SUBSTANCE EVALUATION CONCLUSION

as required by REACH Article 48

and

EVALUATION REPORT

for

Substance name: Triphenyl phosphate (TPP)
EC No. 204-112-2
CAS RN 115-86-6

Evaluating Member State(s): France
(Following the initial assessment by the UK)

Dated: 17 May 2023

Evaluating Member State Competent Authority

Follow-up and conclusion of the substance evaluation process

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Year of evaluation in CoRAP: 2017

Before concluding the substance evaluation, a Decision to request further information was issued on 11 February 2019.

The evaluating Member State concluded the evaluation without any further need to ask for more information from the registrants under Article 46(1) decision.

Further information on registered substances here:

http://echa.europa.eu/web/guest/information-on-chemicals/registered-substances

DISCLAIMER

This document has been prepared by the evaluating Member State as a part of the substance evaluation process under the REACH Regulation (EC) No 1907/2006. The information and views set out in this document are those of the author and do not necessarily reflect the position or opinion of the European Chemicals Agency or other Member States. The Agency does not guarantee the accuracy of the information included in the document. Neither the Agency nor the evaluating Member State nor any person acting on either of their behalves may be held liable for the use which may be made of the information contained therein. Statements made or information contained in the document are without prejudice to any further regulatory work that the Agency or Member States may initiate at a later stage.

Foreword

Substance evaluation is an evaluation process under REACH Regulation (EC) No. 1907/2006. Under this process the Member States perform the evaluation and ECHA secretariat coordinates the work. The Community rolling action plan (CoRAP) of substances subject to evaluation, is updated and published annually on the ECHA web site[1].

Substance evaluation is a concern driven process, which aims to clarify whether a substance constitutes a risk to human health or the environment. Member States evaluate assigned substances in the CoRAP with the objective to clarify the potential concern and, if necessary, to request further information from the registrant(s) concerning the substance. If the evaluating Member State concludes that no further information needs to be requested, the substance evaluation is completed. If additional information is required, this is sought by the evaluating Member State. The evaluating Member State then draws conclusions on how to use the existing and obtained information for the safe use of the substance.

This Conclusion document, as required by Article 48 of the REACH Regulation, provides the final outcome of the Substance Evaluation carried out by the evaluating Member State. The document consists of two parts i.e. A) the conclusion and B) the evaluation report. In the conclusion part A, the evaluating Member State considers how the information on the substance can be used for the purposes of regulatory risk management such as identification of substances of very high concern (SVHC), restriction and/or classification and labelling. In the evaluation report part B, the document provides explanation how the evaluating Member State assessed and drew the conclusions from the information available.

With this Conclusion document the substance evaluation process is finished and the Commission, the Registrant(s) of the substance and the Competent Authorities of the other Member States are informed of the considerations of the evaluating Member State. In case the evaluating Member State proposes further regulatory risk management measures, this document shall not be considered initiating those other measures or processes. Further analyses may need to be performed which may change the proposed regulatory measures in this document. Since this document only reflects the views of the evaluating Member State, it does not preclude other Member States or the European Commission from initiating regulatory risk management measures which they deem appropriate.

[1] http://echa.europa.eu/regulations/reach/evaluation/substance-evaluation/community-rolling-action-plan

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Part A. Conclusion

1. CONCERN(S) SUBJECT TO EVALUATION

Triphenyl phosphate (EC No. 204-112-2; CAS RN 115-86-6; hereafter 'TPP') was originally selected for substance evaluation to clarify concerns about:

- Potential endocrine disruptor
- Consumer use
- High (aggregated) tonnage
- Wide dispersive use

The evaluation of TPP was initiated in 2017 by UK MSCA following its inclusion in the CoRAP list in 2013. It was then transferred to France following the UK's withdrawal from the EU, specifically to assess the potential concern for endocrine disrupting properties.

2. OVERVIEW OF OTHER PROCESSES / EU LEGISLATION

In 2012 ECHA issued a compliance check and in 2021 a testing proposal decision, respectively. The registration dossier was updated accordingly in October 2015 and July 2022. A risk management options analysis of this Substance was completed in 2019 by the French Member State Competent Authority, indicating their intention to evaluate potential endocrine disrupting properties under substance evaluation.

3. CONCLUSION OF SUBSTANCE EVALUATION

The evaluation of the available information on the substance has led the evaluating Member State to the following conclusions, as summarised in the table below.

Table 1

CONCLUSION OF SUBSTANCE EVALUATION	
Conclusions	**Tick box**
Harmonised Classification and Labelling	(X)
Identification as SVHC (authorisation)	X
Restrictions	
Other EU-wide measures	
No need for regulatory follow-up action at EU level	

4. FOLLOW-UP AT EU LEVEL

4.1. Need for follow-up regulatory action at EU level

4.1.1. Identification of endocrine disruptive properties: Harmonised Classification and Labelling

Currently, there is no harmonised classification for TPP but the information available now leads to the conclusion that an entry in CLP-Annex VI is needed for this substance for endocrine disruptors for the environment. The data available on environment shows endocrine activity on non-target organisms with adverse effects on fertility and reproduction in academic studies. These adverse effects are considered sensitive to, but not diagnostic of Estrogen, Androgen, Thyroid, Steroidogenic (EATS) modalities according to the ED EFSA/ECHA guidance 2018 (Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009). Those adverse effects might be the consequences of disturbances in steroid synthesis and gamete quality. Unfortunately, due to some limits of the Fish sexual development test (FSDT) study and its study design, this requested study does not bring any clarification about this mode of action. The ED EG advice indicated support for the identification of this substance as an endocrine disruptor for the environment using a weight-of-evidence approach (ECHA, 2022). Based on a weight of evidence approach and the available guidance for the identification of endocrine disruptors, including the ANSES Endocrine Disruptor (ED) expert Working Group criteria for classification (ANSES, 2016), the eMSCA considers that TPP should be categorized as a presumed endocrine disruptor.

Lastly, the 'one-generation reproductive toxicity study' currently being undertaken under the auspices of the US NTP, whose protocol was completed after discussion with ANSES, will presumably contribute to reduce the remaining uncertainties.

4.1.2. Identification as a substance of very high concern, SVHC (first step towards authorisation)

Based on a weight of evidence approach and the available guidance for the identification of endocrine disruptors, the eMSCA considers that TPP should be identified as SVHC for ED for the environment.

Pending on the outcomes of the 'one-generation reproductive toxicity study' currently being undertaken under the auspices of the US NTP, an update of the proposed SVHC identification for endocrine properties for human health might be considered if deemed necessary.

5. CURRENTLY NO FOLLOW-UP FORESEEN AT EU LEVEL

Not applicable.

6. TENTATIVE PLAN FOR FOLLOW-UP ACTIONS (IF NECESSARY)

Indication of a tentative plan is not a formal commitment by the evaluating Member State. A commitment to prepare a REACH Annex XV dossier (SVHC, restrictions) and/or CLP Annex VI dossier should be made via the Registry of Intentions.

Table 2

FOLLOW-UP		
Follow-up action	**Date for intention**	**Actor**
Harmonised Classification and Labelling	To be initiated in 2024	Member State FR
SVHC identification	To be initiated in 2024	Member State FR

Part B. Substance evaluation

7. EVALUATION REPORT

7.1. Overview of the substance evaluation performed

TPP was originally selected for substance evaluation to clarify concerns about:

- Potential endocrine disruptor
- Consumer use
- High (aggregated) tonnage
- Wide dispersive use.

Table 3

EVALUATED ENDPOINTS	
Endpoint evaluated	**Outcome/conclusion**
Potential endocrine disruptor	**Concern confirmed.** Substance identified as an endocrine disruptor for the environment, process to be initiated.
Consumer use, high (aggregated) tonnage, wide dispersive use	**Concern confirmed.** A release to the environment is likely by widespread use of materials and articles and due to consumer uses.

7.2. Procedure

The evaluation of TPP was initiated in 2017 by UK MSCA following its inclusion in the CoRAP list in 2013. On 11 February 2019, ECHA issued a final decision requesting the inclusion of a Fish Sexual Development Test in the registration dossier by 18 August 2020. Following the withdrawal of the UK from the EU on 1 January 2020, follow-up of substance evaluation was then transferred to France specifically for potential endocrine disrupting properties concern.

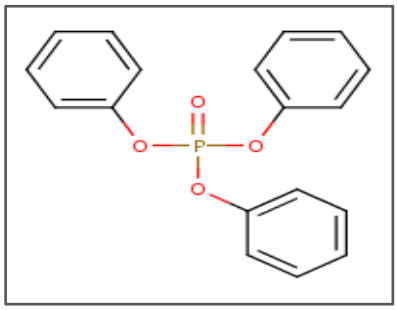
7.3. Identity of the substance

Table 4

SUBSTANCE IDENTITY	
Public name:	Triphenyl phosphate
EC number:	204-112-2
CAS number:	115-86-6
Index number in Annex VI of the CLP Regulation:	None
Molecular formula:	$C_{18}H_{15}O_4P$
Molecular weight range:	326.28
Synonyms:	Triphenyl phosphate, TPP, TPhP

Type of substance : X Mono-constituent ☐ Multi-constituent ☐ UVCB

Structural formula:



7.4. Physico-chemical properties

Table 5

OVERVIEW OF PHYSICOCHEMICAL PROPERTIES	
Property	**Value**
Physical state at 20°C and 101.3 kPa	Colourless, odourless solid
Vapour pressure (at 25°C)	0.000853 Pa (Non-guideline publication using an isoteniscope under a nitrogen atmosphere). Results extrapolated using the Clausius-Clapeyron equation
Water solubility (at 20°C)	1.9 mg/L (non-guideline publication). Other supporting publications/reference sources give similar values. No modern guideline studies.
Partition coefficient n-octanol/water (Log K_{ow}) (At 20°C)	Log Pow 4.63 (non-guideline but similar to shake-flask). Values between 4.5 and 4.7 are reported in various publications
Flammability	Study waived
Explosive properties	Study waived
Oxidising properties	Study waived
Granulometry	OECD Guideline 110 (Particle Size Distribution / Fibre Length and Diameter Distributions). All particles with a mean diameter < 100 µm have a mass fraction of 0.41 %. 0.023 % / 0.019 % (spherical/ cubical) particles of this mass fraction have a mean diameter < 4 µm.
Stability in organic solvents and identity of relevant degradation products	Study waived
Dissociation constant	Study waived
Melting point	49.5 - 50 °C (non-guideline publication). Melting point values within the range 49 - 50. 5 °C are reported in a variety of secondary sources.

Boiling point	414 °C at 101.3 kPa. (Extrapolated according to the Clausius-Clapeyron equation using experimentally derived parameters - non-guideline publication). One publication reported decomposition at or near the boiling point.
Density	The relative density at 50°C is given as 1.21g/cm3 (non-guideline publication).

7.5. Manufacture and uses

7.5.1. Quantities

Table 6

AGGREGATED TONNAGE (PER YEAR)				
☐ 1 – 10 t	☐ 10 – 100 t	☒ 100 – 1000 t	☐ 1000- 10,000 t	☐ 10,000-50,000 t
☐ 50,000 – 100,000 t	☐ 100,000 – 500,000 t	☐ 500,000 – 1000,000 t	☐ > 1000,000 t	☐ Confidential

7.5.2. Overview of uses

TPP is used as a flame retardant and plasticizer in polymer formulations, adhesives and sealants, cosmetics, and personal care products. TPP is used by consumers, in articles, by professional workers (widespread uses), in formulation or re-packing, at industrial sites and in manufacturing. TPP is present as an impurity in many other organophosphate flame retardants or as a constituent of this family of compounds.

Other release to the environment of this substance is likely to occur from: indoor use and outdoor use resulting in inclusion into or onto materials (e.g., binding agent in paints and coatings or adhesives). TPP in articles is also reported as having widespread use with low release outdoors in municipal waste, incineration, and shredding.

The following information is extracted from ECHA dissemination website:

Table 7

USES	Use(s)
Uses as intermediate	-
Formulation	Formulation of plastic and rubber preparations. Formulation of flame retardant/plasticizer preparations and cosmetics.
Uses at industrial sites	Production of plastic and rubber articles (conversion)
Uses by professional workers	Use in adhesives, sealants, and coating products. Use in laboratory chemicals and scientific research and development.
Consumer Uses	Use in adhesives and sealants. Cosmetics and personal care products.

	Other release to the environment of this substance is likely to occur from: indoor use and outdoor use resulting in inclusion into or onto materials (e.g., binding agent in paints and coatings or adhesives).
Article service life	Release to the environment of this substance can occur from: - Industrial use: industrial abrasion processing with low release rate (e.g., cutting of textile, cutting, machining, or grinding of metal). - Outdoor use in long-life materials with low release rate (e.g., metal, wooden and plastic construction and building materials). - Indoor use in long-life materials with low release rate (e.g., flooring, furniture, toys, construction materials, curtains, footwear, leather products, paper and cardboard products, electronic equipment). - No release intended of the substance from complex articles: • vehicles • machinery, mechanical appliances, electrical/electronic articles • fabrics, textiles, and apparel • paper articles • plastic articles.

7.6. Classification and Labelling

7.6.1. Harmonised Classification (Annex VI of CLP)

No current harmonised classification.

7.6.2. Self-classification

- In the registration(s):

Aquatic Acute 1: H400

Aquatic Chronic 1: H410

- The following hazard classes are in addition notified among the aggregated self-classifications in the C&L Inventory:

Aquatic Chronic 2: H411

Aquatic Chronic 4: H413

Skin Irrit. 2: H315

Eye Irrit. 2: H319

Skin Sens. 1A: H317

Muta. 2: H341

Carc. 2: H351.

7.7. Environmental fate properties

Not evaluated by FR-MSCA in the context of this SEV.

7.8. Environmental hazard assessment

Not evaluated by FR-MSCA in the context of this SEV.

7.9. Human Health hazard assessment

Not evaluated by FR-MSCA in the context of this SEV.

7.10. Assessment of endocrine disrupting (ED) properties

7.10.1. Endocrine disruption – Environment

Studies published prior to 2017 were evaluated in detail by the UK eMSCA during the first phase of the evaluation process and this analysis can be found in Annex 1 of the present report. A summary of the studies available at that time related to endocrine disruption properties are summarised in the table 8 below.

Table 8

STUDIES INVESTIGATING ENDOCRINE DISRUPTION PROPERTIES OF TPP UNTIL 2017		
Methodology	**Results**	**Reference**
Binding Activity on CHO K1 and COS-7 cells	Binding activity ERα/ERβ – weak agonist ERα/ERβ – not antagonist AR – not agonist AR – not reliable GR – not agonist GR – weak antagonist TRα1/TRβ1 – no activity	*(Kojima et al. 2013)*
Dual Luciferase Reporter Gene Assay Yeast two Hybrid Assay E-Screen Assay Binding Assay Nominal concentration: between 0.00033 – 3.3 mg/L	↑ activation of ERα in a dose-dependent response (REC_{20} of 2.7 x 10^{-7} M = 32.6 ppb) in CHO-K1 ↑ activation of ERα in a dose-dependent response (REC_{20} of 6.5 x 10^{-7} M) ↑ activation of ERα in a dose-dependent response (REC_{20} of 1 x 10^{-6} M) in MCF-7 cells Tight binding affinity for hERα in docking approach, agonist effect	*(Zhang et al. 2014)*
Binding Activity on CHO K1 and COS-7 cells	Binding activity ERα/ERβ – weak agonist ERα/ERβ – not antagonist AR – not agonist AR – not reliable GR – not agonist GR – weak antagonist TRα1/TRβ1 – no activity	*(Kojima et al. 2016)*
H295R hormone transcript (48h) MVLN Luciferase Assay (72h) Nominal concentration: 0 – 0.001 – 0.01 – 0.1 – 1 mg/L	**H295R cells** ↑ in E2 (1 mg/L), T (1 mg/L), E2/T ratio (0.1 and 1 mg/L) ↑ CYP11A1, CYP11B2, CYP19A1 (1 mg/L) ↓in SULT1E, SULT2A1 (1 mg/L) **MVLN Assay**	*(Liu et al. 2012)*

Adult Zebrafish (Danio rerio) – 4 months old Nominal concentration: 0.04 – 0.2 – 1 mg/L Exposure duration: 14 days	↓ in affinity of E2 for ER (0.001 mg/L), ER antagonism. **Female Zebrafish** <u>Plasma sex hormone</u> ↑ in plasma E2 levels, E2/11-KT ratio (1 mg/L) <u>Transcriptional changes</u> ↑ CYP17, CYP19A (1 mg/L). ↓ in *vtg 1* gene expression (1 mg/L). **Male Zebrafish** <u>Plasma sex hormone</u> ↑ in plasma E2 levels, E2/T ratio, E2/11-KT ratio (1 mg/L) ↓ In T, 11-KT (1 mg/L) <u>Transcriptional changes</u> ↑ *vtg* (0.04, 0.2, 1 mg/L), CYP17, CYP19A (1 mg/L)	
Zebrafish embryos/larvae (Danio rerio) – 120 hpf Nominal concentration: 0 – 0.8 – 4 – 20 – 100 – 500 mg/L from 4 to 120 hpf Based on the acute toxicity test: 0 – 0.02 – 0.2 – 2 mg/L from 4 to 120 hpf (hatching and survival assay) and 0.02, 0.2 and 2 mg/L (gene transcription assay).	↓ in hatching and survival (100 and 500 mg/L). ↑ in CYP1A, NCOR2, CYP1B1, PPARα, PPARgc1a, LPL, IL6, PPARg, TRα, RelA, TGFb1, HSP90aa1, 11*β*HSD, EGFR (2mg/L). ↓ in MR and HPSE[2] (2mg/L) No effects on *vtg* genes expression.	*(Liu et al. 2013 (a))*
Zebrafish embryos/larvae (Danio rerio) GH3 (rat pituitary) FRTL-5 (rat thyroid follicular)[3] Nominal concentration: 0 – 0.001 – 0.01 – 0.1 mg/L Exposure duration: 7 days	**GH3** ↑ in *tshβ, trα, trβ, dio1* (100 µg/L TPP) **FRTL-5** ↑ in *nis* (3, 10 mg/L*), tpo* (10 mg/L*), nkx2.1* (1 and 10 mg/L) ↓ In *tshr, tg* (1 mg/L), **Female Zebrafish** ↑ in malformation rate (500 µg/L), T3, T4, *ttr* (40, 200, 500 µg/L)*, trα* (200 µg/L), *dio1 (500 µg/L), nis, tg, ugt1ab* (200, 500 µg/L*)* ↓ in *crh, trβ* (500 µg/L).	*(Kim et al. 2015)*
Adult zebrafish (Danio rerio) – 4/5 months old Nominal concentration: 0 – 0.04 – 0.2 – 1 mg/L Exposure duration: 21 days	**Female Zebrafish** ↓ in egg number, spawning event and hatchability (0.2 and 1 mg/L). <u>Plasma sex hormone and VTG levels</u> ↑ in plasma E2 levels, E2/11-KT ratio (1 mg/L), E2/T ratio, VTG (0.2 and 1 mg/L) ↓ in testosterone, 11-KT (1 mg/L) <u>Transcriptional changes in Ovaries</u>	*(Liu et al. 2013 (b))*

[2] HPSE: heparanase
[3] *In vitro* tests realized at the same time as the experiment done on Zebrafish embryos/larvae.

	↑ LH*β, LHR and FSHR genes, HMGRA, StAR, 17βHSD,* CYP17, CYP19A. ↓ *HMGRB* <u>Transcriptional changes in brain</u> ↓ *GnRH2, GnRH3, FSHβ* **Male Zebrafish** <u>Plasma sex hormone and VTG levels</u> ↑ in plasma E2 levels and E2/11-KT ratio (0.2 mg/L), VTG (1 mg/L) ↓ in E2/T ratio (0.04 and 0.2 mg/L) <u>Transcriptional changes in testis</u> ↓ CYP11A, CYP17, CYP19A, *LHR, HMGRA, StAR, 17βHSD* <u>Transcriptional changes in brain</u> ↓ *GnRH1, GnRH2*, *GnRH3, FSHβ***,** LH*β*	
Larvae, juvenile and adult Zebrafish (Danio rerio) Nominal concentration: 0 – 0.005 – 0.05 – 0.5 mg/L Exposure duration: 120 days	**Female Zebrafish** ↓ in gonadosomatic index (GSI)[4] (5 and 500 µg/L) ↑ in plasma E2 levels (5 and 500 µg/L) ↑ in plasma cortisol (500 µg/L) ↓ in 11-KT (500 µg/L) <u>Transcriptional changes in brain</u> ↑ in *fshβ* (50 and 500 µg/L), *lhβ and gnrh3* (5 and 500 µg/L)*, erα*, *pomc, mr, T4, T3, trhr2* (in brain at 500 µg/L) <u>Transcriptional changes in Ovaries</u> ↑ in *lhr*, *star, CYP19a* in female ovary 500 µg/L (only *star* at 50 µg/L, 2-fold) **Male Zebrafish** ↓ fish condition factor (CF)[5] (500 µg/L) ↑ in plasma E2 levels (5 µg/L) ↑ in plasma cortisol (5 and 500 µg/L) ↓ in 11-KT (5-500 µg/L) <u>Transcriptional changes in brain</u> ↑ in *pomc* and ↓ in *trh* for male brain (500 µg/L) <u>Transcriptional changes in testis</u> ↓ in *star, CYP17* in male testes 500 µg/L and ↑ in *fshr*, *lhr, 3βhsd, 17βhsd*	*(Liu et al. 2016)*
Japanese Medaka (*Oryzias latipes)* OECD 229 Measured concentration (arithmetic mean of weekly measured concentrations): 0.002 – 0.007 – 0.017 – 0.045 mg/L	**Female Medaka** Fish growth affected (0.045 mg/L) ↓ in VTG (0.007, 0.017, 0.045 mg/L) ↓ number of eggs and fertile eggs (0.045 mg/L) **Male Medaka** HSI affected (0.007, 0.017, 0.045 mg/L)	*JMoE, 2012*

[4] Gonadosomatic index (GSI): gonad weight/body weight
[5] The Condition Factor was calculated by 100 x (body weight in g) / (length in cm).

Exposure duration: 21 days		

Other effects: neurotoxic, metabolic and heart development effects

These studies are included in the document to describe others adverse effects mentioned in the literature and potentially endocrine mediated or due to systemic toxicity.

Table 9

STUDIES INVESTIGATION ON OTHER EFFECTS OF TPP UNTIL 2017 (NEUROTOXIC, METABOLIC AND HEART DEVELOPMENT EFFECTS)		
Methodology	**Results**	**Reference**
Other developmental or neurotoxicity, metabolic or cardiotoxicity data on TPP		
Zebrafish embryos (96 hpf)	↓ in body length (1µM TPP from 5.25 to 96 hpf). ↑ in Pericardial edema, effect on developing heart (2-4 µM TPP from 5.25 to 96 hpf). Blocking heart two-chamber (atrium-ventricle) looping at 24-48 hpf at 4µM, resulting in tube-heart phenotype (dioxin-like phenotype) Pharyngula is the most sensitive stage on heart embryogenesis, exposure result on altered cardiac function. TPP induced cardiotoxicity through AHR-independent pathway ↓ in *cyp1a1.*	*(McGee et al. 2013)*
Battery of assay: • C. Elegans larval development • Zebrafish embryo development • Rat acute neurotoxicity (primary rat neocortical structures) Six concentrations around 0.25 log_{10} units	*C. Elegans* larval development: effects from 0.9 µM (decreased growth and modified morphology) Zebrafish embryonic development: effects from 2 µM (malformations such as edema, small head and eyes, curved spines) Acute neurotoxicity in rat: neural network activity altered from 16.3 µM (decreased of extracellular action potentials)	*(Behl et al. 2015)*
Transgenic Zebrafish embryos (Danio rerio, fli1: egfp; 5 hpf), 11 nominal concentrations between 0.05 and 50 µM (0.016 – 16.3 mg/L) Exposure duration: until 72 hpf.	↑ pericardial area (6.25 to 50 µM) ↓ body length (25-50 µM), *cyp26a1*, in RARα, β, γ, TPP acts as antagonist on the retinoic acid receptor (RAR) inducing developmental toxicity.	*(Isales et al. 2015)*
Zebrafish embryos/larvae (120 hpf)	Change in locomotor activity, ↓ activity dark phase, ↑ activity in light phase (0.4-4 µM) Signs of neurotoxicity	*(Jarema et al. 2015)*

Zebrafish (120 hpf)	Default of acclimatation to dark/light phases (64 µM), hypoactivity Death detected at 0.64 µM at 24 hpf and 0.0064 µM at 120 hpf ↑ in edemas	*(Noyes et al. 2015)*
Zebrafish embryos (2 hpf), 96-day exposure (OECD 236) Nominal concentration: 0 – 0.1 – 0.5 – 1 mg/L	↓ in heart rate (0.50 and 1.0 mg/L), cardiac muscle cells, ventricle, and atrium walls thickness, BMP4, NKX2-5, TBX5 genes, ↑ in Sinus Venosus and Bulbus Arteriosus (SV-BA) distance (0.10 – 1.0 mg/L), blocking cardiac looping. Most sensitive window for TPP effect on heart function is 12-60 hpf.	*(Du et al. 2015)*
C. Elegans Development	Impact larval development at 0.16 µM, reproduction at 6.30 µM, feeding at 40 µM. Inhibition of mitochondrial membrane potential at 0.6 µM as a sign of larval development arrest.	*(Behl et al. 2016)*
Zebrafish liver (7 days)	**Metabolomic effects:** disruption in liver: 19 SCMs were significantly changed; involved in carbohydrate metabolism (glucose, UDP-glucose, glycolate, fumarate, succinate, and lactate), lipid and fatty acid metabolism (choline, acetylcarnitine, esterified cholesterol, arachidonic acid [ARA], timnodonic acid [EPA], linoleic acid and fatty acids , amino acid metabolism (glutamate, glutamine and leucine), and osmolyte metabolism (TMAO, dimethylamine [DMA]). **Transcriptional effect:** 471 and 364 DEGs in 0.050 mg/L and 0.300 mg/L TPP, affected the expression of genes related to carbohydrate and lipid metabolism and to the DNA damage repair system (like p53 signaling pathway). Blood test: ↓ glucose, pyruvate, triglyceride, and total cholesterol in 0.050 mg/L and 0.300 mg/L TPP. **Histopathological liver changes:** vacuolization, enlarged sinusoidal vessels, pyknotic nuclei and loss of nuclei,	*(Du et al. 2016)*
Japanese Medaka (Oryzias latipes) Nominal concentration: 0 – 0.005 – 0.025 – 0.125 – 0.625 mg/L Exposure duration: 5 days	↓ in hatchability (dose and time dependent) relative average speed (625 µg/L), heart rate, body length, relative average speed depending on light phase (125; 625 µg/L). ↑ in time to hatch, gross abnormality rate, body length (625 µg/L). Inhibition of AChE activity (125; 625 µg/L) with down-regulation of ache transcription. Down-regulation of 5 biomarkers genes for developmental neurotoxicity (gap43, α1tubulin, mbp, shha, syn2a, elavl3)	*(Sun et al. 2016)*

Other effects can be observed: exposure of Zebrafish to TPP can impair heart development (Du et al. 2015; Isales et al. 2015; McGee et al. 2013). Exposure of fish embryo to TPP impacted their development and led to the generation of cardiac malformations (McGee et al. 2013; Behl et al. 2015). These developmental malformations

were characterized by modifications of the cardiac muscle wall thickness, the cardiac looping and, in Zebrafish and Japanese Medaka, by a decreased heart rate (McGee et al. 2013; Sun et al. 2016; Isales et al. 2015). TPP can also lead to the generation of cardiac/pericardiac edemas (McGee et al. 2013; Noyes et al. 2015). The substance exhibits neurotoxic effects described by modification of the locomotion in Zebrafish embryo and perturbing the dark/light adaptation mechanisms and the activity (Sun et al. 2016; Noyes et al. 2015; Jarema et al. 2015). Moreover, TPP impacts the liver of fish as identified through impairment of the metabolism of glucose and lipid (Du et al. 2016).

In summary

***In vitro* assays also reported increased activation of ERα activity after exposure to TPP** (Zhang et al. 2014). Sex-dependent changes in transcriptional profiles of several genes of the hypothalamic-pituitary-gonadal (HPG), hypothalamic-pituitary-interrenal (HPI) and hypothalamic-pituitary-thyroid (HPT) axes where observed (Liu et al. 2013 (a); Kim et al. 2015; Liu et al. 2013 (b)). In particular, the expression of *era*, *trh, fshβ, T3, T4* genes was modulated after exposure to TPP. The observed effects indicate an **estrogenicity of TPP in female and male Zebrafish**, with increased plasmatic concentrations of E2 (Liu et al. 2012; Liu et al. 2013 (b); Liu et al. 2016) and, in some cases, an increased plasmatic concentration of vitellogenin (VTG) in Liu et al. 2013 (b)or up-regulation of *vtg1* transcription in males (Liu et al. 2012). A down-regulation of *vtg1* gene in female Zebrafish (Liu et al., 2012) and decreased plasma VTG in Medaka females (JMoE, 2012) was observed. Decreased plasmatic concentrations of 11-ketotestosterone (11-KT) were also reported (Liu et al. 2012; Liu et al. 2013 (b); Liu et al. 2016).

Moreover, in Zebrafish and Japanese Medaka, **TPP had a negative effects on fecundity and hatchability in a dose- and time-dependent manner** (Liu et al. 2013 (a); Liu et al. 2013 (b); Sun et al. 2016). It should be noted that the decrease of hatching occurred at 100 and 500 mg/L with juveniles exposed to TPP in Liu et al. 2013 (a). In Liu et al. 2013 (b), the decrease of hatching occurred at 0.2 and 1 mg/L with adult exposed to TPP. Besides, several morphological abnormalities were observed such as edema, small head and eyes, curved spines etc. (McGee et al. 2013; Isales et al. 2015; Du et al. 2015; Du et al. 2016). These abnormalities and decrease of hatching are considered sensitive to, but not diagnostic of EATS according to the ED EFSA/ECHA guidance 2018 (Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009).

Based on the available data, **it was considered that TPP can interfere with the endocrine system and impair reproduction by impacting egg production and hatchability in Zebrafish**. Nevertheless, it was considered that these data were insufficient to conclude that TPP is an endocrine disruptor according to the OECD conceptual framework for testing and assessment of endocrine disruptors as the data available reach only the level 3 of the OECD conceptual framework on endocrine disruptor (OECD, 2018). Additional *in vivo* data, at the populational level for environment, appeared to be needed to fulfil the ED-definition. **Therefore, performing a test following OECD TG 234 guideline was requested as it could answer to the question related to the ED effects.**

In addition, a literature review was performed by the evaluating MSCA in Scopus database **covering the period from January 2018 to June 2022**. With a non-exhaustive search, 781 publications have been found. Among the 781 publications, only 29 were about the environment, 7 were about the ED concerns.

All recent data post-2017 (OECD TG 234 and 7 publications) are summarised in Table 10.

Table 10

UNPUBLISHED STUDY REPORT AND RELEVANT ACADEMIC STUDIES PUBLISHED FROM 2018 TO JUNE 2022		
Methodology	**Results**	**Reference**
Unpublished study report		
OECD 234 (Fish sexual development test – FSDT) Zebrafish (*Danio rerio*) Measured concentration: 1.11 – 3.01 – 7.76 – 33.3 – 76.8 µg/L (TPP in 1L acetone) Exposure duration: 73 days	**Female:** ↑ VTG (33.3; 76.8 µg/L) ↑ 17-βE2 (3.01; 7.76; 33.3; 76.8 µg/L) **Male:** ↑ VTG (33.3 µg/L) ↑ 11-KT (3.01; 7.76; 33.3; 76.8 µg/L) ↑ Testis maturation stage (76.8 µg/L) No significant change in sex ratio	*Unpublished study report (2021)*
Academic studies		
Japanese quail (*Coturnix japonica*) Exposition by *in ovo* Nominal concentration: 5 – 50 – 100 ng/g (8.3 – 46 – 97.2 ng/g	↓ body length (50 – 100 ng/g) ↓ FT3 in female (100 ng/g) ↓ Epithelial cell height (ECH); thyroid gland activity (ECH:CD) in females (5 – 50 – 100 ng/g) ↑ colloid diameter in males (100 ng/g) ↓ resting metabolic rate in males and females (50 – 100 ng/g)	*Guigueno et al. (2019)*
Japanese Medaka (*Oryzias latipes*) – only females Nominal concentration: 1.6 – 8 – 40 µg/L (131 – 363 – 1773 ng/L) Short-term exposure: 21 days Long-term exposure:100 days	**Female only** <u>After 21 days of exposure</u>: ↑ in plasma 17β-E2 (1.77 µg/L) ↑ in plasma T (1.77 µg/L) ↓ in *vtg2* expression (1.77 µg/L) No change in oocyte maturation, vtg1 & 11-KT <u>After 100 days of exposure</u>: ↓ in plasma 17β-E2 (1.77 µg/L) ↓ in plasma T & 11-KT (all concentrations) ↓ in vtg1 expression (all concentrations) ↓ in vtg2 expression (0.363 – 1.77 µg/L) ↓ number of mature oocyte (all concentrations) ↓ egg production (0.363 – 1.77 µg/L)	*Li et al. (2019)*
Zebrafish adult (*Danio rerio*) Concentration: 0 – 0.04 – 0.2 – 1 mg/L Exposure duration: 14 days	**Female** ↓ crh ; tshβ ; (1 mg/L) ↑ dio2 ; trα ; T4 ; T3 (1 mg/L) **Male** ↑ tshβ (1 mg/L) ↑ crh ; dio 1 (0,2 – 1 mg/L) ↓ dio2 ; trβ (0,2 – 1 mg/L) ↓ T4 & T3 (1 mg/L)	*Liu et al. (2019)*
Zebrafish adult (*Danio rerio*)*:* 5-month adult <u>Acute toxicity</u> Concentration: 0 – 0.5 – 0.7 –	**Female:** ↑ HSI ↑ GSI ↓ Ovary maturation stages: oocytes less mature, with more oocytes at the first two stages. Structure of oocytes more irregular. Inhibition of the ovarian development.	*He et al. (2021)*

1 – 1.2 – 1.5 mg/L Exposure duration: 96h <u>Sub-chronic toxicity (10% of LC_{50})</u> Concentration: 80 µg/L (TPP in DMSO, 0.01% v/v). Exposure duration: 21 days	↓ E_2/T ↓ VTG **Male:** ↑ HSI ↓ GSI ↓ Testis maturation stages (↑ number of immature spermatocytes and ↓ mature spermatocytes). Reproductive parameters: ↓ Fertilization rate ↓ Spawning ↓ cumulative eggs ↓ hatching rate	
Japanese Medaka transgenic (*Oryzias latipes*) – Only male Nominal concentration: 1.6 – 8 – 40 µg/L (134.1 – 299.1 – 1429.5 ng/L) Exposure duration: 100 days	**Male only** ↑ gonadal intersex incidence (1.43 µg/L) ↓ 11-KT & T (1.43 µg/L) ↑ 17β-E2 (0.299 – 1.43 µg/L) Antagonistic activity ↑ severity of abnormal chasing behaviors ↑ courtship behavior ↓ successful mating rate	*Li et al. (2018)*
Amphibians tadpoles (*Hoplobatrachus rugulosus*) • Acute toxicity (96h) • Survival and metamorphosis rate (two weeks) Stade GS35 (Gosner stade) Acute concentration: 1.8 – 2.0 – 2.2 – 2.4 – 2.6 – 2.8 and 3.0 mg/L Sub-chronic concentration: 10 – 50 – 100 – 200 µg/L	<u>Acute toxicity:</u> EC10 = 289 µg/L <u>Sub-chronic toxicity:</u> ↓ survival (50 µg/L) & metamorphosis rates (100 µg/L) ↑ metamorphosis time ↓ SOD & *CuZn-sod* expression (100 – 200 µg/L) ↓ CAT & *cat* expression (100 – 200 µg/L)	*Chen et al. (2022)*
Zebrafish embryos (*Danio rerio*) 1 – 3 – 10 – 30 µM (0.32 – 0.97 – 3.2 – 9.8 mg/L) Exposure duration: 24h	↓ trα (9,8 mg/L) ↓ ttr (9,8 mg/L) ↓ tshβ (9,8 mg/L) ↓ dio1 (3,2 – 9,8 mg/L) ↑ dio2 (3,2 mg/L)	*Lee et al. (2022)*

In the aim of assessing whether TPP fulfils the definition of an endocrine disruptor, a focus has been made on the assessment of studies in which an adverse effect relevant to the assessment of ED properties was observed. Therefore, the OECD 234 study and 2 recent publications (Li et al. 2019 and He et al. 2021) are further described below in details.

The results of the **OECD TG 234 study (Unpublished study report, 2021)** are presented below:

This study aimed to assess the potential endocrine activity and adverse effects of continuous exposure to TPP for 73 days i.e., on the early life stages and sexual differentiation of Zebrafish. Thirty fertilised eggs were used for each test with 4 replicates per concentration. Endpoints that were determined included hatching success and rates, mortalities during the early life stage and the juvenile growth and maturation phase. Sex

ratio was determined macroscopically and by histological examination of the gonads. The steroid hormone 11-ketotestosterone and 17β-estradiol were measured by blood plasma samples respectively for male and female fish and VTG for all fish. Measurements were performed at the end of the study. Additionally, a histopathological examination was performed on the gonads and liver tissues. The exposure doses showed a significant deviation (i.e., 80% or 120%) from the nominal concentrations, the authors report the results in mean measured concentrations of 0; 1.11; 3.01; 7.76; 33.3 and 76.8 µg/L. According to the OECD TG 234 guidelines, all the validity criteria were met.

According to the report, the survival rates observed were 87.5%; 84.2%; 80.0%; 76.7%; 63.3% and 50.8% (control group; 1.11; 3.01; 7.76; 33.3 and 76.8 µg/L respectively). Mortality was mainly observed during the early life stage of Zebrafish until 35 days post-fertilization (dpf), with only few cases of mortality between 35 dpf and test termination (73 dpf). According to the OECD TG 234 guideline, if exposure related mortality occurs, the number of replicates should be reduced appropriately so that fish density between treatment levels is kept as equal as possible, as fish density is extremely important for growth and development. Unfortunately, this reduction of replicate has not been performed at the two highest concentrations where mortality occurs.

A range-finding study was performed at 1.0; 10 and 100 µg/L (measured concentrations: 0.93; 9.18 and 88.3 µg/L respectively) to define a maximum tolerated concentration (MTC). **No effect on hatching success but a reduced fish numbers were observed at 88.3 µg/L**. This concentration was therefore used as the maximum tolerable concentration (MTC) for the main study. The low survival rate observed at the two highest doses in the main study was therefore not expected at this level of dose.

During the early life stage, reduced growth in terms of total length was observed at the highest tested concentration. This reduced size was likely due to the general systemic toxicity of TPP.

However, at 73 dpf, a subsequent exposure to TPP led to the increase in mass of the exposed fish in a concentration-dependent manner with a statistically significant higher for the highest dose in males and the two highest in females (581 mg at the highest dose compared to 454 mg in the controls for females). This increase was likely due to reduction of fish density in treatment conditions, resulting from the reduced post-hatch survival rates. The increase in mass should have been avoided by the reduction of the number of replicates, which has not been done. These variability of the environmental conditions among the groups is impairing the ability of the test to conclude about an adverse effect in terms of population development or sex-ratio.

Regarding the endocrine activity-related endpoints, measured at the end of the exposure only, **a dose-dependent increase in plasma E2 levels was measured in females** (no measurements on males). A significant difference is reported for the highest concentration (x2 compared to the control). A **very strong dose-dependent increase in 11-KT was also measured in treated males** (x3 at the highest dose) although not statistically significant according to the authors. For VTG, no statistically significant differences to controls were retrieved at any treatment level in both sexes. The statistical analyses for these endocrine activity-related endpoints were based on averages of the four replicates for each concentration. When considering all individual measurements for each endpoint, **there is a significant increase of VTG in males at 33.3 µg/L and in females from 33.3 µg/L.** For the steroid hormones, **there is a significant increase from 3.01 µg/L for 11-KT in males and E2 in females**.

Regarding the apical effect, namely sex ratio, no significant difference was observed, but it should be noted that there was a high proportion of females in the control group (64,8%) compared to exposure groups; even if the acceptance criteria related to proportion of sex at termination of the test (30% males – 70% females) is fulfilled. The percentage of females for all the exposed fish increased from 52.6 to 65.4% while the percentage of males decreased from 46.3 and 33.3% according to dose. The number of undifferentiated intersexes is not significantly different (there is only one undifferentiated fish at the 1.11

and 76.8 µg/L concentrations). Importantly, **survival rate may have altered the statistical power of the study to detect a significant effect on sex ratio in these groups**. Given the high mortality rate at high doses and the low percentage of males in those sample, a deficit of males is observed for these high doses, which may alter the representativeness and therefore the interpretation of the measurements performed in males at these doses. It is also not possible to determine whether the mortality affected one sex more specifically (no sex indication was reported for the dead fish), which may have interfered with the assessment of sex ratio.

The histopathological analysis of fish gonad revealed that with increasing concentrations, **the gonads reached a mature stage more rapidly (for males and females but statistically significant at the highest concentration for male only).** The proportion of stage 2 (the most advanced stage found) increases from 55 to 90% for testes. Stage 4 in females increases from 15 (control) to 30% (highest dose). The acceleration in gonadal maturation is consistent with the elevated circulating steroid levels.

Concerning the histological analyses of female's gonads, even though only two doses (3.33 and 76.8 µg/L) and the control group were analysed, a dose-dependent increase in all ovarian pathologies (oocyte atresia, egg debris, granulomatous inflammation) is observed but without statistical significance in any group. These pathologies are indicative of the alteration (acceleration) of the oocyte maturation process and oocyte quality, although not statistically significant.

Regarding males, only the formation of testis-ova was measured which was slightly decreased (not statistically significant) with combined acceleration of maturation of the gonad. However, the percentage of testis-ova male in the control is high (11%) which makes difficult the interpretation of the other doses. Moreover, although testis-ova formation will likely lead to a decrease in fertility or hatching success, the design of FSDT precludes this type of examination since FSDT *per se* did not include the production of a next generation.

In addition to gonad histopathological analysis, liver and heart were examined. Liver from all test groups was examined while for the heart, only two groups were examined (control and 76.8 µg/L). No statistically significant effect on the severity of the hepatic lesions was observed. Nevertheless, liver histopathological analysis revealed a dose-dependent decrease in hepatocyte lipid inclusions in females. In male, a dose-dependent increase in bile duct proliferation and inflammatory foci was observed. The analysis of the liver may reveal a toxic effect, or a more specific effect of lipid metabolism seen in females. For cardiac lesions, no statistics could be performed due to the limited number of replicates and test concentrations.

Overall, this OECD study level 4 (according to the OECD conceptual framework Guidance Document 150 (OECD, 2018)) did not allow a clear identification of EATS-mediated adverse effects, while noting that deviations in the experimental conduct could have impaired the capacity of the test to demonstrate such effect. Nevertheless, mechanistic parameters such as VTG measurements done at the end of the study (73 days) were significantly increased at 33.3 µg/L as well as significant increase of 11-KT from 3.01 µg/L in males and E2 in females. The VTG changes may result, at least partially, from the estrogenic action of TPP. An induction of vitellogenin synthesis was measured in males at 33.3 µg/L but not at the highest dose. A general toxic effect (most of the death occurs at the first day of development) could explain this absence of VTG synthesis at the highest concentration. Other hypotheses should be considered, such as an action on the enzymes of steroidogenesis, especially considering the increase in plasma androgen concentration namely 11-KT. The increasing in 11-KT with the increasing in VTG in male could be explained by a compensatory response. The absence of effect on sex-ratio and the acceleration in gonadal maturation for males (as only statistically significant effect observed) do not allow to conclude on EATS-mediated adverse effects (or not) by TPP exposure in this study.

Finally, it is also important to remember that this experiment was marked by an unexpected systemic effect among exposed groups with a high mortality incidence during the first (21) days of development without any redistribution of the fish which would have allowed to maintain an equal number of fish across the study. These systemic effects may have altered the physiology of the fish and led to other subsequent reaction which may not be representative of a "normal" situation.

In addition of the FSDT study, a focus has been made on the publications where adverse effect on reproduction were observed.

Li et al. (2019) studied the effects of TPP for 21 days on adult (3-month-old) transgenic fish (pMOSP1-EGFP) and on the reproduction of Japanese Medaka by exposure from egg hatch to 100 days (FSDT-like test). Medaka (50 individuals, duplicate experiment) were exposed to TPP in a continuous flow system at 1.6; 8 and 40 µg/L (measured concentrations: 0.131; 0.363, and 1.77 µg/L) with 0.001% DMSO (+ solvent control). A 21-day test was performed with two replicates of 8 female Medakas. Reproduction test was performed with 6 exposed females paired with control males. Oocyte maturation was quantified by a double measurement of oocyte size and *osp1* gene expression by fluorescence. The concentration of the test compound in water was monitored by UPLC-MS/MS measurements of TPP, and two metabolites (DPhP and 4-OH-TPP) were identified.

After 21 days of exposure, no significant effect on oocyte maturation was observed, nor on the hepatic expression of *vtg1* and *2*, except for a decrease in *vtg2* at the highest dose (1.77 µg/L). However, a dose-dependent increase in plasma E2 (significant at the highest dose – 1.77 µg/L) and T (significant at the highest dose – 1.77 µg/L but not 11-KT) concentration was measured.

After 100 days of exposure, **oocytes undergo a delay in maturation characterised by a significant dose-dependent decrease in the number of mature and pre-vitellogenic oocytes** with more than one third of affected females. The highest doses induce more substantial effects involving the **absence of oocyte II** (stage 3). These alterations are accompanied by a **dose-dependent decrease in plasma E2 concentration (significant only at the highest dose), a decrease in plasma T and 11-KT concentration (significant for all concentrations) and a dose-dependent decrease in hepatic expression of the *vtg1* (significant at all doses) and *vtg2* (significant at the two highest doses) genes, in line with previous results of another lab** (JMoE, 2012). Cumulative egg production over three days decreased in a dose-dependent manner and was significantly affected at the two highest doses (with 39 and 51% decrease respectively). The effects observed on egg production and VTG were in line with previous results of another lab (JMoE, 2012). The concentration of TPP is higher in the liver and the ovary than the concentration in muscle and brain. This suggests a potential reproductive toxicity of TPP to females.

In conclusion, the experiment shows that TPP (and/or its metabolites) causes a decrease in fecundity in Medaka after 100 days of exposure to very low concentrations. The authors hypothesised that anti-ER activity would inhibit E2 activity during maturation, reducing it and, in turn, would lead to a delayed maturation and subsequent decrease in plasma steroids in the long term. To this end, the authors show that the metabolite 4-OH-TPP (but not TPP) is able to inhibit E2 activity in the transgenic Medaka model. Finally, the inhibition of maturation would contribute to the decrease in plasma E2.

He et al. (2021) studied acute toxicity with some endocrine indices and effects on reproduction of TPP on adult Zebrafish. Acute toxicity was studied over 96h with a range of concentrations: 0, 0.5, 0.7, 1, 1.2, 1.5 mg/L dissolved with 0.01% v/v DMSO (nominal concentrations, no measurements) on triplicates of 10 fish. The median toxicity (LC50) was calculated to be 976 µg/L. The following studies were conducted with a single dose corresponding to less than 10% of the LC50, *i.e.*, 80 µg/L. The tests included groups of 10 separate male or female fish for 21 days.

In accordance with the acute toxicity test and in line with the range-finding experiment of the FSDT test, exposure to 80 µg/L TPP did not lead to any mortality of the Zebrafish for 21 days.

Exposure to TPP induced a statistically significant increase in the hepatosomatic index (HSI) by a factor of 1.8 and 2.2 for males and females, respectively, as well as a decrease in the gonadosomatic index (GSI) in males and an increase in females (not quantified). These factors are accompanied by histological changes. The testes and ovaries show an increase in the early stages of sex cells and, conversely, a decrease in the more developed stages in both sexes indicating an inhibition of gametogenesis (based on qualitative histological observations).

No effect on plasma hormone levels (T and E2) was observed, although the E2/T ratio was significantly decreased in exposed females. No change in VTG is noted in males but a decrease is observed in females. Exposure to TPP significantly decreased the number of eggs laid (by about -20%), without significant consequences on the fertilisation and hatching rate of the remaining eggs.

In conclusion, the study indicates that TPP (80 µg/L) alters gametogenesis in both sexes of adult Zebrafish resulting in decreased fecundity. The endocrine mechanism could be related to the modulation of the E2/T ratio observed in females.

Conclusion

To give an overall overview of the statistically significant outcomes obtained pending on the duration (21 days to 73 or 100 days) and the period of exposure (juvenile *versus* adult), the following table summarizes all the relevant information available. All the measurements were performed at the adult stage and the reported concentration are those for which the measured parameters are significant.

Table 11

TABLE SUMMARIZING ENDOCRINE DISRUPTION ACTIVITIES OF TPP (ONLY STATISTICALLY SIGNIFICANT OUTCOMES ARE REPORTED)		
Exposition	**Male**	**Female**
Adult exposure – Short term study	• **Zebrafish** Plasma sex hormone and VTG levels ↑ E2 (0.2 mg/L) – *Liu et al. (2013 (b))* ↑ E2/11-KT ratio (0.2 mg/L) *Liu et al. (2013 (b))* ↓ E2/T ratio (0.04 and 0.2 mg/L) – *Liu et al. (2013 (b))* ↑ VTG (1 mg/L) – *Liu et al. (2013 (b))*	• **Zebrafish** Plasma sex hormone and VTG levels ↑ E2 (1 mg/L) – *Liu et al. (2013 (b))* ↓ T, 11-KT (1 mg/L) – *Liu et al. (2013 (b))* ↑ E2/11-KT ratio (1 mg/L) – *Liu et al. (2013 (b))* ↓ E2/T ratio (80 µg/L) – *He et al. (2021)* ↑ E2/T ratio (0.2 and 1 mg/L) – *Liu et al. (2013 (b))* ↓ VTG (80 µg/L) – *He et al. (2021)* ↑ VTG (0.2 and 1 mg/L) – *Liu et al. (2013 (b))* Reproductive parameters

	<u>Reproductive parameters</u> ↑ HSI (80 µg/L) – *He et al. 2021* ↓ GSI (80 µg/L) – *He et al. 2021* ↓ Testis maturation stages (↑ amount of immature spermatocytes and ↓ mature spermatocytes) – *He et al. 2021* • **Japanese Medaka** HSI affected (0.007; 0.017 and 0.045 mg/L) – *JMoE, 2012*	↑ HSI – *He et al. (2021)* ↑ GSI – *He et al. (2021)* ↓ Ovary maturation stages: oocytes less mature, with more oocytes at the first two stages. Structure of oocytes more irregular. Inhibition of the ovarian development – *He et al. (2021)* ↓ Fertilization rate – *He et al. (2021)* ↓ Spawning – *He et al. (2021)* ↓ Cumulative eggs – *He et al. (2021)* ↓ Hatching rate – *He et al. (2021)* ↓ In egg number, spawning event and hatchability (0.2 and 1 mg/L) – *Liu et al. (2013 (b))* • **Japanese Medaka** <u>Plasma sex hormone and VTG levels</u> ↑ 17β-E2 (1.77 µg/L) – *Li et al. (2019)* ↑ T (1.77 µg/L) – *Li et al. (2019)* ↓ VTG (0.007; 0.017 and 0.045 mg/L) – *JMoE, 2012* ↓ *vtg2* (1.77 µg/L) – *Li et al. (2019)* <u>Reproductive parameters</u> ↓ egg number (0.045 mg/L) – *JMoE, 2012*
Juvenile exposure – Long term study	• **Zebrafish** <u>Plasma sex hormone and VTG levels</u> ↑ E2 (5 µg/L) – *Liu et al. 2016* ↑ 11- KT (3.01 ; 7.76 ; 33.3 ; 76.8 µg/L) – FSDT ↓ 11-KT (5-500 µg/L) – *Liu et al. 2016* ↑ Cortisol (5 and 500 µg/L) – *Liu et al. 2016* ↑ VTG (33.3 µg/L) – FSDT <u>Reproductive parameters</u> ↑ Testis maturation stage (76.8 µg/L) - FSDT	• **Zebrafish** <u>Plasma sex hormone and VTG levels</u> ↑ 17-E2 (3.01; 7.76; 33.3; 76.8 µg/L) – FSDT ↑ E2 (5 and 500 µg/L) – *Liu et al. 2016* ↓ 11-KT (500 µg/L) – *Liu et al. 2016* ↑ Cortisol (500 µg/L) – *Liu et al. 2016* ↑ VTG (33.3; 76.8 µg/L) – FSDT ↓ (GSI) (5 and 500 µg/L) – *Liu et al. 2016*

	↓ Fish condition factor (CF)[6] (500 µg/L) – *Liu et al. 2016*	• **Japanese Medaka** <u>Plasma sex hormone and VTG levels</u> ↓ 17β-E2 (1.77 µg/L) – *Li et al. (2019)* ↓ T & 11-KT (all concentrations) – *Li et al. (2019)* ↓ *vtg1* (all concentrations) – *Li et al. (2019)* ↓ *vtg2* (0.363 – 1.77 µg/L) – *Li et al. (2019)* <u>Reproductive parameters</u> ↓ Number of mature oocyte (all concentrations) – *Li et al. (2019)* ↓ Egg production (0.363 – 1.77 µg/L) – *Li et al. (2019)*

All available studies demonstrate that TPP always exerts an effect on the endocrine balance in fish. Depending on the developmental stage, species and concentration, antagonistic and agonistic effects were observed in organisms, leading *in vivo* to modulations of circulating steroid concentrations. With the exception of the studies by He et al. 2021 (no change) and Li et al. 2019 (decrease), TPP induces during medium-term exposure an increase in circulating estradiol concentrations in adult Zebrafish and Medaka (Liu et al. 2013 (b); Liu et al. 2016; Li et al. 2019) and a decrease in 11-KT. Reproductive studies show an alteration of gametogenesis with a reduction of mature testis and ovary stages (except in the FSDT) followed by a decrease in fertility (not measured in the FSDT) and reproductive success (He et al. 2021; Li et al. 2019 and Li et al. 2021). Although these reproduction parameters are sensitive to substances interfering with sex hormone system, they are not considered as "EATS-mediated" as they might be influenced by non-endocrine factors such as systemic toxicity. Nevertheless, they can be used in a weight of evidence approach to draw a conclusion on a specific endocrine pathway. It should be noted that the quality of the FSDT study did not allow to evaluate properly EATS mediated parameters.

It is important to note that the TPP has also an effect on the retinoic acid receptor (RAR). TPP exhibits weak RARα antagonistic activity (Jia et al. 2022; Isales et al. 2015) and leads to ocular and cardiovascular malformations. The RAR signalling pathway is essential for reproduction and embryonic development. Indeed, genetic studies in Zebrafish embryos that are deficient in RA (retinoic acid)-generating enzymes or RARs revealed that RA signalling regulates development on many organs and tissues, including the body axis, spinal cord, forelimb buds, skeleton, heart, eye, pancreas, lung and during the spermatogenesis (Ghyselinck et al. 2019; Clagett-Dame and DeLuca, 2002). There are many additional functions for RA that are supported by *in vivo* genetic loss-of-function studies in Zebrafish. Further studies are needed to identify the key genes regulated by RA signalling. This molecular initiating event may be related to endocrine effects on development. However, in the practice of regulatory evaluation and in the current testing strategies for the detection of ED and their identification, this receptor is still little considered.

[6] The Condition Factor was calculated by 100 x (body weight in g) / (length in cm).

7.10.2. Endocrine disruption - Human health

Not evaluated by FR-MSCA in the context of this SEV.

A 'one-generation reproductive toxicity study' is currently ongoing under the auspices of the US NTP.

7.10.3. Conclusion on endocrine disrupting properties

TPP shows endocrine activity on non-target organisms with adverse effects on fertility and reproduction in academic studies. Those adverse effects can be related to the disturbances in steroid synthesis and gamete quality. Unfortunately, due to some limits of the FSDT study (high mortality rate for all doses tested which may alter the interpretation of results) and the study design (no reproduction endpoints measured), the FSDT does not bring any clarification about this mode of action. The ED EG advice indicated support for the identification of this substance as an endocrine disruptor for the environment using a weight-of-evidence approach (ECHA, 2022). Based on a weight of evidence approach and the available guidance for the identification of endocrine disruptors, including ANSES Endocrine Disruptor (ED) expert Working Group criteria for classification (ANSES, 2016), the eMSCA considers that TPP should be categorized for its endocrine disruptor properties. According to the categories laid down in its' opinion (ANSES, 2016), TPP should be categorized as a presumed endocrine disruptor. Furthermore, the eMSCA considers that TPP should be identified as SVHC for its ED properties for the environment.

Lastly, the 'one-generation reproductive toxicity study' currently being undertaken under the auspices of the US NTP, whose protocol was completed after discussion with ANSES, will presumably contribute to reduce the remaining uncertainties. Pending on its outcomes, an update of the proposed SVHC identification for endocrine properties for human health might be considered if deemed necessary.

7.11. PBT and VPVB assessment

Not evaluated by FR-MSCA in the context of this SEV.

7.12. Exposure assessment

Not evaluated by FR-MSCA in the context of this SEV.

A full exposure assessment was not undertaken by the eMSCA in the context of this substance evaluation. However, a release to the environment is likely based on the information available in the registration dossier indicating that the substance has wide dispersive uses and consumer uses (see Section 7.5 above).

7.13. Risk characterisation

Not evaluated by FR-MSCA in the context of this SEV.

7.14. References

ANSES, 2016. Avis relatif à la définition de critères scientifiques définissant les perturbateurs endocriniens – saisine 2016-SA-0133 – juillet 2016

Ballesteros-Gómez A, Covaci A, Leonards PEG, Reiner EJ, de Boer J. 2017. Identification of impurities of phosphate and brominated flame retardants. Presentation (ref. OP-09) at Brominated Flame Retardants 2017, BFR-2017, conference.

Behl, Mamta, Jui-Hua Hsieh, Timothy J. Shafer, William R. Mundy, Julie R. Rice, Windy A. Boyd, Jonathan H. Freedman, et al. 2015. "Use of Alternative Assays to Identify and Prioritize Organophosphorus Flame Retardants for Potential Developmental and Neurotoxicity." *Neurotoxicology and Teratology* 52 (Pt B): 181–93. doi:10.1016/j.ntt.2015.09.003.

Behl, Mamta, Julie R. Rice, Marjo V. Smith, Caroll A. Co, Matthew F. Bridge, Jui-Hua Hsieh, Jonathan H. Freedman, and Windy A. Boyd. 2016. "Comparative Toxicity of Organophosphate Flame Retardants and Polybrominated Diphenyl Ethers to Caenorhabditiselegans." *Toxicological Sciences* 154 (2): 241–52. doi:10.1093/toxsci/kfw162.Brooke, D N, M J Crookes, P Quarterman, and Simon Burns. 2009. "Environmental Risk Evaluation Report: Triphenyl Phosphate (CAS No. 115-86-6)." SCHO0809BQUK–E–P. http://nora.nerc.ac.uk/8615/.

Chen, J. Y., H. L. Hu, L. Feng, et G. H. Ding. 2022. "Ecotoxicity assessment of triphenyl phosphate (TPhP) exposure in Hoplobatrachus rugulosus tadpoles." Chemosphere 292:133480. doi: 10.1016/j.chemosphere.2021.133480.

Clagett-Dame, M. and DeLuca, H. F. (2002). The role of vitamin A in mammalian reproduction and embryonic development. Annu. Rev. Nutr. 22, 347-381. doi:10.1146/annurev.nutr.22.010402.102745ECristale J, Katsoyiannis A, Sweetman AJ, Jones KC, Lacorte S. 2013. Occurrence and risk assessment of organophosphorus and brominated flame retardants in the River Aire (UK). Environmental Pollution 179 (2013) 194-200

Du, Zhongkun, Guowei Wang, Shixiang Gao, and Zunyao Wang. 2015. "Aryl Organophosphate Flame Retardants Induced Cardiotoxicity during Zebrafish Embryogenesis: By Disturbing Expression of the Transcriptional Regulators." *Aquatic Toxicology* 161: 25–32. doi:10.1016/j.aquatox.2015.01.027.

Du, Zhongkun, Guowei Wang, Shixiang Gao, and Zunyao Wang. 2015. "Aryl Organophosphate Flame Retardants Induced Cardiotoxicity during Zebrafish Embryogenesis: By Disturbing Expression of the Transcriptional Regulators." Aquatic Toxicology 161: 25–32. doi:10.1016/j.aquatox.2015.01.027.

Du, Zhongkun, Yan Zhang, Guowei Wang, Jianbiao Peng, Zunyao Wang, and Shixiang Gao. 2016. "TPhP Exposure Disturbs Carbohydrate Metabolism, Lipid Metabolism, and the DNA Damage Repair System in Zebrafish Liver." Scientific Reports 6 (February). doi:10.1038/srep21827.

ECHA, 2022. Summary report of the 23rd ED expert group meeting: b03ea820-5fee-03fe-c7d7-09e8aed422a8 (europa.eu)

ED EFSA/ECHA guidance 2018 (Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009).

Environment Agency 2009. Brooke D N, Crookes M J, Quarterman P and Burns J. Environmental risk evaluation report: Triphenyl phosphate (CAS no. 115-86-6). 2009. Environment Agency, Bristol, UK.

Ghyselinck, N. B., et G. Duester. 2019. "Retinoic acid signaling pathways." Development 146 (13). doi: 10.1242/dev.167502.

Greaves AK, Letcher RJ, Chen D, McGoldrick DJ, Gauthier LT, Backus SM. 2016. Retrospective analysis of organophosphate flame retardants in herring gull eggs and relation to the aquatic food web in the Laurentian Great Lakes of North America. Environmental Research 150 (2016) 255–263

Greaves AK, Letcher RJ (2016). A Review of Organophosphate Esters in the Environment from Biological Effects to Distribution and Fate. Bull Environ Contam Toxicol DOI 10.1007/s00128-016-1898-0.

Guigueno, M. F., J. A. Head, R. J. Letcher, N. Karouna-Renier, L. Peters, A. M. Hanas, et K. J. Fernie. 2019. "Early life exposure to triphenyl phosphate: Effects on thyroid function, growth, and resting metabolic rate of Japanese quail (Coturnix japonica) chicks." Environ Pollut 253:899-908. doi: 10.1016/j.envpol.2019.05.110.

Hallanger IG, Sagerup K, Evenset A, Kovacs KM, Leonards P, Fuglei E, Routti H, Aars J, Strøm H, Lydersen C, Gabrielsen G. 2015. Organophosphorous flame retardants in biota from Svalbard, Norway. Marine Pollution Bulletin 101 (2015) 442–447

He, J., X. Yang, et H. Liu. 2021. "Enhanced toxicity of triphenyl phosphate to zebrafish in the presence of micro- and nano-plastics." Sci Total Environ 756:143986. doi: 10.1016/j.scitotenv.2020.143986.

Isales, Gregory M., Rachel A. Hipszer, Tara D. Raftery, Albert Chen, Heather M. Stapleton, and David C. Volz. 2015. "Triphenyl Phosphate-Induced Developmental Toxicity in Zebrafish: Potential Role of the Retinoic Acid Receptor." *Aquatic Toxicology* 161: 221–30. doi:10.1016/j.aquatox.2015.02.009.

Japanese Ministry of Environment. 2012. Two page English summary of the Tier 1 in vivo Test of Triphenyl phosphate (CAS no. 115-86-6). Unpublished.

Jarema, Kimberly A., Deborah L. Hunter, Rachel M. Shaffer, Mamta Behl, and Stephanie Padilla. 2015. "Acute and Developmental Behavioral Effects of Flame Retardants and Related Chemicals in Zebrafish." Neurotoxicol Teratol. 2015 Nov-Dec;52(Pt B):194-209. doi: 10.1016/j.ntt.2015.08.010.

Jia, Y., H. Zhang, W. Hu, L. Wang, Q. Kang, J. Liu, T. Nakanishi, Y. Hiromori, T. Kimura, S. Tao, et J. Hu. 2022. "Discovery of contaminants with antagonistic activity against retinoic acid receptor in house dust." J Hazard Mater 426:127847. doi: 10.1016/j.jhazmat.2021.127847.

Kim J-W, Isobe T, Chang K-H, Amano A, Maneja RH, Zamora PB, Siringan FP, Tanabe S. 2011. Levels and distribution of organophosphorus flame retardants and plasticizersin fishes from Manila Bay, the Philippines. Environmental Pollution 159 (2011) 3653 – 3659

Kim, Sujin, Joeun Jung, Inae Lee, Dawoon Jung, Hyewon Youn, and Kyungho Choi. 2015. "Thyroid Disruption by Triphenyl Phosphate, an Organophosphate Flame Retardant, in Zebrafish (Danio Rerio) Embryos/Larvae, and in GH3 and FRTL-5 Cell Lines." *Aquatic Toxicology* 160 (March): 188–96. doi:10.1016/j.aquatox.2015.01.016.

Kojima H, Takeuchi S, Itoh T, Iida M, Kobayashi S, Yoshida T. 2013. In vitro endocrine disruption potential of organophosphate flame retardants via human nuclear receptors. Toxicology 314 (2013) 76– 83

Kojima H, Takeuchi S, Van den Eede N, Covaci A. 2016. Effects of primary metabolites of organophosphate flame retardants on transcriptional activity via human nuclear receptors. Toxicology Letters 245 (2016) 31–39

Krivoshiev BV, Dardenne F, Covaci A, Blust R, Husson SJ. Assessing in-vitro estrogenic effects of currently-used flame retardants. 2016. Toxicology in Vitro, Vol. 33, 153–162

Lee, J. S., Y. K. Kawai, Y. Morita, A. Covaci, et A. Kubota. 2022. "Estrogenic and growth inhibitory responses to organophosphorus flame retardant metabolites in zebrafish embryos." Comp Biochem Physiol C Toxicol Pharmacol 256:109321. doi: 10.1016/j.cbpc.2022.109321.

Li, Y., C. Wang, F. Zhao, S. Zhang, R. Chen, et J. Hu. 2018. "Environmentally Relevant Concentrations of the Organophosphorus Flame Retardant Triphenyl Phosphate Impaired Testicular Development and Reproductive Behaviors in Japanese Medaka (Oryzias latipes)." Environmental Science and Technology Letters 5 (11):649--654. doi: 10.1021/acs.estlett.8b00546.

Li, Y., R. Chen, J. He, H. Ma, F. Zhao, S. Tao, J. Liu, et J. Hu. 2019. "Triphenyl Phosphate at Environmental Levels Retarted Ovary Development and Reduced Egg Production in Japanese Medaka (Oryzias latipes)." Environmental Science and Technology 53 (24):14709--14715.

Liu, Xiaoshan, Kyunghee Ji, and Kyungho Choi. 2012. "Endocrine Disruption Potentials of Organophosphate Flame Retardants and Related Mechanisms in H295R and MVLN

Cell Lines and in Zebrafish." *Aquatic Toxicology* 114–115: 173–81. doi:10.1016/j.aquatox.2012.02.019.

Liu, Chunsheng, Qiangwei Wang, Kang Liang, Jingfu Liu, Bingsheng Zhou, Xiaowei Zhang, Hongling Liu, John P. Giesy, and Hongxia Yu. 2013(a). "Effects of Tris(1,3-Dichloro-2-Propyl) Phosphate and Triphenyl Phosphate on Receptor-Associated MRNA Expression in Zebrafish Embryos/Larvae." *Aquatic Toxicology* 128–129 (March): 147–57. doi:10.1016/j.aquatox.2012.12.010.

Liu, Xiaoshan, Kyunghee Ji, Areum Jo, Hyo-Bang Moon, and Kyungho Choi. 2013(b). "Effects of TDCPP or TPP on Gene Transcriptions and Hormones of HPG Axis, and Their Consequences on Reproduction in Adult Zebrafish (Danio Rerio)." *Aquatic Toxicology* 134–135: 104–11. doi:10.1016/j.aquatox.2013.03.013.

Liu, Xiaoshan, Dawoon Jung, Areum Jo, Kyunghee Ji, Hyo-Bang Moon, and Kyungho Choi. 2016. "Long-Term Exposure to Triphenylphosphate Alters Hormone Balance and HPG, HPI, and HPT Gene Expression in Zebrafish (Danio Rerio)." *Environmental Toxicology and Chemistry* 35 (9): 2288–96. doi:10.1002/etc.3395.

Liu, X., Y. Cai, Y. Wang, S. Xu, K. Ji, et K. Choi. 2019. "Effects of tris(1,3-dichloro-2-propyl) phosphate (TDCPP) and triphenyl phosphate (TPP) on sex-dependent alterations of thyroid hormones in adult zebrafish." Ecotoxicol Environ Saf 170:25-32. doi: 10.1016/j.ecoenv.2018.11.058.

McGee, Sean P., Alex Konstantinov, Heather M. Stapleton, and David C. Volz. 2013. "Aryl Phosphate Esters Within a Major PentaBDE Replacement Product Induce Cardiotoxicity in Developing Zebrafish Embryos: Potential Role of the Aryl Hydrocarbon Receptor." Toxicological Sciences 133 (1): 144–56. doi:10.1093/toxsci/kft020.

Möller A, Xie Z, Caba A, Sturm R, Ebinghaus R (2011). Organophosphorus flame retardants and plasticizers in the atmosphere of the North Sea. Environmental Pollution 159 (2011) 3660 - 3665

Muir, D. C. G., A. L. Yarechewski, and N. P. Grift. 1983. "Environmental Dynamics of Phosphate Esters. III. Comparison of the Bioconcentration of Four Triaryl Phosphates by Fish." Chemosphere 12 (2): 155–66. doi:10.1016/0045-6535(83)90159-5.

Noyes PD, Haggard DE, Gonnerman GD, and Tanguay RL. 2015. Advanced Morphological — Behavioral Test Platform Reveals Neurodevelopmental Defects in Embryonic Zebrafish Exposed to Comprehensive Suite of Halogenated and Organophosphate Flame Retardants. Toxicological Sciences, 145(1), 2015, 177–195

OECD 2009a. Emission Scenario Documents On Coating Industry (Paints, Laquers and Varnishes). Series On Emission Scenario Documents, No. 22. OECD, Paris.

OECD 2009b. Emission Scenario Document On Plastic Additives. Series On Emission Scenario Documents, No. 3. OECD, Paris.

OECD 2012. Guidance Document On Standardised Test Guidelines For Evaluating Chemicals For Endocrine Disruption. Series on Testing and Assessment No. 150. OECD, Paris, 2012.

OECD 2018. Guidance Document On Standardised Test Guidelines For Evaluating Chemicals For Endocrine Disruption. Series on Testing and Assessment No. 150. OECD, Paris, 2018.

Oliveri AN, Bailey JM, Levin ED. 2015. Developmental exposure to organophosphate flame retardants causes behavioural effects in larval and adult Zebrafish. Neurotoxicology and Teratology 52 (2015) 220–227

Pers comm. 2018. Email exchange between eMSCA and Japanese authorities in March and April 2018.

Scanlan LD, Loguinov AV, Teng Q, Antczak P, Dailey KP, Nowinski DT, Kornbluh J, Lin XX, Lachenauer E, Arai A, Douglas NK, Falciani F, Stapleton HM, Vulpe CD. 2015. Gene Transcription, Metabolite and Lipid Profiling in Eco-Indicator Daphnia magna Indicate Diverse Mechanisms of Toxicity by Legacy and Emerging Flame-Retardants. Environ. Sci. Technol., 2015, 49 (12), pp 7400–7410

Sitthichaikasem Sutham. 1978. Some toxicological effects of phosphate esters on Rainbow Trout and Bluegill. Ph.D. dissertation, Iowa State University, Ames, Iowa. Testing laboratory : Dept. of Animal Ecology, Iowa State University.

Sun, Liwei, Hana Tan, Tao Peng, Sisi Wang, Wenbin Xu, Haifeng Qian, Yuanxiang Jin, and Zhengwei Fu. 2016. "Developmental Neurotoxicity of Organophosphate Flame Retardants in Early Life Stages of Japanese Medaka (Oryzias Latipes)." Environmental Toxicology and Chemistry 35 (12): 2931–40. doi:10.1002/etc.3477.

Sundkvist AM, Olofsson U and Haglund P. 2010. Organophosphorus flame retardants and plasticizers in marine and fresh water biota and in human milk. J. Environ. Monit. (2010) 12, 943-951

UNEP, 2002. OECD SIDS Initial Assessment Report of Triphenyl phosphate for SIAM 15. UNEP. Available at: https://hpvchemicals.oecd.org/UI/SIDS_Details.aspx?id=FE97BB78-9F35-4515-8D31-476089A5865A

Unpublished study report (2021), Zebrafish (Danio rerio), Sexual development Test.

van der Veen I & de Boer J. 2012. Phosphorus flame retardants: Properties, production, environmental occurrence, toxicity and analysis. Chemosphere 88 (2012) 1119–1153

Wang, G., Du, Z., Chen, H., Su, Y., Gao, S., Mao, L., 2016. Tissue-specific accumulation, depuration, and transformation of triphenyl phosphate (TPHP) in adult Zebrafish (Danio rerio). Environ. Sci. Technol. 50, 13555-13564.

Wang G, Shi H, Du Z, Chen H, Peng J, Gao S. Bioaccumulation mechanism of organophosphate esters in adult Zebrafish (Danio rerio). 2017. Environmental Pollution 229, 177-187

Yuan S, Li H, Dang Y, Liu C. (2018). Effects of triphenyl phosphate on growth, reproduction, and transcription of genes of Daphnia magna. Aquatic Toxicology, Vol. 195, pp 58-66, 2018.

Zhang, Quan, Meiya Lu, Xiaowu Dong, Cui Wang, Chunlong Zhang, Weiping Liu, and Meirong Zhao. 2014. "Potential Estrogenic Effects of Phosphorus-Containing Flame Retardants." *Environmental Science & Technology* 48 (12): 6995–7001. doi:10.1021/es5007862.

Zhang Q, Ji C, Yin X, Yan L, Lu M, Zhao M. 2016. Thyroid hormone-disrupting activity and ecological risk assessment of phosphorus-containing flame retardants by in vitro, in vivo and in silico approaches. Environmental Pollution 210 (2016) 27-33.

7.15. Abbreviations

11-KT: 11-ketotestosterone

AR: Androgen receptor

CAT : Catalase

CF: Condition factor

E2: Estradiol

ER: Estrogen receptor

FSDT: Fish Sexual Development Test

GR: Glucocorticoid receptor

GSI: Gonadosomatic index

HPG: Hypothalamic-pituitary-gonadal

HPI: Hypothalamic-pituitary-interrenal

HPSE: Heparanase

HPT: Hypothalamic-pituitary-thyroid

HSI: Hepatosomatic index

MTC: Maximum Tolerated Concentration

RA: Retinoic acid

RAR: Retinoic acid receptor

REC: Relative effect concentration

RIC: Relative inhibitory concentration

RXR: Retinoic X receptor

SOD: Superoxide dismutase

SV-BA: Distance between Sinus Venosus and Bulbus Arteriosus

T: Testosterone

ThR: Thyroid receptor

TSH: Thyroid stimulating hormone

VTG: Vitellogenin

Annex 1: Assessment of endocrine disrupting (ED) properties for the Environment

This Annex presents the studies published prior to 2017 that were evaluated in detail by the UK eMSCA during the first phase of the evaluation process.

The Registrants have provided a report "*Evaluation of potential* endocrine disrupting properties of triphenyl phosphate – Environmental Focus", dated 04/05/17. This summarises a literature search that they performed to locate data relevant to endocrine disruption (ED), and an assessment of the findings and conclusion with respect to the ED properties of TPP. Studies were located consistent with levels 1, 2 and 3 of the OECD Conceptual Framework (CF) Guidance Document 150 (OECD, 2012).

The OECD CF level 1 data include the available systemic aquatic ecotoxicity data in the registration dossier, together with two studies from the literature, i.e., Du et al. (2015) and Scanlan et al. (2015). Du et al. (2015) is a Zebrafish embryo toxicity test. Scanlan et al. (2015) is an acute Daphnia toxicity study with the inclusion of mRNA expression profile, summarised in Section 7.8.1.2.1. The Registrant's summary indicates that three genes (DM06382-1/2/3) were down-regulated. The chronic fish studies described in the registration dossier are also included, together with an *in silico* (sic) study by Zhang et al. (2016) where TRβ receptor binding is reported (this in vitro study is described later in this section).

OECD CF level 2 data are indicated to be ToxCast data and Weiss et al. (2015). These report the results of androgen receptor (AR), estrogen receptor (ER) and thyroid receptor (ThR) binding assays. The Registrant reports that 8 of the 16 assays indicated ER-mediated activity, although all interaction was above the reported cytotoxicity threshold (3.45 µM, 1.13 mg/L). One out of the 8 androgen assays showed AR-mediated activity, again above the cytotoxicity threshold. No binding was observed in the three ThR assays. The Registrants report that Weiss et al. (2015) investigated TTR binding and no TR interaction for TPP was found. Test concentrations are not indicated.

OECD CF level 3 data are reported by the Registrant to be X Liu et al. (2013) and X Liu et al. (2016). These data are discussed in more detail below.

For the environment, the eMSCA has performed a literature search and located additional data summarised in Table A1 and described in more detail in the following text.

Table A1 summary of ED results located by eMSCA

Ref.	Species & development stage	Gene expression or binding activity	Hormone concentrations	In vivo effects	Comments
Kim et al. (2015)	GH3 cell line 48-h	tshβ ↑ (0.1 mg/L) trα↑ (0.01 and 0.1 mg/L) trβ↑ (0.1 mg/L) dio1↑ (0.1 mg/L) dio2 no change			Well plate. Static.
	FRTL-5 cell line 24-h	*nis↑ (3, 10 mg/L) tg↓ (1 mg/L) *tpo↑ (10 mg/L) tshr↓ (1 mg/L) pax8 no change nkx2.1↓ (1 mg/L)			
	Danio rerio embryo 7-d	crh↓ (0.5 mg/L) tshβ no change trα↑ (0.2 mg/L) trβ↓ (0.5 mg/L) dio1↑ (0.5 mg/L) dio2 no change ttr↑ (0.04 mg/L) ugt1ab↑ (0.2 mg/L) *nis↑ (0.2 mg/L) tg ↑(0.2 mg/L) tshr (no change) pax8 (no change) nkx2.1 (no change)	T3↑ (0.04 mg/L) T4↑ (0.04 mg/L) Both had flat d/r	NOEC ≥ 0.500 mg/L (mortality) NOEC 0.200 mg/L (cardiac malformation)	Beaker (800 mL). 50% solution renewal. Semi-static (24-h).
C Liu et al. (2013)	*Danio rerio* embryo 120-h	TRα↑ (2 mg/L) PPARgc1a↑ (2 mg/L) NCOR (no change) NCOR2↑ (2 mg/L) C1D (no change) HDAC3 (no change)	Not measured	none	Well plate. Semi-static (48-h).

Ref.	Species & development stage	Gene expression or binding activity	Hormone concentrations	In vivo effects	Comments
		FUS↑(2 mg/L) <u>ER1-</u> ER1 (no change) ER2a (no change) ER2b↑ (2 mg/L) VTG1 (no change) VTG2 (no change) VTG4 (no change) VTG5 (no change) NCOA1 (no change) NCOA2 (no change) NCOA3 (no change) PGR (no change) CCND1 (no change) Other gene expression for AhRs-, PPARα-, GR- and MR- not reported here. Authors state PPARα- and TRα-centred gene networks were the primary targets for TPP.			
X Liu et al. (2012)	H295R cell line 48-h	CYP11A1↑, CYP11B2↑, CYP19A1↑ (1 mg/L) SULT1E1↓, SULT2A1↓ (1 mg/L) HSD3β2 (unchanged)	E2 ↑, T ↑, E2/T ↑ (1 mg/L)		Well plate. Static. Only one independent run.

Ref.	Species & development stage	Gene expression or binding activity	Hormone concentrations	In vivo effects	Comments
	MVLN cell line 72-h	No binding to E2 receptor (1 mg/L) Anti-E: reduction in binding affinity (0.001 mg/L)			Well plate. Static. OECD development of this test method has been dropped, and assay considered to be invalid.
	Danio rerio adult 14-d	CYP17 ♂♀↑, (1 mg/L) CYP19A ♂♀↑ (1 mg/L) VTG-1 ♂↑ (0.04 mg/L); ♀↓ (1 mg/L)	♂ E2↑, T↓, 11-KT↓, E2/T↑, E2/11-KT↓ (1 mg/L) ♀ E2↑, E2/11-KT↑ (1 mg/L); no change: T, 11-KT, E2/T	NOEC ≥1 mg/L (mortality)	Semi-static (48-h).
X Liu et al. (2013)	*Danio rerio* adult 21-d	<u>Brain</u> GnRH2 ♀↓ (1 mg/L) ♂↑ (0.04 mg/L) GnRH3 ♀↑♂↓ GnRHR1 ♀♂ (no change) GnRHR2 ♀ (no change) ♂↑ GnRHR3 ♀↓♂↑ GnRHR4 ♀↑♂ (no change) LHβ ♀↑♂↓ FSHβ ♀↑♂↑	♂ E2↑, T↓, 11-KT↓, E2/T↑, E2/11-KT↓ (all 1 mg/L) ♀ E2/T (0.2 mg/L), E2↑, 11-KT↓E2/11-KT↑, T↓ (1 mg/L)	CF: NOEC ≥ 1mg/L GSI: NOEC ≥ 1mg/L HSI: NOEC ≥ 1mg/L VTG: ♂↑ (1 mg/L) ♀↑ (0.2 mg/L) Fecundity LOEC: 0.2 mg/L	Broadly in line with OECD TG 229. 2 L aquaria. Semi-static (48-h).

Ref.	Species & development stage	Gene expression or binding activity	Hormone concentrations	In vivo effects	Comments
		CYP19B ♀↑♂↑ AR ♀↑♂↓ Erα ♀↑♂↑ ER2β1 ♀↑♂↑ <u>Gonad</u> LHR ♀↑♂↓ FSHR ♀↑♂ (no change) HMGRA ♀↑♂↓ HMGRB ♀↓♂ (no change) StAR ♀↑♂↓ CYP11A ♀ (no change), ♂↑ CYP17A ♀↑♂↑ CYP19A ♀↑♂↑ 3βHSD ♀♂ (no change) 17βHSD ♀↑♂↓			
X Liu et al. (2016)	*Danio rerio* embryo to	<u>Brain</u>	♂ E2↑ (0.005), 11-KT↓ (0.005 mg/L),	♂CF: NOEC = 50 µg/L	Beaker then aquaria tanks. Semi-static (48-h).

Ref.	Species & development stage	Gene expression or binding activity	Hormone concentrations	In vivo effects	Comments
	adult 120-d	fshβ ♀↑ (0.05 mg/L) ♂ (no change) Ihβ ♀↑ (0.005 mg/L) ♂↑ (0.5 mg/L) gnrh3 ♀↑ (0.005 mg/L) ♂↓ (0.5 mg/L) erα♀ (0.5 mg/L) ♂ (no change) <u>gonad</u> fshr ♀ (no change) ♂↑ (0.005 mg/L) ihr ♀♂ (0.5 mg/L) star ♀↑ (0.05 mg/L) ♂ ↑(0.5 mg/L) cyp17 ♀ (no change) ♂↓ (0.5mg/L) cyp19a ♀↑ (0.5 mg/L) ♂ (no change) 3βhsd ♀ (no change) ♂↑ (0.5 mg/L) 17βhsd ♀ (no change) ♂↑ (0.05 mg/L)	♀ E2↑ (0.05), 11-KT↓(0.5 mg/L) E2/11-KT (same pattern)	♀CF: NOEC ≥500 µg/L ♂ GSI: NOEC ≥500 µg/L ♀ GSI: NOEC = 5 µg/L NOEC ≥ 1 mg/L (mortality) NOEC ≥ 1 mg/L (growth)	

Ref.	Species & development stage	Gene expression or binding activity	Hormone concentrations	In vivo effects	Comments
		pomc ♀♂↑ (0.5 mg/L) mr ♀↑ (0.5 mg/L) ♂ (no change) trh ♀ (no change) ♂ ↓ (0.5 mg/L) trh2 ♀ (0.5 mg/L) ♂ (no change)	♂ Cortisol↓ (0.05, 0.5 mg/L) ♀ Cortisol↑(0.5 mg/L) T3 ♀ ↑ (0.5 mg/L) ♂ (no change) T4 ♀ ↑ (0.5 mg/L) ♂ (no change)		

JMoE, 2012	*Oryzias latipes* 21-d	Not measured	Not measured	Mortality: ♂ NOEC ≥0.045 mg/L ♀ NOEC ≥0.045 mg/L Growth: ♂ NOEC ≥0.045 mg/L ♀ NOEC 0.017 mg/L HSI: ♂ NOEC 0.002 mg/L ♀ NOEC ≥0.045 mg/L GSI: ♂ NOEC ≥0.045 mg/L ♀ NOEC ≥0.045 mg/L Secondary sexual char. ♂ NOEC ≥0.045 mg/L "other" observations ♂ NOEC ≥0.045 mg/L ♀ NOEC ≥0.045 mg/L VTG ♂ NOEC ≥0.045 mg/L ♀↓ NOEC = 0.002 mg/L, LOEC = 0.007 mg/L No. eggs/female/d ♀ NOEC = 0.017 mg/L No. fertile eggs/female/d ♀ NOEC = 0.017 mg/L	OECD TG 229. Test concentration: 2.13, 7.19, 17.1 and 44.9 µg/L

Ref.	Species & development stage	Gene expression or binding activity	Hormone concentrations	In vivo effects	Comments
				Fertility rate (%) ♀ NOEC ≥0.045 mg/L	
Zhang et al. (2016)	Dual-luciferase report gene assay (24-h)	Trβ binding activity			No TRβ agonistic activity or TRβ antagonistic activity was observed for TPP. Only one independent run.
	GH3 cell line	Trβ binding activity			
Kojima et al. (2013)	CHO K1 and COS-7 cells	Binding activity: ERα/ERβ – v. weak agonist ERα/ERβ – not antagonist AR – not agonist AR – not reliable GR– not agonist GR – weak antagonist TRα1/TRβ1 – no activity			Three independent runs. Activity reported as REC_{20} or RIC_{20}.
Kojima et al. (2016)	CHO K1 and COS-7 cells	Binding activity: ERα/ERβ – v. weak agonist ERα/ERβ – not antagonist AR – not agonist			Three independent runs. Activity reported as REC_{20} or RIC_{20}. Metabolites of TPP also tested but not reported in this table.

Ref.	Species & development stage	Gene expression or binding activity	Hormone concentrations	In vivo effects	Comments
		AR – not reliable GR – not agonist GR – weak antagonist TRα1/TRβ1 – no activity			
Toxcast data		8 of the 16 assays indicated ER mediated activity, although all interaction was above the reported cytotoxicity threshold (3.45 µM, 1.13 mg/L). 1 out of the 8 androgen assays showed AR mediated activity, again above the cytotoxicity value. No binding was observed in the three ThR assays.			

* Result exceeds water solubility of TPhP; ** interpreted to be a LOEC; effect values in italics reflect values in an inconsistent dose-response

X Liu et al. (2012) investigated the endocrine disruption potential of six organophosphate flame retardants including TPP. They used two *in vitro* systems (H295R and MVLN cell lines) and an *in vivo* test using Zebrafish (*Danio rerio*) to assess effects on gene transcription. The test was performed on the three chemicals that were most responsive in the *in vitro* tests, which included TPP.

An MTT bioassay was performed to ensure non-cytotoxic doses in the *in vitro* studies. Using a >80 % cell viability compared to control, cytotoxicity due to TPP was determined to occur at 10 mg/L[7].

The H295R cell assay was therefore performed using nominal TPP concentrations of 0.001, 0.01, 0.1 and 1 mg/L plus a control, with three replicates per concentration. No chemical analysis was performed. Cells were exposed to TPP for 48 h in 24-well plates and sex hormones and gene transcriptors were then analysed. Statistically significant increases in the CYP11A1, CYP11B2, CYP19A1 gene transcription (up-regulation) were seen at the highest nominal concentration (1 mg/L). No effects were seen on the HSD3β2 gene transcription. Statistically significant effects were also seen at the highest concentration (1 mg/L) for SULT1E1 and SULT2A1 gene transcription (both downregulated). Both sets of gene transcriptions are linked by the authors to increased E2 concentrations (i.e., an estrogenic response). For the sex hormone concentrations, statistically significant increases in E2 and testosterone (T) were seen at the highest concentration of TPP (1 mg/L) but not at lower concentrations. A statistically significant increase in the E2/T ratio was also observed at the two highest concentrations (0.1 and 1 mg/L).

Estrogenic binding affinity was measured with a MVLN cell line using 96-well plates. The same TPP concentrations were used as for the H295R cell assay. After a 72-h exposure the luciferase activity was measured, and results expressed relative to E2. Anti-estrogenicity was measured in the same system but using co-treated chemical and E2. Binding affinity to the E2 receptor was not detected for TPP in the MVLN cells. A statistically significant reduction in binding affinity of E2 to the estrogen receptor was seen at 0.001 mg/L of TPhP (with effects observed at all concentrations). The eMSCA is aware that the MVLN assay was proposed for the OECD TG programme but considered to be invalid. Therefore, the results are not considered further in this assessment.

Adult Zebrafish (*Danio rerio*) were exposed to nominal TPP concentrations of 0.04, 0.2 and 1 mg/L for 14 days. The study used wild-type Zebrafish around 4 months old, which were acclimated for ">10 days" at 24+/-2 °C. The test was performed using a semi-static exposure (renewal every two days). Three replicates (three fish in each replicate) were used for each sex (i.e., male, and female fish were tested separately) making a total of eighteen fish per concentration. The tests appear to have been performed in aquaria (as acclimation occurred in these vessels) but the test volume is not indicated. After a 14-d exposure period blood plasma, gonad and liver samples were taken from anesthetized fish. This allowed measurements of three sex hormones in the blood, and transcription of related genes (CYP17, CYP19A and VTG-1) in the gonads and liver. In both male and female fish, statistically significant effects on the gonad gene transcription (up-regulation) were only seen at the highest test concentration (1 mg/L). A statistically significant effect on the VTG-1 gene transcription (up-regulation) was seen at all three test concentrations in male fish, and the highest concentration in female fish (down-regulation). For the sex hormone concentrations, statistically significant effects were seen at the highest concentration (1 mg/L) in males for E2 (increase), T (decrease), 11-KT (decrease), ratio of E2/T (increase), E2/11-KT (decrease). In females, statistically significant effects were seen in E2 (increase) and E2/11-KT (increase). The article notes that no significant mortality was observed during exposure but does not provide any detail of whether sub-lethal effects were seen. The authors note the upregulation of VTG-1 coupled with increased E2/T and E2/11-KT ratios strongly suggest potential estrogenicity. For the female fish, where VTG-

[7] This is above the measured water solubility of the substance (1.9 mg/L), although lower concentrations were used in the main assays.

1 was down regulated with a decreased E2/T ratio but increased E2/11-KT ratio they suggest that this requires further study.

C Liu et al. (2013) investigated the effects of two organophosphate flame retardants (TPP and tris(dichloropropyl)phosphate (TDCPP)) on mRNA expression in Zebrafish (*Danio rerio*, AB strain) embryos over a 120-h period using 6-well plates. Expression of mRNA for 48 genes in six receptor-centred gene networks[8] (AhRs-, PPARα-, TRα-, ER1-, GR-, and MR-) were investigated. Tests using several positive control substances[9] at specific concentrations were also performed. These appear to have been used principally to provide context to the gene expression results. The tests used 20 eggs per well, with 4 replicate wells at each concentration. Test solutions were renewed at 48-h intervals. In an initial experiment, nominal TPP concentrations of 0, 0.8, 4, 20, 100 or 500 mg/L were used to assess hatching and survival. Exposure concentrations of TPP in the 0.8 mg/L nominal solutions were measured using LC-MS/MS following filtration (0.22 µm membrane filter). Mean concentrations of TPP were recorded as 0.83 (4-h), 0.79 (48-h expired solution), 0.80 (48-h fresh solution) and 0.00 mg/L (120-h). No significant effects were observed up to 20 mg/L, but 100 % mortality was observed at 100 and 500 mg/L. Based on these mortality results, further testing to assess gene transcription was performed using nominal concentrations of 0.02, 0.2 and 2 mg/L. The article provides the following measured concentrations of TPP in this part of the experiment: 0.02, 0.19, 1.80 (48-h fresh solutions); 0.00, 0.00, 1.39 mg/L (120-h). RNA was isolated from larvae, and qRT-PCR was used to assess alterations in gene expression. From a graph in the paper, survival rate in the controls exceeded 90 % throughout the test. No significant effects on mortality, hatching or malformation were observed at any concentration of TPP.

Little effect was observed on gene transcription at 0.02 and 0.2 mg/L (two different genes affected out of the 48 monitored at each concentration). At 2 mg/L effects were seen on a number of genes mainly in the PPARα-, TRα-, GR-, and MR-centred networks. For GR-centred networks up and down-regulation was observed. No effects were seen on VTG genes (VTG1, VTG2, VTG4 and VTG5) in the ER centred network. The TRα-centred network included genes associated with T3 and T4 thyroid hormones. PPARα is nuclear receptor protein and involved with lipid homeostasis. GR- and MR-centred networks address glucocorticoids and mineralocorticoids which are steroid hormones regulating physiological functions such as glucose metabolism and mineral balance.

The authors considered TPP to be less potent than TDCPP, which could be a consequence of TPP being easily metabolized by Zebrafish larvae. They concluded the PPARα- and TRα-centred gene networks were the primary targets for TPP.

X Liu et al. (2013) studied the effects of TDCPP and TPP on reproductive endpoints and gene transcriptions of the hypothalamus-pituitary-gonad axis in adult Zebrafish (*Danio rerio*) exposed over 21 days. This study is included in the Registrants' analysis of ED effects. Wild-type adult male and female Zebrafish aged 4-5 months old were acclimated for 40 days before being separated by sex for 7 days prior to the test. One male and one female fish were then randomly selected to add to 2 L "mating chambers" for the test (it is not clear whether any spawning substrate was provided). The study was performed with 12 fish per concentration, split into pairs of fish in six 2 L tanks. Fish were exposed to nominal TPP (or TDCPP) concentrations of 0, 0.04, 0.2 and 1 mg/L based on the results of

[8] Aryl hydrocarbon receptors (AhRs)-, peroxisome proliferator-activated receptor alpha (PPARα)-, estrogenic receptors (ERs)-, thyroid hormone receptor alpha (TRα)-, glucocorticoid receptor (GR)-, and mineralocorticoid receptor (MR)-centered gene networks.

[9] Perfluorooctanesulfonic acid potassium salt (PFOS), Benzo[a]pyrene (B[a]P), 17β-estradiol (E2), 3,3',5-triiodo-lthyronine (T3), dexamethasone (DEX), fludrocortisone acetate (FCA), and 3-amino-benzoic acid ethyl ester, methanesulfonate salt (MS-222).

a range-finding test.

At the end of the test, fish condition factor[10] (CF), gonadosomatic and heptasomatic indexes (GSI and HSI, gonad weight/body weight and liver weight/body weight) were measured. Blood was collected to allow analysis of VTG and sex hormones (E2, T, 11-KT). Total RNA was extracted from brains and gonads to allow gene transcription of 22 genes to be assessed. Spawned eggs were removed from each tank daily and quantified. 50 eggs randomly selected from each mating chamber were observed for fertilisation and hatching success. Media was renewed every 48-h, and water quality was checked weekly (for example pH and dissolved oxygen). The test was performed at 27 °C.

The authors used GCMS to provide measured concentrations (limit of detection for TPP stated to be 0.12 ng/mL) over one 48-h renewal interval. For the nominal concentrations of 0.04, 0.2 and 1.0 mg/L, measured concentrations at the start and end of the renewal period (0, 48-h) were: 0.03, 0.00; 0.14, 0.00; and 0.89, 0.38 mg/L, respectively. The paper does not indicate what the values represent (for example a mean across the test period of all tanks, one tank, etc.).

No mortality and no significant[11] effect on CF, GSI and HSI occurred at any concentration (based on observations of five fish per sex per concentration for these endpoints). There is no indication of the level of mortality (if any) during the acclimation period.

For the measurement of sex hormones in the fish, a significant increase in E2 was observed in female fish exposed to 1 mg/L (nominal) TPP and in male fish at 0.2 mg/L (but not 1 mg/L, so there was no clear dose-response). A statistically significant decrease in T and 11-KT were seen in female fish at 1 mg/L, but no effects were seen in male fish for either hormone.

A statistically significant increase in the E2/T ratio was seen in both male (0.04 and 0.2 mg/L) and female fish (0.2 and 1.0 mg/L). A statistically significant increase in the E2/11-KT ratio was seen in both male (0.2 mg/L only) and female fish (1.0 mg/L). Significant increases in plasma VTG concentrations were observed in female fish at 0.2 and 1 mg/L, and in male fish at 1 mg/L. The authors note that the VTG changes are consistent with the hormone concentration changes and suggest an estrogenic effect.

With respect to fecundity, there was a statistically significant effect on cumulative egg numbers at both 0.2 and 1 mg/L and statistically significant decreases in the number of eggs per female (1 mg/L), the number of spawning events (0.2 and 1 mg/L) and hatchability (0.2 and 1 mg/L). No statistically significant effect was observed on fertilisation success.

X Liu et al. (2016) studied the effects of TPP on Zebrafish (*Danio rerio*) sex hormones and gene expression during long-term exposure to the chemical. This covered larval, juvenile, and adult life stages. The study is included in the Registrants' analysis of ED effects. Wild-type Zebrafish were acclimated for at least one month before eggs were collected. Embryos were collected with 4-h post fertilization and exposed to nominal concentrations of 0, 0.005, 0.050 and 0.500 mg/L of TPP for 120 days post-fertilisation (dpf). There were 3 replicates per treatment, and each replicate (beaker) contained 100 embryos and 300 mL of test solution. After hatch and swim bladder inflation, fry were transferred to 3 L beakers until 30 dpf, when they were moved to 15 L tanks through to the end of exposure at 120 dpf. Half of the exposure solution was renewed every 48 h throughout exposure. Water quality was checked weekly (for example pH and dissolved oxygen). The test was performed at 27 °C. Mortality, CF, GSI and HSI were assessed at

[10] The Condition Factor was calculated by 100 x (body weight in g) / (length in cm)^3.
[11] $p < 0.05$ for this study

120 dpf, with CF also assessed at 14 and 40 dpf. Sex hormones and gene expression[12] were assessed at 120 dpf. Concentrations of E2, testosterone (T), 11-KT, T4, T3, cortisol[13] were determined from blood plasma[14] (5 fish per sex per treatment), and brain and gonad samples were collected to permit gene transcription assessment. There was no statistical difference in the ratio of male and female fish amongst the treatment groups, including the controls, although the actual ratios are not provided.

The authors used GC/MS to provide measured concentrations (limit of detection for TPP = 0.12 ng/mL [0.00012 mg/L]) during one 48-h renewal period at 40 dpf. Measurements were made at 0, 6, 18, 24, 32 and 48 h from each of the three tanks per treatment. For the nominal concentrations of 0.005, 0.050 and 0.500 mg/L, the paper shows in a graph that measured concentrations dropped significantly over the 48-h period. A table of individual concentrations is not provided. From the graph, measured starting concentrations were ~0.050 and ~0.550 mg/L for the two higher concentrations. The eMSCA has not been able to determine the lowest concentration from the graph. The paper states that by 24-h measured concentrations were 0.0008 (below l.o.d.), 0.0055 and 0.1308 mg/L, and by 48-h nominal the 0.005 and 0.050 mg/L were below detection, and 0.011 mg/L for the 0.500 mg/L nominal concentration. Using additional analysis, the authors found that there was no statistical difference for the decline in test concentration over 48-h with and without fish (therefore the decline could not be attributed to organism adsorption or bioaccumulation).

No distinct malformations were observed at any concentration, and the authors state that there were no significant[15] differences in mortality between treatments (although the level of mortality observed, including controls, is not specified). The CF and GSI was unaffected up to 0.500 mg/L for the larval (14 dpf) and juvenile (40 dpf) fish. In adult fish (n= 5 per sex), CF (i.e., growth) in male fish was significantly affected at 0.500 mg/L and GSI in females at 0.005 and 0.500 mg/L. No other effects for CF, GSI or HSI were observed.

For the HPG axis, statistically significantly elevated levels of E2 were observed in female fish at 0.005 and 0.500 mg/L, and in male fish at 0.005 mg/L (but not 0.050 or 0.500 mg/L). A significant decrease in 11-KT levels was seen in female fish at the highest test concentration (0.500 mg/L) and in all test concentrations for male fish (0.005, 0.050 and 0.500 mg/L). The E2/11-KT ratio showed the same pattern as 11-KT effects. For the gene transcription in the brain, more effects were observed in female fish than males. For ERα, only up-regulation at the highest concentration in females was observed. For the seven hypothalamic-pituitary-gonad (HPG) genes associated with the gonads, a similar pattern was seen, with effects mainly occurring at the highest concentration, and more in male fish than female fish.

For the hypothalamic-pituitary-interrenal (HPI) axis, a statistically significant increase in cortisol was seen at the highest concentration (0.500 mg/L) in female fish, and a decrease at 0.050 and 0.500 mg/L in male fish. For the associated gene transcription (i.e., genes related to stress mediation), significant up-regulation was seen for the *pomc* gene in male and female fish at the highest concentration and for *mr* gene at the highest concentration in female fish only. The authors note the difference between male and female fish. Other than a possibility of general stress at 0.500 mg/L (based on the *pomc* change), the authors

[12] Follicle-stimulating hormone receptor (fshr), lhr, 3β-hydroxysteroid dehydrogenase (3βhsd), 17β-hydroxysteroid dehydrogenase (17βhsd), cytochromeP450 17 (cyp17), adrenocorticotrophin precursor proopiomelanocortin (pomc) mineralocorticoid receptor (mr), thyrotrophin-releasing hormone receptor 2 (trhr2) and the thyrotrophin-releasing hormone (trh)

[13] The authors note that while this axis is mainly responsible for the fish's response to stress, it plays a role in steroidogenesis.

[14] There appears to be a typo as earlier in the article 3 fish per sex per treatment are indicated to have been used for this.

[15] $p < 0.05$ for this study.

did not explain the difference between male and female fish, other than other factors (unspecified) factors influencing plasma cortisol levels.

For the hypothalamic-pituitary-thyroid (HPT) axis, statistically significant increases were seen on T3 and T4 concentrations at the highest test concentration (0.500 mg/L) in female fish, but not at lower concentrations nor in any concentration in male fish. Of the two thyroid genes effects were seen at the highest concentration in male fish for one (*trh* – down-regulation), and in female fish for the other (*trhr2* – up-regulation).

The authors note that "generally, changes in transcript levels were less pronounced in the present study compared with those reported from our previous study" [X Liu et al, 2013].

The eMSCA has been provided with an English summary of an OECD TG 229 study performed by the Japanese Ministry of Environment as part of their EXTEND 2010 endocrine disruption programme[16] (**JMoE, 2012**). The eMSCA has had follow up correspondence with the Japanese authorities (pers. comm. 2018) to provide some additional information.

The test was triggered by a positive result in an *in vitro* assay[17] for estrogenicity (a second androgenicity assay was negative) for TPP in the EXTEND programme. The OECD TG 229 test used Japanese Medaka *Oryzias latipes* and was conducted in 2012. It was not performed to GLP. The endpoints measured were mortality, growth (length and weight), fecundity (number of eggs, number of fertile eggs, fertility rate), GSI, HSI, VTG (l.o.d. = 1 ng/mg liver) and secondary sexual characteristics (papillary processes – male fish). The test used four concentrations and a control, with nominal test concentrations of 20.0, 64.0, 200 and 640 µg/L. There is no mention of a solvent vehicle. Measured concentrations were significantly lower than nominal being around 10 %: 2.13, 7.19, 17.1 and 44.9 µg/L, respectively (these are an arithmetic mean of weekly measured concentrations). Twenty-four fish per concentration were used (4 replicates of 3 female and 3 male fish per tank). The test used flow-through conditions. The test temperature range was 24.9-26.4 °C and DO range was 7.33-8.38 mg/L (>60%). Fecundity was observed for 7 days acclimation period prior to the start the test, with the "best tanks" selected for the test.

Statistically significant effects ($p < 0.05$) on length and growth were observed in female fish at the highest concentration (44.9 µg/L, measured). One female fish also died at this concentration, although this was not statistically significant. Statistically significant effects on the number of eggs and fertile eggs were seen at 44.9 µg/L. Statistically significant effects on male fish HSI were seen at the three highest concentrations (NOEC = 2.13 µg/L) but no effects were observed in female fish. A statistically significant decrease in VTG was observed in female fish at the three highest concentrations, but no effects were observed in male fish. No growth effects were observed in male fish. No effects were observed on fertility rate, GSI, secondary sexual characteristics nor "other observations" at any concentration.

The summary concludes that adverse reproductive effects were observed in Japanese Medaka. TPP was concluded as not being an estrogenic compound as there was no increase in the male hepatic VTG level at sub-lethal concentrations. The paper states that the study was not designed to detect anti-androgenic activity (presumably as no gonad histopathology was performed[18]). Due to the significant decrease in female hepatic VTG

[16] http://www.env.go.jp/chemi/end/substances.html

[17] Summaries of the tests are available on the EXTEND website in Japanese.

[18] OECD GD 150 indicates that the assay may in some cases have low statistical power or sensitivity to detect anti-androgenic activity through effects on secondary sexual characteristics

level, it was concluded that anti-estrogenic activity should be tested in a tier 1[19] *in vitro* test.

Subsequently, the Japanese authorities have confirmed that a Medaka estrogen receptor alpha reporter gene assay was performed in 2013, which was negative for anti-estrogenicity (pers. comm. 2018). A tier 1 assessment was then performed as part of the EXTEND 2016 programme. The supposition of Japanese authorities is the observed reverse of effects between the *in vitro* and *in vivo* tests[20] was a result of metabolism. Priority for further testing of TPP (which would be a MEOGRTS in the Japanese programme) was low due to the high concentration at which effects occurred in the OECD TG 229 compared to the low concentration in the Japanese environment (pers. comm. 2018).

Kim et al. (2015) studied the thyroid disruption effects of TPP (99% purity) on GH3 and FRTL-5 cells lines, and Zebrafish (*Danio rerio*) embryos.

Prepared GH3 cells were dosed with nominal concentrations of 0, 0.001, 0.010 and 0.100 mg/L TPP, using 24-well plates, with T3 used as a positive control. Each treatment was run in triplicate[21], with a 48-h exposure period. Prepared [described in article, but not here] FRTL-5 cells were dosed with nominal concentrations of 0, 1, 3 and 10 mg/L TPP, onto 24-well plates, with TSH (thyroid-stimulating hormone) used as a positive control. Each treatment was run in quadruplicate, with a 24-h exposure period. For both *in vitro* assays, the exposure concentration is determined using the WST-1 cell proliferation assay although there is no further information or measurement described in the paper. qRT-PCR was used for gene transcription of 6 genes in the GH3 assay, 7 in the FRTL-5 assay and all 13 in the Zebrafish larvae. Cells were not indicated to have been bleached prior to testing.

For the embryo test, an unspecified number of wild-type Zebrafish were randomly placed into 1 L glass beakers containing 800 mL of test solution. Nominal concentrations of 0, 0.040, 0.200 and 0.500 mg/L were used based on a range finding test. There was no positive control or chemical analysis. DMSO (0.005 %) was used as a vehicle. There were 6 replicates per treatment, and 50 % of the exposure solution was renewed daily. Water quality (including dissolved oxygen) was measured regularly although the frequency and values are not provided. The test was performed at 25 °C using a 14/10-h light/dark period. The authors used a 7-d exposure period as development of most organs and a relatively high level of thyroid hormones maintained was anticipated by 7 dpf. Embryo and larval survival, hatchability, malformation rate (although the method by which this was judged is not described) and weight were recorded over the study. At 7 dpf 100 larvae per replicate were homogenised and used for T3 and T4 quantification (limit of detection 0.1 ng/mL and 1.2 ng/mL, respectively). There was no mention of a reference substance being performed in the laboratory.

In the Zebrafish embryos, there was no significant effect on survival and hatchability, with embryo survival noted to range between 92.8 and 96.7 % over the test period. There was a statistically significant increase in malformations (yolk sac and pericardial oedema) at 0.500 mg/L. A statistically significant increase in T3 and T4 concentrations was observed at all 3 TPP concentrations, although the dose response is flat for both hormones. For T3, the actual incremental increases do not appear to be much above the limit of detection[22] -

[19] NB: tier 1 in this context refers to the Japanese ED test strategy rather than OECD CF

[20] I.e. positive estrogenic, negative anti-estrogenic *in vitro* assays compared to negative estrogenic, positive anti-estrogenic *in vivo*.

[21] Stated to be quadruplicate in the supplementary information.

[22] The graphs are quoted as ng/g, whereas the l.o.d. is quoted as ng/mL.

around 2.6 ng/g compared to 2.0 ng/g.

Gene expression changes in the *in vitro* assays had several statistically significant changes: *tshβ*, *tra*, *trβ* and *dio1* (GH3) and *nis*, *tg*, *tpo*, *tshr* and *nkx2.1* (FRTL5) although some occurred above the water solubility in the FRTL5 (*nis* and *tpo*). In the Zebrafish embryos, *crh*, *tra*, *trβ*, *nis*, *tg*, *dio1*, *ttr* and *ugt1ab* were all significantly up regulated.

Zhang et al. (2016) studied the thyroid hormone disrupting effects of nine organophosphate flame retardants including TPP (99.5% purity) in *in silico*, *in vitro* and *in vivo* assays.

The *in vitro* assay was a dual-luciferase report gene assay using CHO-K1. Following preparation on a 96-well plate, the cells were exposed to five concentrations of each chemical as well as a T3 control. After 24-h exposure, the luciferase and *Renilla* luciferase activity was detected using a fluorescence spectrophotometer. The test concentrations of TPP were not reported but appear to be between 10-9 and 10-5 M (0.00033 - 3.3 mg/L). DMSO was used as a vehicle (<0.1% v/v) for both studies. No chemical analysis was performed. Cells were not reported to be bleached prior to testing. Results were reported relative to T3. No cytotoxicity, TRβ agonistic activity or TRβ antagonistic activity was observed for TPP.

To confirm the results of the dual-luciferase report gene assay, a T-screen was performed with a GH3 cell line using a concentration of 10-6 M of each chemical (0.33 mg/L TPP). Exposure was performed using 96-well plates in the presence of the organophosphate (agonistic activity) and additionally with T3 (antagonistic activity). A T3 control was run, but no chemical analysis was performed.

None of the chemicals exhibited agonistic activity, but two showed TRβ antagonistic activity. TPP exhibited no activity. As TPP exhibited no effects in the *in vitro* assays no further testing of the chemical was performed by the authors for the *in vivo* (Frog Embryo Teratogenesis Assay-Xenopus, FETAX) assay or *in silico* modelling (molecular docking).

Kojima et al. (2013) studied the effects of 11 organophosphate flame retardants, including TPP (>97 % purity), in a cell-based assay to assess agonistic and antagonistic activities against human nuclear receptors: estrogen receptor (ERα & ERβ), androgen receptor (AR), glucocorticoid receptor (GR) and thyroid hormone receptor (TRα1 or TRβ1), retinoic acid receptor (RAR)α, retinoic X receptor (RXR)α, PPARα/γ and PXR. CHO-K1 (Chinese hamster) cells (ER, AR, GR, and TR) and simian kidney COS-7 cells (RAR, RXR, PPAR and PXR) were added to 96-well microtitre plates. Cells were not indicated to have been bleached prior to testing. The concentrations used were: 1E-07, 3E-07, 1E-06, 3E-06, 1E-05 and 3E-05 M (which equate to 0.033, 0.098, 0.33, 0.98, 3.26, 9.79 mg/L for TPP. DMSO was used as the vehicle at 0.1 %. Plates were incubated for 24 h, although the temperature at which this conducted at is not specified[23]. The paper states that the data presented are the mean of "at least three independent experiments".

Results were assessed against positive controls in each assay. The potency of the receptor agonistic activity was estimated from a dose-response curve of the luminescence intensity of the assay. Results for the test chemicals were reported based on 20% of the total response of the positive control dose-response (called a relative effective concentration, REC_{20}). In a similar way antagonistic activity was expressed as RIC_{20} relative inhibitory concentration). To allow comparison of the results, data from positive controls are also

[23] Nor in the two papers referenced in Kojima et al. (2013) where further details on the methodology are referenced.

provided from the authors' previous papers. These were E2, RIF, TAM, HF and RU-48624 and are of varying potency (i.e., care is needed when comparing for example the relative estrogenic activity of TPP to the anti-androgenic activity).

For agonistic activity to the ERα and ERβ receptors, this assay showed that TPP showed estrogenic activity (nominal concentrations of 4.9 and 6.5 µM – equivalent to 1.6 and 2.1 mg/L, respectively), but around 5,600,000-fold and 1,000,000-fold lower respectively than that of the reference substance E2. TPP did not exhibit any antagonistic ERα or ERβ binding activity, nor agonistic activity to AR or GR. Weak antagonistic activity was shown for AR (17 µM – 5.6 mg/L, and 944-fold lower than the positive control, HF), however from the dose response curve in the paper, only the top concentration was statistically significant. Weak antagonistic activity was also shown for GR (15µM – 4.9 mg/L, and 263-fold lower than the positive control, RU-486) by TPP. No agonistic or antagonistic activity against TRα1 or TRβ1, or agonistic activity against (RAR)α or (RXR)α receptors. For PXR compared to RIF (positive control), TPP exhibited a 9-fold lower agonistic activity (2.8 µM – 0.91 mg/L). Slight PPARγ agonistic activity was shown by TPP but below the 20 % threshold. No PPARα activity was shown. TPP was not cytotoxic to the COS-7 or CHO-K1 cells at any concentration (3E-5 M), which is contrary to the slight cytotoxicity observed in Kojima et al (2016) described below.

Consideration of metabolites of TPhP

Since effects have been observed in test systems in which the parent compound appears to have been rapidly depleted, it is possible that a transformation product might be involved.

Kojima et al. (2016) followed up their earlier paper (Kojima et al., 2013) by characterising the agonistic and antagonistic activity of 12 metabolites of 6 organophosphate flame retardants, including 4 metabolites of TPP, and the parent chemicals, to a number of human nuclear receptors: ERα/β, AR, GR, TRα1, retinoic acid receptor (RAR)α, retinoic X receptor (RXR)α, PPARα/γ and PXR, using CHO-K1 and COS-7 cells. The authors cite several earlier papers that indicate human liver preparations metabolise TPP to HO-p-TPhP, DPhP and HO-DPhP. HO-m-TPhP has not been confirmed as a human liver metabolite but has been observed in studies using chicken embryonic hepatocytes. The purity of TPP and DPhP were stated to be >99 %, while 4-hydroxylphenyl diphenyl phosphate (HO-p-TPhP), 3-hydroxylphenyl diphenyl phosphate (HO-m-TPP) and 4-hydroxylphenyl diphenyl phosphate (HO-DPhP), were stated to be >90 % pure (what constituted the remaining 10% is not stated). The concentrations used were: 1E-07, 3E-07, 1E-06, 3E-06, 1E-05 and 3E-05 M (which equate to 0.033, 0.098, 0.33, 0.98, 3.26, 9.79 mg/L for TPhP), together with a vehicle control (DMSO, 0.3%). The paper states that the data presented are the mean of "at least three independent experiments". The cells were incubated (temperature not stated) for 24-h in 96-well microtiter plates. Cells were not indicated to have been bleached prior to testing.

The potency of the receptor agonistic activity was estimated from a dose-response curve of the luminescence intensity of the assay, with results for the test chemicals reported based on 20% of the total response of the positive control dose-response (called a relative effective concentration, REC_{20}). In a similar way antagonistic activity was expressed as RIC_{20} relative inhibitory concentration). To allow comparison of the results, data from positive controls are also provided from the authors' previous papers. These were E2, RIF,

[24] 17b-Estradiol (E2; >97% pure), 5a-dihydrotestosterone (DHT; 95% pure), hydrocortisone (HC; >98% pure), 9-cis retinoic acid (9- cis RA; 98% pure), hydroxyflutamide (HF; >99% pure). 3,30,5-Triiodo-L-thyronine (T3; 99% pure), all trans-retinoic acid (at-RA; >98% pure), rifampicin (RIF; >97% pure), ciprofibrate (>99% pure), rosiglitazone (>99% pure), and mifepris-tone (RU-486; 98% pure).

TAM, HF and RU-486[25] and are of varying potency (i.e., care is needed when comparing for example the relative estrogenic activity of TPP to the anti-androgenic activity).

TPP, HO-p-TPhP and HO-m-TPhP were found to be slightly cytotoxic (at the highest concentration of 3E-5 M) to the CHO-K1 cells based on their β-galactocidase activity. The paper does not indicate how "slight" is defined.

In the ERα agonistic assay, a dose-response curve is provided. TPP, HO-p-TPhP and HO-m-TPhP were all active, but information on which effects were statistically significant is not provided. Based on the REC_{20} values, HO-p-TPhP exhibited an estrogenic activity via ERα 100,000-fold lower than E2, but about 10-fold higher than TPP and about 6-fold above HO-m-TPhP. In the ERβ agonistic assay TPP exhibited an estrogenic activity 57,000-fold lower than E2. HO-p-TPhP and HO-m-TPhP were noted as inducing about a 2- and 20- fold higher responses than TPhP. None of the chemicals exhibited AR, GR or TRα1 agonistic activity, DPhP and HO-DPhP did not exhibit activity in any agonistic assay.

In the antagonistic assays, none of the chemicals exhibited ERα antagonistic activity. TPP exhibited no activity in the ERβ assay at any concentration, while HO-p-TPhP induced ERβ activity at both 1E-05 and 3E-05 M (latter cytotoxic), and HO-m-TPhP only at 3E-05 M (cytotoxic). Based on the RIC_{20}, compared to TAM (reference for estrogenic antagonistic activity) for HO-p- TPhP was 900-fold lower activity, and for HO-m- TPhP 2500-fold lower (cytotoxic).

TPP induced activity at 1 E-05 and 3E-05 M (latter cytotoxic) in the AR antagonistic assay, and 3E-05 M (cytotoxic) in the GR assay. HO-p-TPhP and HO-m-TPhP induced the same effects in the AR and GR antagonistic assays (at 1 E-05 and 3E-05 M – again the latter was cytotoxic). Based on the RIC_{20} values, the anti-androgenic activities of all three substances were stated to be 650-fold lower than that of HF, which the authors used as a known AR antagonist. There was no antagonistic activity against ERα or TRa1. DPhP and HO-DPhP did not exhibit activity in any antagonistic assay.

None of the chemicals exhibited (RAR)α, (RXR)α, PPARαor PPARγ activity. PXR activity was shown by TPhP, HO-p-TPhP and HO-m-TPhP at greater than 20% the activity of RIF. These were similar and around 7-fold lower than RIF. HO-p-TPhP and HO-m-TPhP were found to be slightly cytotoxic (at the highest concentration of 3E-5 M) to the COS-7 cells based on their β-galactocidase activity. The statistical significance of the different concentrations is not provided.

[25] 17b-Estradiol (E2; >97% pure), 5a-dihydrotestosterone (DHT; 95% pure), hydrocortisone (HC; >98% pure), 9-cis retinoic acid (9- cis RA; 98% pure), hydroxyflutamide (HF; >99% pure). 3,30,5-Triiodo-L-thyronine (T3; 99% pure), all trans-retinoic acid (at-RA; >98% pure), rifampicin (RIF; >97% pure), ciprofibrate (>99% pure), rosiglitazone (>99% pure), and mifepris-tone (RU-486; 98% pure).