosterone. According to the authors, inhibiting effects of BPA and BPAF on hormone production were probably mediated by down-regulation of steroidogenic genes in H295R cells.
In opinion of the MSC, this is a well planned non-guideline study (Klimisch 2). BPAF induced progesterone and reduced testosterone supporting inhibition of steroidogenesis.

Lin *et al.* (2021) investigated the endocrine disrupting potential of ten bisphenols including BPAF and BPA in H295R and MVLN (Bioluminescent MCE-7-derived cell line) cell bioassays at concentrations of 0.39, 1.56 and 6.25 nM. In the MVLN assay, seven bisphenols induced luciferase activity and the ER transactivation activity was highest for BPAF compared to the other bisphenols tested.
In opinion of the MSC, this is a well-planned non-guideline study (Klimisch 2). In the MVLN luciferase gene reporter assay, BPAF was the most potent substance activating the ER compared to other BPs (including BPA) and this supports the estrogenic MoA.

In addition, Sauer *et al*., 2021 was included in the BPF Art. 77 report and should also have been included in the BPAF Art. 77 report as BPAF shows anti-androgenic activity in the CALUX *in vitro* reporter assay in this study. According to this study, the TTR binding potency of BPAF is similar to BPF and greater than the binding potency of BPA.

In vivo EAS modality:

The MSC opinion includes additional studies investigating EAS-modalities of BPAF that were not included in the report by the dossier submitter.

In a study by Mu *et* al. (2018), zebrafish embryos were used to assess the lethality, developmental effects and estrogenic activity of bisphenol analogues including BPAF. The authors observed that the lethality of bisphenol analogues decreased in order of BPAF > BPA > BPF > BPS. BPAF and BPF induced significant effects on zebrafish embryos, including decreased heart rate, hatching inhibition, and teratogenic effects. The binding potentials of bisphenol analogues toward zebrafish ERs decreased in the following order: BPAF > BPA > BPF. *In vivo* estrogenic activity tests showed that BPAF, BPA, and BPF significantly enhanced the protein levels of ER alpha along with the mRNA levels of esrl, esr2a, esr2b, and VTGl in zebrafish embryos. Esr2b showed the strongest response to BPAF and BPA exposure. In conclusion, this study demonstrates that BPAF showed the highest lethality, developmental effects and estrogenic activity *in vivo* followed by BPA and BPF.

In a study by Qiu *et al*. (2021), the effects of BPA and other bisphenols (including BPF, and BPAF) on the reproductive neuroendocrine system were evaluated during zebrafish embryonic and larval development at concentrations of 1 and 100 µg/L. The study showed that the numbers of gonadotropin-releasing hormone 3 neurons in zebrafish embryos increased after 100 µg/L BPA analog treatment and exposure to BPA or its analogues at 1 or 100 µg/L increased the expression of reproductive neuroendocrine-related genes and the levels of typical hormones such as LH, FSH, E2, and growth hormone. Moreover, the effects were observed to be associated with increases in the activities of ER alpha, ER beta, and *cyp19a* genes. The respective estrogen receptors and aromatase antagonists significantly attenuated the stimulation of LH beta, FSH beta, LH and FSH expression in this study, thereby proving that BPA analogs affect the reproductive neuroendocrine system via ERs and aromatase pathway. Furthermore, the reproductive neuroendocrine toxicity of BPAF was similar to that of BPA.

Cai *et al*. (2020) investigated whether low concentrations (1, 10 and 100 nM) of BPAF could disrupt gonadal differentiation and subsequent development using Xenopus laevis. Tadpoles were exposed to BPAF or E2 (positive control) in a semi-static exposure system. All concentrations of BPAF caused changes in testicular morphology at different developmental stages compared with the controls. BPAF resulted in decreased size and number of gonadal metameres in testes such as the ovarian cavity at stages 53 and 66 and poorly developed seminiferous tubules. At the molecular level, BPAF inhibited expression of male genes in testes at stage 53. Correspondingly, BPAF, like E2, inhibited cell proliferation in testes at stage 50. These results show that low concentrations of BPAF inhibited testicular differentiation and subsequent development in *Xenopus laevis* along with feminizing effects to some degree.

Arancio *et al*. (2019) used *Xenopus laevis* to compare effects on early embryo cell division and development following exposure to BPA at concentrations of 1, 10, 25 and 50 µM and BPAF at concentrations of 0.03, 0.3, 3 and 25 µM. Directly after *in vitro* fertilizations, embryos were exposed to BPA, BPAF, di-n-butyl phthalate or E2 for up to 96 hours. BPA (1-50 µM) and BPAF (0.003-25 µM) caused disrupted cleavage divisions, and slowed cytokinesis, and cellular dissociation within 1-6 h. Flexures of the spinal cord, shorter body axis/tail, craniofacial malformations and significant mortality occurred with environmentally relevant doses of BPAF (LC50 = 0.013 µM). The study showed that BPAF had the greatest potency and toxicity compared to BPA and estradiol.

One study on avian *in vitro*/embryonic stages was identified. To determine effects of BPAF exposure on avian embryonic viability, development and hepatic mRNA expression at mid-incubation and term (day 11 and day 20), Sharin *et al.* (2021) performed an egg injection study. Test chemicals including BPAF were injected into the air cell of unincubated, fertilized chicken eggs at concentrations ranging from 0-114 µg/g egg for BPAF. Embryonic concentrations of BPAF decreased at mid-incubation and term compared to injected

concentrations suggesting embryonic metabolism. Exposure to BPAF at a concentration 114 µg/g significantly decreased embryonic viability at day 11 (mid-incubation). Exposure to 114 µg/g BPAF resulted in increased gallbladder mass after 20 days in embryos. Expression of hepatic genes related to xenobiotic metabolism, lipid homeostasis, and response to estrogen were also altered at both developmental stages. This study supports estrogenic activity in avians of BPAF.

The *in vitro* and *in vivo* evidence identified by the rapporteur provide additional supporting evidence of the estrogenic/anti-androgenic MoA proposed by the dossier submitter.

In vivo T-Modality:
The MSC opinion includes three additional studies investigating the T-mediated effects of BPAF and they show clear effects on e.g. T-hormone levels (Chen et al. 2022, Kwon et al. 2016, Tang et al. 2015). They are briefly summarized below.

Chen *et al.* (2022) used a 7 day zebrafish embryotoxicity test to study the potential thyroid disruption of BPAF at concentrations of 12.5 and 125 µg/Lµg/L, indicating that both the metabolism and transport of thyroid hormones were perturbed. The thyroid hormone receptor levels decreased significantly upon exposure to ≥12.5 µg/L BPAF in this study, implying that BPAF acts as a TR antagonist.

Kwon *et al.* (2016) used adult zebrafish to investigate thyroid endocrine disruption by BPAF alone at a concentration of 24.7 mg/L or in combination with sulfamethoxazole (SMX). Microarray and parallel experiments were performed to characterize gene transcription profiles related to thyroid and metabolism by mixture exposure. BPAF alone or in combination with SMX affected genes related to thyroid hormone production and receptor activity, thyroid gland development and deiodinase activity. Significant down-regulation of thyrotropin-releasing hormone (**TRH)** and thyroid-stimulating hormone (**TSH**) β genes in the brain suggested a negative feedback response resulting in increased thyroxine levels. The study indicates that BPAF exposure alters transcription of genes associated with the thyroid endocrine system.

For elucidating the disruptive effects of BPAF on thyroid function and expression of genes along the HPT axis in zebrafish embryos, (Tang et al., 2015) examined total triiodothyronine (**T3**), total tetraiodothyronine (**T4**), free T3 and free T4 levels following 168 hour post-fertilization exposure to BPAF concentrations of 0, 5, 50 and 500 µg/L. The results showed that whole-body T3, T4, free T3 and free T4 levels decreased significantly with the BPAF treatment, indicating endocrine disruption of the thyroid system. The expression of thyroid-stimulating hormone- and thyroglobulin genes increased after exposure to 50 µg/L BPAF in seven-day-old larvae. The expressions of thyronine deiodinases type 1, type 2 and transthyretin mRNAs were also significantly up-regulated, which were possibly associated with a deterioration of thyroid function according to the authors.
This study demonstrates that BPAF exposure triggers thyroid endocrine toxicity by altering the whole-body contents of thyroid hormones and changing the transcription of the genes involved in the HPT axis in zebrafish larvae.

The additional studies included in the MSC opinion after the public consultation indicate that BPAF also affects the thyroid system *in vivo*.

Plausible link between adverse effects and endocrine activity:

The MoA analysis leads the MSC to the conclusion that BPAF acts via an estrogenic and/or anti-androgenic MoA. The molecular initiating event is binding and activation of the ER(s) as identified in several *in vitro* studies. This leads to the effects observed *in* several *in vivo* studies including VTG gene induction in male fish and decreased testosterone levels and

increased E2 levels all supporting an estrogenic/anti-androgenic MoA. BPAF induced reproductive toxicity, leading to gonad histological changes. Although a decrease in fecundity was not significant, a decrease in fertilization and hatchability and an increase in malformation and mortality in F1 suggest that BPAF-induced reproductive effects are population relevant. These population related reproductive effects were often accompanied with an induction in VTG in male fish and changes in sex hormones. Overall, the available evidence suggests that BPAF binds to and transactivate ERs, leading to adverse reproductive effects in fish. These observations suggest that BPAF is an endocrine disruptor for the environment.
Furthermore, the additional studies included in the MSC opinion after the public consultation indicate that BPAF also affects the thyroid system *in vivo*.

Read across to eight BPAF salts
The dossier submitter proposes read across to eight salts of BPAF due to the occurrence of BPAF as the counter anion in these substances and argues that the substances can be expected to dissociate to the cation and the anion (BPAF) under environmentally relevant conditions and that, under physiological conditions, dissociation of the salts is also expected. Based on the nature of the substances, it is concluded that the ED properties for the environment relevant for BPAF apply to the salts as well.

Conclusion on ED properties of BPAF and eight BPAF salts:

Lines of evidence (LoE)-) - EAS modalities:
There is strong evidence that BPAF is an endocrine disruptor for the environment. *In vitro* as well as *in vivo* data support that BPAF binds to and transactivates estrogen receptors and therefore induces vitellogenin in male fish an estrogenic/anti-androgenic mechanism. Two studies showed that BPAF induced histological changes in zebrafish gonads (Yang *et al.* 2016), leading to a decrease in fertilisation and hatchability as well as an increase in malformation and mortality in F1 (Shi *et al.*, 2015). These population relevant reproductive effects are accompanied with an induction of vitellogenin and a change in sex hormones and therefore are considered to be mediated by the estrogen receptor pathway. Additional studies on the endocrine disrupting properties of BPAF were identified by the rapporteur during its assessment. These studies were not included by the dossier submitter but overall, they provide additional supporting evidence of an estrogenic activity and/or anti-androgenic activity of BPAF.

Lines of evidence – T modality:
The MSC opinion includes additional relevant studies showing some interaction of BPAF on the thyroid system. None of these studies were included in the assessment by the dossier submitter. The studies support BPAF as a thyroid hormone system (THS) active substance but the adversity has not been clearly addressed. However, the available evidence suggest that BPAF may also interfere with the thyroid hormone system. In this regards, BPAF seems to mimic BPA in this respect where THS-activity has also been observed in several studies.

Overall, the MSC agree with the conclusions of the dossier submitter that there is scientific evidence to conclude that BPAF and the eight BPAF salts can be identified as ED to the environment according to the WHO/IPCS definition based on the available evidence.

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List of Abbreviations

AhR	Aryl-hydrocarbon receptor
AR	Androgen receptor
ARN	Assessment of Regulatory Needs
BPA	Bisphenol A (EC 201-245-8, CAS 80-05-7)
BPAF	Bisphenol AF (EC 216-036-7, CAS 1478-61-1)
BPB	Bisphenol B (EC 201-025-1, CAS 77-40-7)
BPF	Bisphenol F (EC 210-658-2, CAS 620-92-8)
BPS	Bisphenol S (EC 201-250-5, CAS 80-09-1)
DHT	Dihydrotestosterone
dph	days post hatching
dpf	days post-fertilisation
E2	Estradiol
EAG	Expert Advisory Group
EC	Effective concentration
ECHA	European Chemicals Agency
ED	Endocrine Disruptor
EDC	Endocrine Disrupting Compound
EFSA	European Food Safety Authority
ER	Estrogen receptor
FSH	Follicle Stimulating Hormone
FSHR	Follicle Stimulating Hormone receptor
GFP	Green Fluorescent Protein
GnRH	Gonadotropin releasing hormone
GSI	Gonadosomatic index
HeLa	Human cell line
HepG2	Human liver cell line
hpf	hours post-fertilisation
HPG	Hypothalamic-pituitary-gonadal
HSI	Hepatosomatic index
HPT	Hypothalamic-pituitary-thyroid
IC	Inhibitory concentration
JRC	Joint Research Center
LC	Lethal concentration
LC-MS	Liquid chromatography/mass spectroscopy
LDH	Lactate dehydrogenase
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
LOEC	Lowest Observed Effect Concentration
MAPK	Mitogen-activated Protein Kinases
MoA	Mode of action
MSC	Member State Committee
(MS)CA	(Member State) Competent Authority
MVLN	Bioluminescent MCE-7-derived cell line
NF	Nieuwkoop and Faber (developmental staging for xenopus laevis)
NO(A)EL	No observed (adverse) effect level
OECD	Organisation for Economic Co-operation and Development
(q)PCR	(quantitative) Polymerase chain reaction
RAC	Committee for Risk Assessment
RMOA	Risk Management Option Analysis
SEv	Substance Evaluation
SMX	Sulfamethoxazole
SVHC	Substance of very high concern
T	Thyroid

T3	Triiodothyronine
T4	Thyroxine
THR	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone
TH	Thyroid hormone
THS	Thyroid hormone system
TR	Thyroid hormone receptor
TTR	Transthyretin
VTG	Vitellogenin
WHO	World Health Organisation
WoE	Weight of Evidence