Substance Name: Triphenyl phosphate

EC Number: 204-112-2

CAS Number: 115-86-6

MEMBER STATE COMMITTEE SUPPORT DOCUMENT FOR IDENTIFICATION OF

TRIPHENYL PHOSPHATE

AS A SUBSTANCE OF VERY HIGH CONCERN BECAUSE OF ITS ENDOCRINE DISRUPTING PROPERTIES (ARTICLE 57(F) - ENVIRONMENT)

Adopted on 9 October 2024

This document has been prepared according to template: TEM-0049.04

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ABBREVIATIONS:

A: androgenic
adcy3 : adenylyl Cyclase 3
ALDH: aldehyde dehydrogenase
AO: adverse outcome
AR: androgen receptor
3β-HSD: 3β-hydroxysteroid dehydrogenase
17β-HSD: 17β-hydroxysteroid dehydrogenase
BBzP: benzyl butyl phthalate
BCF: bioconcentration factor
BOD: biochemical oxygen demand
cAMP: cyclic adenosine monophosphate
CF: condition factor
CHO: chinese hamster ovary cells
COS-7: simian kidney cells
CYP11A1: cholesterol side-chain cleavage
CYP11B2: aldosterone synthase
CYP17: 17α-hydroxylase
CYP19: cytochrome P450 aromatase
DEGs: differentially expressed genes
DEP: diethyl phthalate
DEPs: differentially expressed proteins
DHEA: dehydroepiandrosterone
DiBP: diisobutyl phthalate
Dio1: thyroxine deiodinase, type I
Dio2: thyroxine deiodinase, type II
dpf/h: days post fertilisation/hatch
DS: Dossier submitter
DT50: degradation half-life time
dw: dry weight
E: estrogenic
E2: 17β-Estradiol
E2-F: fluorescent derivative of 17β-Estradiol
EAS: Estrogen/Androgen/ Steroidogenesis (modalities)
EATS: Estrogen/Androgen/Thyroidal/Steroidogenesis (modalities)
EC ED EAG: Expert Advisory Group of the European Commission on Endocrine Disruptor
EC20: 20% effective concentration
EC50: half maximal effective concentration
ECHA: European Chemicals Agency
ED: endocrine disruptor
EDC: endocrine disrupting chemical
EDC-WG: ANSES' Thematic Working group on Endocrine Disruptors
EFSA: European Food Safety Authority
EGFR: epidermal growth factor receptor
ELoC: equivalent level of concern
ER: estrogen receptor
FRTL: rat thyroid follicular cell strain
FSDT: fish sexual development test
G15: GPR30 inhibitor
GC/MS: gas chromatography/ mass spectrometry
GH3: rat pituitary-derived cell line that expresses the Pit-1 transcription factor.
GPER: G protein-coupled estrogen receptor
GS: Gosner stage
GSI: gonadosomatic index (gonadal weight/body weight x 100)
H295R: Human adrenocarcinoma cell line
HEK293: immortalised human embryonic kidney cells
HepG2: Human liver cancer cell line

HG5LN: HeLa cells with the (GAL4RE) 5-betaGlobin-Luciferase-SV40-Neomycin plasmid
HMGR: 3-Hydroxy-3-methylglutaryl-coenzyme A reductase
hpf: hours post-fertilisation
HPLC: high performance liquid chromatography
HPSE: Heparanase
HSD3β2: 3β-hydroxysteroid dehydrogenase type 2
HSI: hepatosomatic index
IC50: concentration required to inhibit the cell viability by 50%
JRC: Joint Research Centre
KEs: key events
Koc: organic carbon-water partition coefficient
Kow: octanol/water partition coefficient
kPa: kilopascal
11-KT: 11-ketotestosterone
LC: liquid chromatography
LC50: concentration inducing 50% lethality
Lhcgr: luteinising hormone/choriogonadotropin receptor
LOD: limit of detection
LOEC: lowest observed effect concentration
LOQ: limit of quantification
MA-10: mouse Leydig cell line tumor
MAPK: mitogen-activated protein kinase
MBzP: monobenzyl phthalate
MCF-7: breast cancer cell line (Michigan Cancer Foundation-7)
MEP: monoethyl phthalate
MIE: molecular initiating event
MoA: mode of action
MR: mineralocorticoid receptor
MS: mass spectrometry
MSCA: member state competent authority
MTC: maximum tolerated concentration
MVLN ERα-positive and hormone-responsive human breast carcinoma cell line derived from the MCF-7 cell line, stably transfected with an ER-controlled luciferase reporter gene construct
NAM: no analytical measurement
nd: not detected
nER: nuclear estrogen receptor
NIS: sodium/iodide symporter
NKA: Na+/K+ ATPase
Nkx2.1: NK2 homeobox 1 also known as thyroid transcription factor 1 (TTF-1).
NOEC: no observed effect concentration
NR: not reported.
Pax8: paired box gene 8
PI3K-Akt: phosphoinositide 3-kinase/protein kinase B
POMC: proopiomelanocortin receptor
PPARγ: peroxisome proliferator-activated receptor-γ
PXR: pregnane X receptor
QSAR: Quantitative Structure-Activity Relationship
RT-PCR: real-time polymerase chain reaction
RA: retinoic acid
RAR: retinoic acid receptor
RBA: relative binding affinity
RDH: retinal dehydrogenase
RPE: relative proliferative effect
S: steroidogenic
SKBR3: human breast cancer cell line isolated by the Memorial Sloan–Kettering Cancer Center in 1970
SPM: suspended particulate matter

SRC: steroid receptor co-activators
SSC: secondary sex characteristics
StAR: steroidogenic acute regulatory protein
SULT: sulfotransferase
SVHC: substance of very high concern
T3 : 3,5,3-triiodo-L-thyronine
T4: L-thyroxine
T: testosterone
Tg: thyroglobulin
TPhP: triphenyl phosphate
TPO: thyroperoxidase
TR: thyroid hormone receptor
TSH: thyroid-stimulating hormone (thyrotropin)
TSHR: thyroid-stimulating hormone receptor (thyrotropin receptor)
TSPO: translocator protein
TTR: transthyretin receptor
VTG: vitellogenin (protein)
vtg: vitellogenin (gene)
WHO/IPCS: International Program on Chemical Safety of the World Health Organisation
wpf: week post fertilisation
ww: wet weight

IDENTIFICATION OF A SUBSTANCE OF VERY HIGH CONCERN ON THE BASIS OF THE CRITERIA SET OUT IN REACH ARTICLE 57

Substance name: Triphenyl phosphate (TPhP)

EC number: 204-112-2

CAS number: 115-86-6

- The substance is identified as a substance of equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of Regulation (EC) No 1907/2006 (REACH) according to Article 57(f) of REACH Regulation.

Summary of how the substance meets the criteria set out in Article 57 of the REACH Regulation

TPhP is identified as a substance of very high concern in accordance with Article 57(f) of Regulation (EC) 1907/2006 (REACH) because it is a substance with endocrine disrupting properties for which there is scientific evidence of probable serious effects to the environment, which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 REACH.

Endocrine activity

The available *in vitro* information demonstrates the capacity of TPhP to produce agonist activity on nuclear estrogen receptors ERα and ERβ of several vertebrate species including rat, mouse, fish, chicken, frog and turtle as evidenced by ER transactivation in reporter cell lines. In addition, TPhP can induce ER-regulated gene expression, and related physiological cell responses (e.g., increased cell proliferation). Two recent studies show that TPhP can also activate GPER. The available H295R assays on human adrenal carcinoma cells show that TPhP affects steroidogenesis by increasing estrogen levels (17ß-estradiol) and by increasing expression of genes involved in this pathway like CYP19 and 3β-HSD2. *In vivo* fish studies indicate that CYP19A is significantly upregulated by exposure to TPhP. Significant alteration of plasmatic concentrations of E2 and E2/T ratio and E2/11-KT ratio can result from this modification in the steroidogenesis pathway. The degree of perturbation of circulating steroid concentrations depends on the fish developmental stage, species and tested concentrations. The observations of VTG concentrations, that are consistent with perturbation of E2 concentrations, suggest an EAS activity of TPhP in female and male zebrafish, with altered concentration of VTG. Therefore, TPhP exerts an effect on the endocrine balance in fish. It has EAS activity as clearly shown both *in vitro* and *in vivo*.

Adverse effects

In vivo studies on fish reproduction show an alteration of gametogenesis in both sexes with a disruption of testis and ovary maturation followed by a decrease in reproductive success in terms of fecundity (decrease in egg production, spawning events fertility (impaired hatchability) and fertilisation rate). Therefore, TPhP shows adverse effects on fertility and reproduction in fish, observed in several studies at levels without concurrent systemic effects.

Plausible link between adverse effects and endocrine activity

The consistency between the observed adverse effects and EAS activity provides evidence that EAS modalities are plausibly biologically linked to the adverse effects. Depending on the developmental stage, exposure period, reproductive status, species and concentration, antagonist and agonist effects are observed in organisms, leading *in vivo* to perturbations of circulating steroid concentrations in most of the analysed studies.
The effect observed on reproduction in fish (fecundity and fertility) can affect population stability and is considered as an adverse effect relevant at population level.

Based on all available scientific evidence, it can be concluded that TPhP fulfils the WHO/IPCS definition of an endocrine disruptor.

Equivalent level of concern

The very high concern raised by this property is substantiated by the severity and irreversibility of the effects on organisms and populations that may have long term consequences, the large variety of species that may be adversely affected and the difficulties to quantify a safe level of exposure with regard to the endocrine mediated effects.

Conclusion

In conclusion, there is scientific evidence that TPhP causes probable serious effects to the environment due to its endocrine disrupting properties, which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of the REACH Regulation.

Registration dossiers submitted for the substance: Yes

Justification

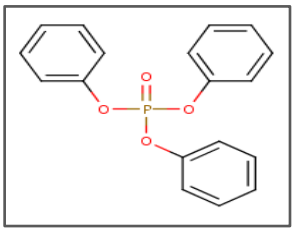
1. Identity of the substance and physical and chemical properties

Name and other identifiers of the substance

Table 1: Substance identity

EC number:	204-112-2
EC name:	triphenyl phosphate
CAS number (in the EC inventory):	115-86-6
CAS number:	115-86-6
IUPAC name:	triphenyl phosphate
Index number in Annex VI of the CLP Regulation	None
Molecular formula:	$C_{18}H_{15}O_4P$
Molecular weight range:	326.28
Synonyms:	TPhP

Structural formula:



Composition of the substance

Name: Triphenyl phosphate

Description: Colourless, odourless solid.

Substance type: mono-constituent

Identity and composition of degradation products/metabolites relevant for the SVHC assessment

Not relevant for the SVHC assessment of the substance.

Identity and composition of structurally related substances (used in a grouping or read-across approach)

Not applicable.

Physicochemical properties

Table 2: Overview of physicochemical properties

Property	Description of key information	Value [Unit]	Reference/source of information
Physical state at 20°C and 101.3 kPa	Visual inspection	Colourless, odourless solid.	Data quoted from the FR conclusion document* published in: https://echa.europa.eu/documents/10162/916779d9-ec10-07fa-f178-9562bdd7dedc
Melting/freezing point	Non-guideline publication	49.5 - 50 °C. Melting point values within the range 49 - 50.5 °C are reported in a variety of secondary sources.	
Boiling point	Extrapolated according to the Clausius-Clapeyron equation using experimentally derived parameters - non-guideline publication.	414 °C at 101.3 kPa. One publication reported decomposition at or near the boiling point.	
Vapour pressure	Non-guideline publication using an isoteniscope under a nitrogen atmosphere. Results extrapolated using the Clausius-Clapeyron equation.	0.000853 Pa at 25°C	
Density	Non-guideline publication.	The relative density at 50°C is given as 1.21g/cm^3.	
Water solubility	Non-guideline publication.	1.9 mg.l^{-1} at 20°C. Other supporting publications/reference sources give similar values. No modern guideline study.	
Partition coefficient n-octanol/water (log value)	Non-guideline but similar to shake-flask.	Log Pow 4.63. Values between 4.5 and 4.7 are reported in various publications	
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.	

* Physicochemical properties quoted in the Conclusion document were based on the analysis of physicochemical properties extracted from the registration dossier of the lead registrant as disseminated in ECHACHEM (https://chem.echa.europa.eu/100.003.739/dossier-view/9b2f054a-c82b-4cc1-9393-415a4f7781c1/4c37f1df-d04d-4911-8afc-4a9bf3b52bf0_4c37f1df-d04d-4911-8afc-4a9bf3b52bf0?searchText=204-112-2).

2. Harmonised classification and labelling

No current harmonised classification for TPhP.

3. Environmental fate properties

The environmental fate data presented in this section are extracted from the registration dossier of the lead registrant as disseminated in ECHACHEM[1] and are provided as contextual information for the SVHC assessment of the substance that focus on endocrine disruptor (ED) properties for the environment. These data have not been further evaluated.

Degradation

Abiotic degradation

Hydrolysis

The main study gave half-lives of 3 days at pH 9, 19 days at pH 7 and >28 days at pH 5 at 25°C (Mayer et al., 1981). One study indicates that 100 % of the TPhP is hydrolysed after 10 minutes at pH 13 (Ishikawa et al., 1985). Another study indicates that at pH 4 the rate of disappearance is too slow to measure (Howard et al., 1979). All studies show that under alkaline conditions TPhP is rapidly degraded, while under acidic conditions TPhP is relatively stable.

Phototransformation in air

From AOPWIN v1.92, a calculation based on a 12 hour-day and a concentration of 1.5x10E6 OH/cm^3 gave a half-life of TPhP of 11.85 hours.

Phototransformation in water

Two studies (Ishikawa et al., 1992; Wan and Wong, 1994) are cited for phototransformation in water, and the registrant concluded that TPhP was rapidly degraded when irradiated at 254 nm. The key test was performed at pH 3 and pH 10. However, since the stratospheric ozone layer prevents ultraviolet light of less than 290 nm from reaching the earth's surface, only light at wavelengths between 290 and 750 nm can result in photochemical transformations in the environment.

Phototransformation in soil

No relevant information available.

Biodegradation

Biodegradation: screening tests

Eight biodegradation screening studies are summarised in the registration dossier. These include tests of both ready and inherent biodegradation, as well as several river and pond sediment assays. The key study is CITI (1992), which is an OECD 301C test where biodegradation between 83-94% (based on BOD after 28 days was observed).

[1] https://chem.echa.europa.eu/100.003.739/dossier-view/9b2f054a-c82b-4cc1-9393-415a4f7781c1/4c37f1df-d04d-4911-8afc-4a9bf3b52bf0_4c37f1df-d04d-4911-8afc-4a9bf3b52bf0?searchText=204-112-2

Biodegradation in water and sediment

An OECD 303A (sewage treatment plant simulation study) is included assessing aerobic degradation (Unpublished study report, 1982). The registrant concludes that TPhP is readily degraded in water/sediment systems.

Biodegradation in sediment

No data available

Biodegradation in soil

Two simulation tests for biodegradation in soil (aerobic and anaerobic conditions) are summarised in the registration dossier (Anderson et al., 1993). They calculated a half-life (DT50) of 37 days in aerobic conditions and 21 days in anaerobic conditions.

Environmental distribution

Adsorption/desorption

Four studies in the registration dossier provide information on adsorption/desorption (Anderson et al., 1993; Mayer et al., 1981; Boethling & Cooper, 1985; Huckins et al., 1991). The registrant notes that the range of values (Koc between 2514 and 5500) indicates that the substance will be relatively immobile in soil. The key data is Anderson et al. (1993) using three soils, which gave measured Koc values between 2414 and 3561.

Volatilisation

The Henry's law constant has been calculated by QSAR using HENRYWIN v3.2. The estimated Henry's law constant is 0.004 Pa.m^3/mol.

Distribution modelling

The registrant includes Mackay Level 1 modelling (Unpublished study report, 2002), which provides the following distribution:

Air:	0.7%
Water:	14.3%
Soil:	43.9%
Sediment:	41%
Suspended sediment:	0.07%
Biota:	0.03%
Aerosol:	0%

The following inputs were used: temperature = 25 °C, vapour pressure = 8.35 x 10E-4 Pa, water solubility = 1.90 g/m^3, log Kow = 4.59.

Environmental occurrence data

This section integrates data from i) the Norman EMPODAT database, ii) the French Naïades database and, iii) a review of the scientific literature.

Norman EMPODAT database

Norman EMPODAT is a database of geo-referenced monitoring and bio-monitoring data in Europe on emerging substances in the following matrices: water, sediment, biota, SPM, soil, sewage sludge and air. This database cannot be used as representative of contaminated areas associated to industrial activities, but rather as an initial overview of the global state of various environmental media.

Surface water - results

24 580 samples, mainly from three countries (France, Germany and Netherlands) are provided in this database. It should be noted that the variability of the detection and of the quantification limits restrains the data exploitation. 8% of data was reported with no information about the limit of quantification, 85% of values with LOQ > = 0.01 µg.l^{-1} and 24% of values with LOQ >= 0.1 µg.l^{-1}.

Table 3: Results for Surface water compartment

Descriptive data analysis for surface water – (2002 – 2020) Total individual values > LOQ: 1835 (7.47% of total analysis (24580))	
Parameter	**Concentration Value (µg.l^{-1})**
90th Percentile	5.42E-02
Mean	2.79E-02
Median	1.50E-02
Max	1.74

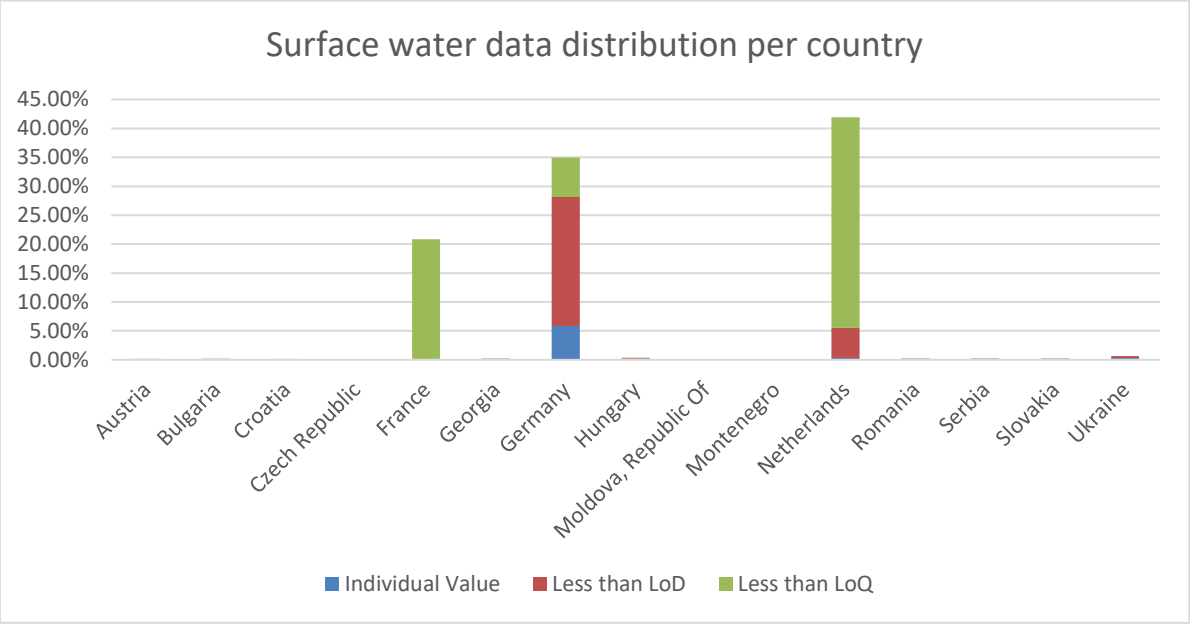


Figure 1: Percentage of surface water data (Total number of analysis) distribution per country

Sediment - results

Forty samples from eight countries (Moldova, Ukraine, Bulgaria, Georgia, Germania, Hungary, Romania, and Montenegro) are provided in this database for TPhP with 4 values above the LOD (1.5 µg/kg $_{dw}$). The other 36 samples had a LOD of 5 µg/kg $_{dw}$.

Table 4: Results for sediment compartment

Descriptive data analysis for sediment – values above the LOD								
COUNTRY	**Ecosystem / Matrix: sediment**	**Concentration value**	**Unit**	**Month**	**Year**	**River basin name**	**Limit of Detection (LOD): [µg/kg $_{dw}$]**	**Analytical method**
Germany	Sediments - River water	6	µg/kg dry weight	7	2019	Danube	1.5	HPLC-MS or MS/MS
Hungary		9						
Bulgaria		9						
Ukraine		5						

Waste water – results

The data for waste water covers the year 2007 and 3 consecutive years (2017-2018-2019). The range of the detection limits and the quantification limits in µg.l^{-1} are [8E-05; 5E-02] and [2.5E-04; 6E-02] respectively.

Table 5: Results for waste water

Descriptive data analysis for waste water Total individual value (> LOQ): 29 (28.2% of total analysis) Total number of analysis: 103	
Parameter	**Concentration Value (µg.l^{-1})**
90th Percentile	7.86E-02
Mean	3.52E-02
Median	1.77E-02
Max	1.96E-01

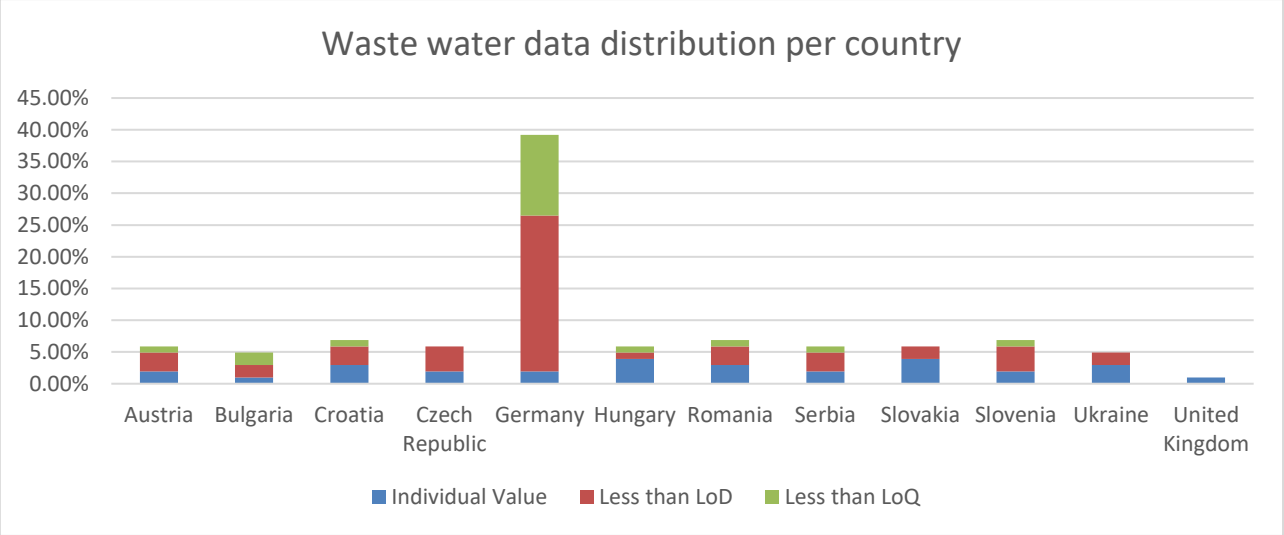


Figure 2: Percentage of waste water data (total number of analysis) distribution per country

Sewage sludge - results

Eight samples from Sweden only are provided in this database for TPhP. No information about the limit of quantification or detection is available.

Table 6: Results for sewage sludge

Descriptive data analysis for sewage sludge						
Country	**Type**	**Value**	**Unit**	**Month**	**Year**	**Analytical method**
Sweden	Sewage sludge - Mixed	54	µg/kg dry weight	11	2007	GC-MS or MS/MS
	Sewage sludge - Mixed	265	µg/kg dry weight	10	2007	GC-MS or MS/MS
	Sewage sludge - Mixed	54	µg/kg dry weight	10	2007	GC-MS or MS/MS
	Sewage sludge - Mixed	136	µg/kg dry weight	10	2007	GC-MS or MS/MS
	Sewage sludge - Municipal	107	µg/kg dry weight	10	2007	GC-MS or MS/MS
	Sewage sludge - Industrial	32	µg/kg dry weight	2	2008	GC-MS or MS/MS
	Sewage sludge - Mixed	104	µg/kg dry weight	10	2007	GC-MS or MS/MS
	Sewage sludge - Municipal	1306	µg/kg dry weight	10	2007	GC-MS or MS/MS

Biota – results

Among the 205 samples available in this database for TPhP, 2 samples on marine mammals and raptors are above the LOD (range value [0.00125; 5] $\mu g.kg_{ww}^{-1}$).

Table 7: Results for biota

Descriptive data analysis for biota (205 samples with 2 samples > LOD)						
Country	**Sample matrix**	**Value**	**Unit**	**Year**	**Species group**	**Species name**
Belgium	Biota - Territorial (marine) water	0.99	µg/kg wet weight	2021	Marine mammals	*Phocoena phocoena*
Belgium	Biota - Terrestrial	0.81	µg/kg body weight	2021	Raptors	*Buteo*

Naïades database

Naïades is a database collecting French data only. It is an observatory on the quality of river and water bodies. 4705 samples are included in the database for TPhP between 2020 and 2022, including 4647 values below the limit of quantification (LOQ) and 58 values above the LOQ (range: 0.02-0.1 µg.l^{-1}). These values (> LOQ) were measured in a single French region and by a single analysis laboratory.

Table 8: Descriptive data analysis for Naïades (suite)

Descriptive data analysis for value > LOQ (58 values)	
Parameter	**Raw water concentration (µg.l^{-1})**
90th Percentile	0.12
Mean	0.06
Median	0.046
Max	0.22

Scientific literature related to occurrence data

A scientific literature review was conducted up to May 2023. The systematic literature search was performed in Scopus database. A single concept strategy search was applied to retrieve all relevant information on TPhP by using its Chemical Abstracts Service Registry Number (CAS No 115-86-6), scientific chemical names, and common names, as recommended in the EDC guidance (ECHA/EFSA, 2018). 812 entries were recorded from 23 studies that included monitoring data in an environmental media (water, sediment, biota) in Europe.

Table 9: Descriptive data analysis from Literature review

Descriptive data analysis from Literature review								
Country	**Type of environmental media**	**Type of value**	**Value**	**Unit**	**LOD**	**Analytical method**	**Year**	**REF**
WATER								
Spain	Nalon river	Mean (min-max)	1.7 (1.6-2.4)	$ng.l^{-1}$	1.6 $ng.l^{-1}$	GC-MS	2012 (11 samples)	Cristale et al., 2013
Spain	Arga river	Mean (min-max)	2.8 (1.6-7.2)	$ng.l^{-1}$	1.6 $ng.l^{-1}$	GC-MS	2012 (8 samples)	Cristale et al., 2013
Spain	Besos river	Mean (min-max	12.5 (01.6-35)	$ng.l^{-1}$	1.6 $ng.l^{-1}$	GC-MS	2012 (13 samples)	Cristale et al., 2013
Sweden	21 rivers	Mean	< LOD	$ng.l^{-1}$	37 $ng.l^{-1}$	GC-MS-MS	2013 (25 samples)	Gustavsson et al., 2018
Greece	River water Dissolved fraction	Mean (min-max) Detection frequencies	137 (40-258) 100%	$ng.l^{-1}$	NR	GC-MS-MS	2019-2020 (12 samples)	Pantelaki et al., 2021
Greece	River water Particulate fraction	Mean (min-max) Detection frequencies	100 (46-219) 100%	$ng.l^{-1}$	NR	GC-MS-MS	2019-2020 (12 samples)	Pantelaki et al., 2021
Greece	Coastal water Dissolved fraction	Mean (min-max) Detection frequencies	135 (41-260) 100%	$ng.l^{-1}$	NR	GC-MS-MS	2019-2020 (18 samples)	Pantelaki et al., 2021
Greece	Coastal water Particulate fraction	Mean (min-max) Detection frequencies	47 (34-81) 100%	$ng.l^{-1}$	NR	GC-MS-MS	2019-2020 (18 samples)	Pantelaki et al., 2021
Greece	Streams Dissolved fraction	Mean (min-max) Detection frequencies	279 (45-1142) 100%	$ng.l^{-1}$	NR	GC-MS-MS	2019-2020 (12 samples)	Pantelaki et al., 2021
Greece	Streams Particulate fraction	Mean (min-max) Detection frequencies	281 (38-980) 100%	$ng.l^{-1}$	NR	GC-MS-MS	2019-2020 (12 samples)	Pantelaki et al., 2021
SEDIMENT								
United Kingdom	Worcester-Birmingham Canal	Mean (min-max)	4 (0.1-26)	ng/g dry weight	NR	GC-MSD	2019-2021 (12 samples)	Onoja et al., 2023
United Kingdom	River Severn	Mean (min-max)	1 (0.3-8)	ng/g dry weight	NR	GC-MSD	2019-2021 (12 samples)	Onoja et al., 2023
United Kingdom	River Sowe	Mean (min-max)	1 (0.4-2)	ng/g dry weight	NR	GC-MSD	2019-2021 (12 samples)	Onoja et al., 2023

United Kingdom	River Tame	Mean (min-max)	2 (0.3-9)	ng/g dry weight	NR	GC-MSD	2019-2021 (12 samples)	Onoja et al., 2023
Spain	Nalon	Mean (min-max)	15 (15-15)	µg/kg dry weight	15 µg/kg dw	GC-MS	2012 (8 samples)	Cristale et al., 2013
Spain	Arga	Mean (min-max)	22.17 (15-44)	µg/kg dry weight	15 µg/kg dw	GC-MS	2012 (6 samples)	Cristale et al., 2013
Spain	Besos	Mean (min-max)	37.71 (19-63)	µg/kg dry weight	15 µg/kg dw	GC-MS	2012 (7 samples)	Cristale et al., 2013
Italy	Adige River	Max	9.69	ng/g dry weight	0.08 ng/g dw	Pressurised liquid extraction-Turbulent Flow Chromatography-LC-MS/MS	2015 (20 samples)	Giulivo et al., 2017
Greece	Evrotas River	Max	0.67	ng/g dry weight	0.08 ng/g dw	Pressurised liquid extraction-Turbulent Flow Chromatography-LC-MS/MS	2014-2015 (12 samples)	Giulivo et al., 2017
Slovenia	Sava River	Max	< LOD	ng/g dry weight	0.08 ng/g dw	Pressurised liquid extraction-Turbulent Flow Chromatography-LC-MS/MS	2014-2015 (20 samples)	Giulivo et al., 2017
France	Gulf of Lion	Mean (Min-Max)	1.57 (0-5.17)	ng/g dry weight	NR	GC-MS	2018 (12 samples)	Alkan et al., 2021
BIOTA								
France	Mussels (*Mytilus spp*) and oysters (*Crassostrea gigas*)	Mean or Median (Min-Max)	1.12 (0.17-8.01)	ng/g dry weight	53 pg/g dw	LC-ESI-MS/MS	2014-2021	Aminot et al., 2023
Italy	Adige River (Fish)	Max	30.3	ng/g lipid weight	1.30 ng/g lw	Pressurised liquid extraction-Turbulent Flow Chromatography-LC-MS/MS	2015 (13 samples)	Giulivo et al., 2017
Greece	Evrotas River (Fish)	Max	< LOD	ng/g lipid weight	1.30 ng/g lw	Pressurised liquid extraction-Turbulent Flow Chromatography-LC-MS/MS	2015 (4 samples)	Giulivo et al., 2017
Slovenia	Sava river (Fish)	Max	< LOD	ng/g lipid weight	1.30 ng/g lw	Pressurised liquid extraction-Turbulent Flow Chromatography-LC-MS/MS	2015 (10 samples)	Giulivo et al., 2017

Denmark	Western Iceland (Denmark Strait) Fin Whale	Mean	59.2	ng/g lipid weight	NR	Ultra-sonication extraction-LC-MS/MS	2015 (20 whales)	Garcia-Garin et al. 2020
Western Mediterranean area	Western Mediterranean Sea *Sardina Pilchardus*	Mean (Min-Max)	2.42 (ND-9.39)	ng/g wet weight	0.12 ng/g ww (LOQ)	Ultra-sonication extraction-TFC-HPLC-MS/MS	2019	Sala et al., 2022
Western Mediterranean area	Western Mediterranean Sea *Engraulis Encrasicolus*	Mean (Min-Max)	0.66 (ND-2.28)	ng/g wet weight	0.12 ng/g ww (LOQ)	Ultra-sonication extraction-TFC-HPLC-MS/MS	2019	Sala et al., 2022
Western Mediterranean area	Western Mediterranean Sea *Merluccius Merluccius*	Mean (Min-Max)	ND	ng/g wet weight	0.12 ng/g ww (LOQ)	Ultra-sonication extraction-TFC-HPLC-MS/MS	2019	Sala et al., 2022
Mediterranean sea (Catalan coast)	Turtle	Mean (Min-Max)	0.15 (ND-1.08)	ng/g wet weight	0.12 ng/g ww (LOQ)	Ultra-sonication extraction-TFC-HPLC-MS/MS	2014-2017	Sala et al., 2021
Mediterranean sea (Balearic Island)	Turtle	Mean (Min-Max)	0.31 (ND-1.34)	ng/g wet weight	0.12 ng/g ww (LOQ)	Ultra-sonication extraction-TFC-HPLC-MS/MS	2014-2017	Sala et al., 2021
Mediterranean sea (Balearic Island)	Prey of turtle	Mean (Min-Max)	1.25	ng/g wet weight	0.12 ng/g ww (LOQ)	Ultra-sonication extraction-TFC-HPLC-MS/MS	2014-2017	Sala et al., 2021

NR: not reported.

Human occurrence

In addition to indirect exposure *via* the environment, humans are exposed *via* several consumer articles or in the workplace (widespread uses), in formulation or repacking, on industrial sites and in manufacturing. TPhP is also present as an impurity in many other organophosphate flame retardants or as a constituent of this family of compounds.

Human biomonitoring data demonstrate also the presence of TPhP or its metabolites in human milk (Sundkvist et al., 2010; Kim et al., 2014; Zheng et al., 2021), human placenta (Ding et al., 2016; Zhao et al., 2017), blood serum (Ya et al., 2019; Zhao et al., 2016) and urine (Carignan et al., 2016; Li N. et al., 2019a). TPhP has also frequently been detected in indoor house dust samples in Europe (Belgium and Sweden) and outside Europe (Japan, Philippines and USA) with detection frequencies up to 98% (Kanazawa et al., 2010; Kim et al., 2013; Marklund et al., 2003; Stapleton et al., 2009; Van den Eede et al., 2011). It can be concluded that TPhP reaches diverse environmental compartments and biota of remote areas.

Summary on occurrence and environmental distribution

Several literature studies demonstrate that TPhP can be found in Europe and that it is ubiquitous in several environmental compartments (water, sediment, sewage sludge, indoor dust/air). Therefore, many environmental species and humans are exposed more or less continuously to TPhP and exposure cannot be avoided.

Bioaccumulation

Bioaccumulation in aquatic organisms (pelagic & sediment organisms)

The registration dossier of the Lead Registrant cites 9 fish bioaccumulation studies (Muir et al., 1980; Mayer et al., 1981; Sasaki et al., 1981; Muir et al., 1982; Sasaki et al., 1982; Muir et al., 1983; Boethling & Cooper, 1985; Kuehl and Haebler , 1995; Lo et al., 2000), although only three were assessed to be valid of which two are conventional tests (the third was blubber samples from dead Common bottlenose dolphins). These two conventional tests provide bioconcentration factor (BCF values of 110 and 144).

Bioaccumulation in terrestrial organisms (soil dwelling organisms, vertebrates)

No relevant information available.

Summary

Considering data coming from the registration dossier of the lead registrant, the substance is rapidly degraded in water under alkaline conditions, TPhP photodegrades rapidly in the atmosphere (DT50= 11.85 h) and TPhP would be readily biodegraded in water/sediment systems. The data provided in the registration dossier of the Lead registrant would indicate that TPhP is relatively immobile in soil.

4. Human health hazard assessment

Human health data for TPhP were not reviewed and thus not included in this document.

5. Environmental hazard assessment

Please note that the environmental toxicity data presented in this section are extracted from the registration dossier of the Lead Registrant as disseminated in ECHACHEM[2] and are provided as contextual information for the SVHC assessment of the substance that focuses on ED properties for the environment. These data have not been further evaluated. However, the data used as a basis for the environmental assessment of the potential endocrine disrupting properties of TPhP have been evaluated and are described in section 5.

Aquatic compartment (including sediment)

Fish

Short-term toxicity to fish

Twelve studies are reported in the registration dossier. All of these studies are old (generally performed more than thirty years ago) and mostly used static exposure conditions. Three include chemical analysis, although the results are only reported for one.

The registrant judges the majority of the tests to be Klimisch reliability 2 with two considered unreliable (Klimisch 3 and 4). The reliable studies report a range of LC_{50} values between >0.32 and 1.26 $mg.l^{-1}$, with the majority below 1 $mg.l^{-1}$ (Mayer et al., 1981; Palawski et al., 1983; Huckins et al., 1991; Sasaki et al., 1981). The lowest, reliable value is a 96-h LC_{50} of 0.36 $mg.l^{-1}$ for Rainbow Trout *Oncorhynchus mykiss* (Palawski et al., 1983). However, the key study selected by the registrant is Mayer et al. (1981), which, reported a 96-h LC_{50} of 0.40 $mg.l^{-1}$ for *O. mykiss* based on nominal concentrations.

Long-term toxicity to fish

There are five long-term studies in the registration dossier, all of which are judged to be Klimisch reliability 2 by the registrant. However, two of these are 96 h tests which should not be considered as part of the chronic dataset. Of the remaining three tests, the key study for long-term toxicity to fish (Sitthichaikasem, 1978) started with 10-days old sac frys and lasted 30 days, and is considered part of an Early Life Stage Test. No NOEC was obtained with the TPhP concentrations tested.

Aquatic invertebrates

Short-term toxicity to aquatic invertebrates

A test on the acute toxicity of TPhP to *Daphnia magna* was conducted according to the US guideline EPA-660/3-75-009. Static exposure for 96 h resulted in a LC_{50} value of 1.0 $mg.l^{-1}$ (nominal concentration). Lower effective concentrations were found for another crustacean, *Mysidopsis bahia*, in the same test on acute toxicity. Static exposure for 96 h resulted in a LC_{50} value of 0.18 $mg.l^{-1}$ (nominal concentration) (Mayer et al., 1981).

Further invertebrate toxicity studies investigated the acute effects of TPhP on scud (*Gammarus pseudolimnaeus*) and midge larvae (*Chironomus riparius*) (Huckins et al., 1991). Static exposure for 96 h resulted in a LC_{50} value of 0.25 $mg.l^{-1}$ and 0.36 $mg.l^{-1}$ respectively (nominal concentrations). Further, Lo et al. (2000) investigated the toxicity of TPhP to golden apple snail

[2] https://chem.echa.europa.eu/100.003.739/dossier-view/9b2f054a-c82b-4cc1-9393-415a4f7781c1/4c37f1df-d04d-4911-8afc-4a9bf3b52bf0_4c37f1df-d04d-4911-8afc-4a9bf3b52bf0?searchText=204-112-2

(*Pomacea canaliculata*) and reported a relatively lower toxicity than identified for crustaceans in other studies (72 hr, LC_{50} 38.2 $mg.l^{-1}$).

Long-term toxicity to aquatic invertebrates

The chronic toxicity of TPhP to aquatic invertebrates (*Daphnia magna*) was tested according to OECD Guideline 211 (*Daphnia magna* Reproduction test). After 21 days a NOEC of 0.254 $mg.l^{-1}$ (mean measured concentration) was obtained based on reproduction (Unpublished study report, 2000).

Algae and aquatic plants

The registrant reports nine tests. Eight of these tests appear to be the same as those reported in Environment Agency (2009). The registrant judges all of the tests to be Klimisch reliability 2, which is consistent with Environment Agency (2009).

The registrant considers the test (three species and three different media) conducted by Millington et al. (1988) according to an OECD guideline 201 to be the key study. The authors of the test only reported LOEC values. The most critical values were obtained for *Desmodesmus subspicatus* and *Pseudokirchneriella subcapitata.* The data reports LOECs between 0.5 and 5.0 $mg.l^{-1}$ depending on the growth medium. Mayer et al. (1981) determined the toxicity of TPhP to *Desmodesmus subspicatus* and a 96h-EC_{50} was determined to be 2 $mg.l^{-1}$.

Sediment organisms

No relevant information available.

Other aquatic organisms

No relevant information available.

Terrestrial compartment

Toxicity to soil macro-organisms

No relevant information available.

Toxicity to terrestrial plants

No relevant information available.

Toxicity to soil micro-organisms

No relevant information available.

Toxicity to other terrestrial organisms

No relevant information available.

Atmospheric compartment

No relevant information available.

Microbiological activity in sewage treatment systems

The registrant used an activated sludge assay as key study, according to OECD guideline 301C (CITI, 1992). Activated sludge (30 $mg.l^{-1}$ as concentration of suspended solid) in a volume of 300 mL was exposed to TPhP (100 $mg.l^{-1}$) for 28 days at 25°C. Degradation was determined by BOD. Under the used conditions, TPhP did not adversely affect the microorganisms present, since TPhP biodegraded to the extent of 83 - 94 %. The key values should be understood as > 100 $mg.l^{-1}$ as no effect was observed up to the test concentration of 100 $mg.l^{-1}$.

Toxicity to birds

No relevant information available.

Mammalian wildlife

No relevant information available.

6. Endocrine disruption (Environment)

General approach for the assessment of endocrine properties

Framework of the evaluation

To evaluate whether or not TPhP fulfils the WHO/IPCS definition (WHO/IPCS, 2002) of an endocrine disruptor as interpreted by the EC ED EAG (JRC, 2013), both *in vitro* and *in vivo* data were taken into account, in order to demonstrate:

- Adverse effects;
- Endocrine mode of action;
- Biological plausible link between adverse effects and endocrine mode of action.

As highlighted in EDC guidance developed by ECHA and the European Food Safety Authority (EFSA) to identify EDC under the plant protection products and the biocidal product regulations published in 2018 (ECHA/EFSA, 2018), the 'endocrine mode of action' in the second bullet point should be interpreted as 'endocrine activity', i.e., the substance has the potential to alter the function(s) of the endocrine system. Consequently, the third bullet point should be interpreted as biological plausible link between adverse effects and endocrine activity.

Specificity, i.e., that adverse effects were not non-specific secondary consequences of general toxic effects, and population relevance were also considered in the assessment.

The structure and the assessment of data are mainly based on the OECD Revised Guidance Document 150 on standardised test guidelines for evaluating chemicals for endocrine disruption (OECD, 2018).

Two different aspects are assessed separately:

- Evidence for endocrine activity;
- Effects on apical endpoints that provide evidence that a substance exerts adverse effects owing to its endocrine activity.

Information sources and strategy for endocrine disruptor identification

Literature review

A literature review was performed following the principles displayed in the EDC guidance (ECHA/EFSA, 2018). The EDC guidance provides a tiered approach to assess the adversity of chemicals on vertebrates, and to link it with an estrogenic (E), androgenic (A), thyroid hormone (T), or steroidogenesis-related (S) mode of action (the so-called EATS modalities). The evidence is first assembled by using a literature review and weight of evidence approach. Then, the EATS-mediated adversity and the endocrine activity are assessed. The detailed methodology is presented in the following sections.

A literature review was conducted up to May 2023. The literature review on TPhP endocrine disruptive properties was focused on fish, amphibian and *in vitro* mechanistic studies. The literature search was performed in Scopus database.

A single concept strategy search was applied to retrieve all relevant information on TPhP by using its Chemical Abstracts Service Registry Number (CAS No 115-86-6), scientific chemical names, and common names, as recommended in the EDC guidance (ECHA/EFSA, 2018).

Studies were included in the literature review based on their relevance when they met all of the following criteria: a) peer-reviewed research articles or primary reports of research findings that presented original data; b) endocrine activity or adversity assessed in *in vitro*, *ex vivo*, or *in vivo* studies in vertebrate species; and c) English-language articles. The relevance filtering was first

based on title and abstract screening, and second, on full-text screening. When checking title and abstract was insufficient to decide if the paper was relevant and should be included in the review, full-text screening was applied (e.g., TPhP not explicitly mentioned in the abstract). Two distinct reviewers shared the two screening phases during the literature search and resolved any conflicts or discrepancies by complementary full-text screening and by discussion.

In order to provide further support and transparency to the robustness of the evaluation of the data, ToxRtool was used to assess the reliability of the *in vivo* experimental studies that were considered as the most informative to reach the conclusions. ToxRtool[3] was developed by the European Commission's Joint Research Center in 2009 (Segal et al., 2015) and builds on Klimisch categories by providing additional criteria and guidance for assessing the reliability of (eco)toxicological studies. It is applicable to various types of experimental data, endpoints and studies (study reports, peer-reviewed publications). ToxRtool scores 1 and 2 are defined as reliable without restrictions and reliable with restrictions, respectively. ToxRtool 3 is assigned as not reliable. In consequence they should not be used as key studies, but depending on the shortcomings of the study it may still be useful in weight of evidence approaches or as supportive information. The ToxRtool score of each experimental study is justified by the description of possible limitations, when relevant.

Assessment of the evidence

The present analysis was performed in collaboration with the ANSES Thematic Working group on Endocrine Disruptors (EDC-WG)[4]. The studies were retrieved from the literature review as well as from the registration dossier and were considered on the basis of their relevance (see criteria of selection based on relevance above), reliability and adequacy for the analysis. Studies were qualitatively weighted based on expert judgement to produce a conclusion on the selected adverse effects and their ED MoA.

Analysis of the results

The data were grouped into three categories in accordance with the OECD conceptual framework (OECD, 2018) and EU EDC guidance (ECHA/EFSA, 2018):

a) *in vitro* mechanistic parameters;

b) *in vivo* mechanistic parameters;

c) parameters providing information on adversity.

Based on the adverse effects identified, results were further integrated into lines of evidence, defined as a "set of relevant information grouped to assess a hypothesis," using a weight-of-evidence approach (ECHA/EFSA, 2018).

In silico and *in vitro* information indicative of endocrine activity

Nineteen studies gathered in the literature and included in the review provide *in vitro* mechanistic information on the capacity of TPhP to interact with the endocrine system. Five studies investigated *in silico* by modelling potential interactions with receptors. Most of the studies focus on TPhP estrogenic activity.

Table 10: Summary table of *in silico* and *in vitro* mechanistic data (chronologic order)

STUDIES INVESTIGATING ENDOCRINE DISRUPTION PROPERTIES OF TPhP

[3] https://eurl-ecvam.jrc.ec.europa.eu/about-ecvam/archive-publications/toxrtool
[4] https://www.anses.fr/en/content/endocrine-disruptors.

Methodology	Results (statistically significant)	Modality	Reference
Molecular Docking and Molecular Dynamic Simulation using Discovery Studio 2.5/Ligand fit module.	Tight binding affinity for hERα in docking approach (agonist effect)		Zhang et al., 2014
Molecular dynamics analysis	TPhP disturbed conformational changes to affect the structural stability of TRβ ligand binding domain leading to potential disruption of the transcriptional activity of TR.		Wang et al., 2021
Molecular docking (CDOCKER)	With ERα, TPhP formed a pi-sigma bond with Arg394 as E2 which in addition bond to His524 and Glu353. With GPER, TPhP formed a pi-sigma bond with Tyr142 while E2 formed hydrogen bonds with Tyr123 and Glu275, and Van der Waals forces with Cys205. G1, a GPER agonist, had a hydrogen bond and a pi-pi bond with Gln138 and Phe208. The CDOCKER interaction energy with TPhP was - 29.60 kcal/mol.		Ji et al., 2022
Molecular dynamics simulation	Signs of TPhP binding to GPER leading to the change of its structural conformation for a possible activation		Guan et al., 2022
Molecular dynamics simulation	Signs of TPhP binding to GPER leading to the change of its structural conformation for a possible activation	E	Ji et al., 2022
AR competitive binding assay (18 to 20h) (Rat AR protein) Dose: 4.28 nM to 0.428 mM.	IC_{50}: 1.50E-05 M AR Relative binding affinity (RBA): 0.0205% (moderate binding)	A	Fang et al., 2003
H295R cell bioassay (48h) Dose: 0 – 0.001 – 0.01 – 0.1 – 1 $mg.l^{-1}$ Dose: 0 – 0.01 – 0.1 – 1 $mg.l^{-1}$	<u>Hormone synthesis</u> 17β-estradiol (E2): ↑ at 1 $mg.l^{-1}$ Testosterone (T): ↑ at 1 $mg.l^{-1}$ E2/T Ratio: ↑ at 0.1 and 1 $mg.l^{-1}$ <u>Steroidogenic genes</u> CYP11A1: ↑ at 1 $mg.l^{-1}$ CYP11B2: ↑ at 1 $mg.l^{-1}$ CYP19A1: ↑ at 1 $mg.l^{-1}$ HSD3β2: no effect <u>SULT genes</u> SULT1E1 ↓ at 1 $mg.l^{-1}$ SULT2A1 ↓ at 1 $mg.l^{-1}$	S	Liu et al., 2012

Transcriptional assay on MVLN cell (72h) Dose: 0 – 0.001 – 0.01 – 0.1 – 1 - 10 mg.l^{-1}	Agonist activity to E2 receptor was not detected for TPhP tested alone. TPhP co-exposed to E2 decreased E2 binding activity for hER (from 0.001 mg.l^{-1}).	E	
Cell-based transactivation Assay			Kojima et al., 2013
CHO-K1 cells (24h) Dose: 0.10 to 30 µM.	Erα: REC_{20} = 4.9 µM (weak agonist) Erβ: REC_{20} = 6.5 µM (weak agonist) AR: RIC20 = 17 µM (weak antagonist) GR: RIC20 = 15 µM (weak antagonist)	E A	
COS-7 cells (24h) Dose: 0.10 to 30 µM.	PPARγ: weak agonist (15% at concentration of 30 µM) PXR: REC_{20} = 2.8 µM (agonist)	Other	
Dual Luciferase Reporter Gene Assay / CHO-K1 cells (24h) Dose: 0.1 nM to 1 µM.	Erα: REC_{20} of 0.27 µM (↑ activation of Erα in a dose-dependent manner)	E	Zhang et al., 2014
Yeast two-hybrid assay (4h) Dose: 0.1 nM to 1 µM.	Erα: REC_{20} of 0.65 µM (↑ activation of Erα in a dose-dependent manner)		
E-Screen Assay / MCF-7 cells (human breast cancer cell line, immortalised cells, ERα positive), (5 days) Dose: 0.1 nM to 1 µM.	Maximum cell proliferation: 1 µM Relative proliferative effect: 37.5%		
GH3 (rat pituitary) cell assay (48h) Dose (µg.l^{-1}) : 0 – 1 (0.0003 µM) – 10 (0.031 µM) – 100 (0.31 µM) FRTL-5 (rat thyroid follicular) cell assay (24h) Dose (mg.l^{-1}) : 0 – 1 (3.1 µM) – 3 (9.2 µM) – 10 (31 µM)	*Tshβ* ↑ at 0.31 µM *Trα* ↑ at 0.031 & 0.31 µM *trβ* ↑ at 0.31 µM *dio1* ↑ at 0.31 µM *dio2* no effect *nis* ↑ at 9.2 & 31 µM *tg* ↓ at 3.1 µM *tpo* ↑ at 31 µM *tshr* ↓ at 3.1 µM *nkx2.1* ↓ at 3.1 & 31 µM *pax8* no effect	Thyroid	Kim et al., 2015

Cell-based transcriptional Assay CHO-K1 cells (24h) Dose: 0.1 to 30 µM COS-7 cells (24h) Dose: 1 to 30 µM	 Erα: REC_{20} = 4.6 µM (weak agonist) Erβ: REC_{20} = 7.3 µM (weak agonist) AR: RIC20 = 11 µM (weak antagonist) GR: RIC20 = 12 µM (weak antagonist) PPARγ: weak agonist (15% at concentration of 30 µM) PXR: REC_{20} = 2.8 µM (agonist)	 E A Other	Kojima et al., 2016
MCF-7 (human breast cancer cell line, immortalised cells, ERα and GPER positive), flow-cytometric proliferation assay (24h) Dose: 10 µM to 1 mM	Relative proliferative effect (RPE) = 87% (E2 100%) EC20 = 88 µM (E2 8E-07 µM)	E	Krivoshiev et al., 2016
MA-10 mouse Leydig tumour cells (48h) Dose: 1 to 100 µM	qRT-PCR *(Expression of Key Genes Involved in Steroidogenesis)* Lhcgr: no effect Adcy3: no effect Star: no effect TSPO: no effect Cyp11A1: no effect Hsd3*β*: no effect Progesterone production: no effect	S Other	Schang et al., 2016
Transthyretin (TTR) binding experiment (2h) Dose: approx. 6 nM to 32 µM	IC_{50}: 1083 nM (significant binding ability to TTR compared to T4 = IC_{50} of 291 nM)	Thyroid	Zhao et al., 2017
Yeast two-hybrid assay with Japanese medaka Erα (mERα) Dose: approx. 0.1 to 100 µM (4h)	IC50 = 29 µM (antagonist activity)	E	Li et al., 2018
Yeast two-hybrid assay with human Erα (4h) 0.5 nM to 0.1µM.l^{-1} MVLN cell assay with human ERE (72h) 0.1 nM to 20µM.l^{-1} E-SCREEN assay (proliferation MCF7, human breast cancer cell line, immortalised cells, ERα and GPER positive)	hERα: Antagonist activity (RIC20 = 2E-05 mol/L) compete with E2 for binding to Erα hERE: Agonist response (EC50 = 1.45E-05 mol/L) = Erα agonist to activate the ERE pathway Promotes cell proliferation = agonist activity (REC20 = 5.01E-07 M/L, max 77.08%). This proliferative effect was significantly inhibited when cells were pretreated with G15 (a GP30 inhibitor).	E	Ji et al., 2020

Multiplexed reporter assay (the Ecotox FACTORIAL) 40 µM (24h)	Activation of Zebrafish ER2*β*, frogER1, turtleER1, chickenER1, hERα Activation of chickenAR No effect on ZebrafishER1 and ER2α, frogER2, hER*β*, No effect on AR (fish, frog, turtle, human) No effect on fishTRα and TR*β*, frogTRα, turtleTRα, hTRα and hTR*β* Activation of hPPARγ, mPPARγ, zPPARγ	E A E A Thyroid Other	Medvedev et al., 2020
HG5LN cell assay (24h) Dose (µM): 0.1 to 10 µM	<u>Transcriptional activation bioassay:</u> Activation of hPPARγ (EC50: nd; % max. activity: 28%) Activation of mPPARγ (EC50: nd; % max. activity: 24%) Activation of xPPARγ: (EC50: nd; % max. activity: 21%) Activation of zfPPARγ (EC50 = 3.5 µM; % max. activity: 104%)	Other	Garoche et al., 2021
Multiplexed reporter assay (the Ecotox FACTORIAL) 22.2 µM	Agonist activity on mPPARγ, hPPARγ, zPPARγ	Other	Houck et al., 2021
Reporter gene assay: HEK293 cell assay (39h) with medaka estrogen receptor 1(mEsr1) HepG2 cell assay (39h) with medaka androgen receptor β (mArβ)	mEsr1: - Agonist activity (EC_{50} = 9.7E-06 M). - No antagonist activity up to 1.0 E-05 M. mARβ: No agonist or antagonist activity up to 1.0 E-05 M.	E A	Kawashima et al., 2021
Transcriptomic analysis on L02 cells (HL-7702, human normal liver cells) for 24h. 20 and 200µM	<u>Gene expression</u> ↑ THRB, RXRA, DIO2 and PIK3R3 (from 20 µM) ↓ TP53, NOTCHI1 (from 20 µM) ↓ AKT3, DIO2, THRA, SLCO1C1 and CASP9 (200 µM) ↑ MDM2, BAD (200 µM)	Thyroid	Wang et al., 2021
H295R cell assay (48h) Dose (µM) : 0.1 – 1 – 2 and 10	<u>Hormone levels:</u> *E2* ↑ at 1, 2 and 10 µM *E2/T ratio* ↑ at 1, 2 and 10 µM *Progesterone* ↑ at 2 and 10 µM *Estrone* ↓ at 2 and 10 µM *Testosterone* ↓ at 1, 2 and 10 µM	S	Ji et al., 2022

	Transcriptional genes[5] *CYP17* ↑ at 2 and 10 µM *CYP19* ↑ at 1, 2 and 10 µM *3β-HSD2* ↑ at 2 and 10 µM *17β-HSD1* ↑ at 1, 2 and 10 µM *HMGR*[6] ↑ at 1, 2 and 10 µM *StAR*[7] ↑ at 10 µM *SULT2A1* ↓ at 1, 2 and 10 µM *CYP11A1* No sign. effect *17β-HSD4* No sign. effect		
MVLN (hERα +) cell assay (72h) Dose (µM) : 0.1 – 0.2 – 1 – 2 – 10 – 20	Receptor activation hERα Agonist activity EC50 = 1.2E-05 M	E	
SKBR3 (ERα-, GPER+) cell assay (24h) Dose (µM) : 0.1 – 1 and 10	GPER-cAMP activation: - Interaction with GPER at 1.0E-05 µM *via* cAMP formation - The agonist effects of TPhP on cAMP production were significantly inhibited when TPhP was co-exposed with G15. - The E2-control group responded as expected.	E	
ERα- SRC (steroid receptor co-activators) recruitment assay (1 h) Dose 0.01 µM to 1000 µM.	ERα agonist interaction with SRC1–1: EC50 of 2.93E-04 mol/L ERα agonist interaction with SRC3–3: EC50 of 1.30E-05 mol/L, No effect on the other 8 SRCs peptides	E	
Competitive binding assay: SKBR3 cells (ERα-, GPER+) (10 min) Dose: 1 nM to 100 µM	TPhP inhibits the binding of E2-F to GPER (probe) (IC50 4.2 µM)	E	
Transcriptomic and proteomic analysis (SKBR3 cells)	64 genes were enriched for both mRNA and protein after TPhP exposure. Among these overlaps, 52 DEGs[8]/DEPs[9] were found to be up-regulated or down-regulated together. Combined transcriptome and proteome analysis showed that TPhP could exert its estrogen-like proliferation-promoting effect through the interaction of the PI3K-Akt[10] signalling pathway, MAPK[11] signalling pathway, and EGFR[12] signalling pathway, which are downstream pathways mediated by GPER.		Guan et al., 2022

[5] Fold above 1.75 are reported in this table.
[6] HMGR: 3-Hydroxy-3-methylglutaryl-coenzyme A reductase.
[7] StAR: Steroidogenic acute regulatory protein.
[8] DEGs: differentially expressed genes.
[9] DEPs: differentially expressed proteins.
[10] PI3K-Akt: phosphoinositide 3-kinase/protein kinase B.
[11] MAPK: mitogen-activated protein kinase.
[12] EGFR: epidermal growth factor receptor.

ToxCast Pathway (AUC)	Estrogen Agonist: 6.41E-02 = equivocal Antagonist: 3.14E-02 = equivocal Androgen Agonist: negative Antagonist: 3.79E-02 = equivocal	E A	CompTox Chemicals Dashboard v2.4.1

Estrogen pathway

Overall, 12 studies investigating the estrogenic activity of TPhP *in vitro* were identified and analysed. Four studies provided *in silico* modelisation. The main results are summarised below and further discussed in the conclusion section.

In silico, the ToxCast ER Model gave equivocal results for TPhP in respect of estrogen receptor agonist activity (predictive score: 6.41E-02) and antagonist activity (predictive score: 3.14E-02).

TPhP has been tested in different types of *in vitro* assay including binding assays, transactivation in reporter gene assays, regulated gene expression and G protein-coupled estrogen receptor (GPER) signalling pathways.

It appears that TPhP has **agonist activity on human ERα and / or ERβ receptor and antagonist activity on E2 to ER** (Liu et al., 2012; Kojima et al., 2013; Zhang et al., 2014; Kojima et al., 2016; Medvedev et al., 2020; Ji et al., 2022). Toxcast models showed equivocal agonist and antagonist activities. The agonist activity is also reported for the medaka ER1 (Kawashima et al., 2021) and for the zebrafish ER2β, frog ER1, turtle ER1 and chicken ER1 (Medvedev et al., 2020). In some yeast two-hybrid assays with human and medaka ERα, an antagonist effect is shown when TPhP was co-exposed with E2 indicating **a competition between TPhP and E2 for binding to ERα** (Li et al., 2018; Ji et al., 2020). Zhang et al. (2014) also demonstrated a binding affinity of TPhP to hERα with a docking approach. Moreover, TPhP can induce the ERE pathway as demonstrated by a dose-response agonist effect in MVLN cells (transfected with the human ERE-luciferase reporter gene) (Ji et al., 2020).
This estrogenic activity is supported by proliferation assays on MCF-7 cells, ERα and GPER positive cell line, that show TPhP promotes cell proliferation (Zhang et al., 2014; Krivoshiev et al., 2016; Ji et al., 2020).

In Guan et al. (2022), the authors explored the effects of TPhP on the key downstream signalling pathways mediated by the G-coupled protein ER (GPER) through combined transcriptome and proteome analysis. These relationships were also tested on a SKBR3 cell-based fluorescent competitive binding assay. The authors report that **TPhP inhibits the binding of E2 to GPER** with an IC_{50} of 4.2 µM. Combined transcriptome and proteome analyses show that **TPhP could exert its estrogen-like proliferation-promoting effect** through the interaction of the PI3K-Akt signalling pathway, MAPK signalling pathway, and EGFR signalling pathway, which are downstream pathways mediated by GPER.

In Ji et al. (2022), the interaction of TPhP with GPER was confirmed by molecular docking and their activation on GPER-mediated signalling was investigated in SKBR3 cells. In fact, the assay on SBRK3 cells shows that **TPhP can interact with GPER and activate cAMP formation,** which is one of the rapid signalling pathways induced by GPER activation. However, this activation is 10-times less efficient than the reaction induced by E2. This activation *via* GPER is also confirmed by the results obtained with a TPhP co-exposure with the specific GPER30 inhibitor, G15, which inhibits the effects of TPhP in the assay.

Conclusion: The available *in vitro* information demonstrate the agonist activity of TPhP on nuclear ERα and ERβ estrogen receptors of several vertebrate species including human, rat, mouse, fish, chicken, frog and turtle as evidenced by ER transactivation in reporter cell lines, increased promoter occupancy, induction of ER-regulated gene expression and related physiological cell responses (e.g., increased cell proliferation). Studies showed also an antagonist activity of TPhP on E2 effect to ER. Two recent studies showed that TPhP could also activate GPER.

Androgen pathway

TPhP binding capacity to the androgen receptor (AR) has been investigated in the study by Fang et al. (2003). The results indicate a moderate binding of TPhP to the AR.

The ToxCast AR Model gave negative result for TPhP in respect of androgen receptor agonist activity (predictive score: 0.00) and equivocal result for antagonist activity (predictive score: 3.79E-02).

Weak AR antagonist effects were observed in hamster CHO-K1 cells (Kojima et al., 2013; 2016) but no agonist or antagonist activity is detected with medaka ARβ (Kawashima et al., 2021).

Conclusions: The data available indicate that TPhP can moderately bind to the AR and could induce a weak anti androgenic effect in vertebrate cells.

Thyroid pathway

The expression of thyroid hormone-related gene expression was investigated in rat pituitary GH3 cells and in rat thyroid follicular FRTL-5 cells (Kim et al., 2015). In GH3 cells, TPhP exposure led to an up-regulation of *tra* from the medium tested dose (0.031 µM) and of *tshß, trβ* and *dio1* gene expression at the highest concentration tested (0.31 µM). In FRTL-5 cells, TPhP exposure led to a down-regulation of *tg* and *tshr* expression at 3.1 µM, an up-regulation of *nis* (9.2 µM and 31 µM), and *tpo* gene expression (31 µM). In cells from fish, frog, turtle and human, TPhP exposure led to no effects on TRα and TR*β* at 40 µM (Medvedev et al., 2020).

Conclusions: The results on GH3 cells and FRTL-5 cells show that TPhP can interact with thyroid hormone receptors in the rat pituitary or thyroid gland cells and could thus lead to stimulation of the synthesis of thyroid hormones.

Steroidogenesis

The steroidogenesis activity of TPhP was assessed in human adrenal cortico-carcinoma (H295R) cells (Liu et al., 2012; Ji et al., 2022). These two assays show that TPhP affects steroidogenesis

by increasing estrogen levels (17ß-estradiol: E2) (see

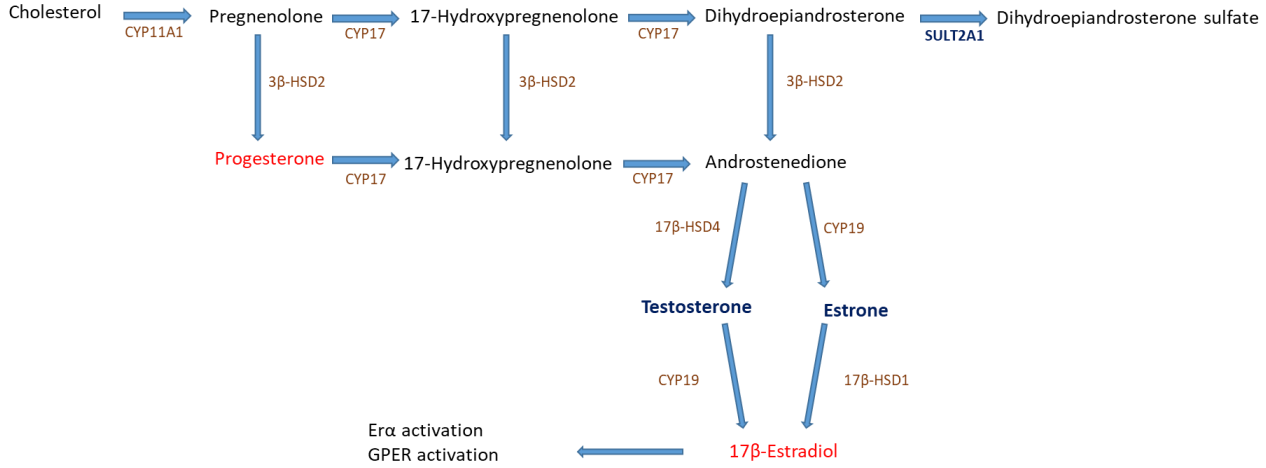


Figure 3).

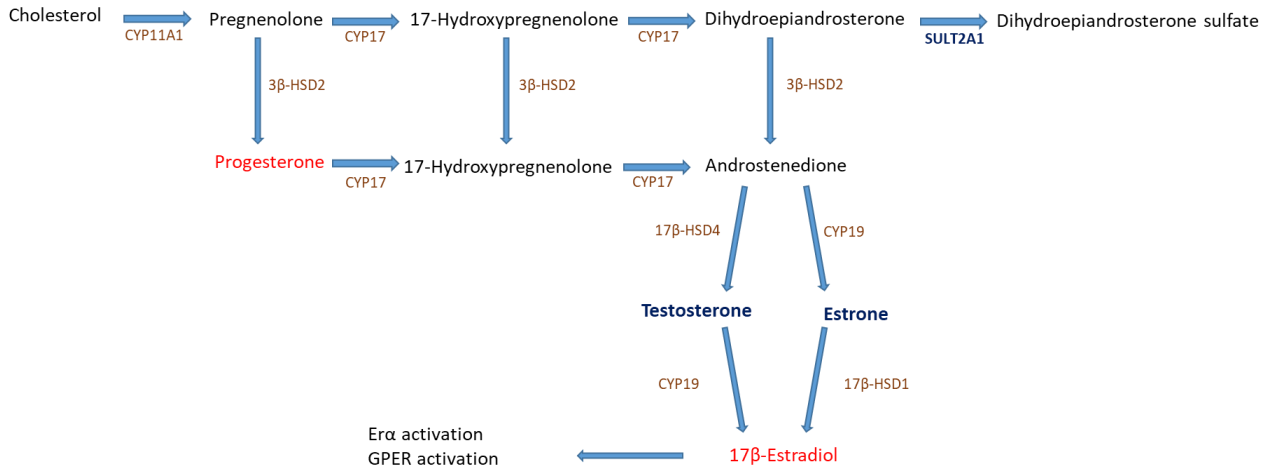


Figure 3: Proposed mechanism framework of estrogen disrupting effects for TPhP from the publication of Ji et al. (2022). Hormones in red indicates a significant increase of levels and blue indicates a significant decrease of levels (from Ji et al., 2022).

TPhP leads to an increase of E2 and E2/T ratio (Liu et al., 2012; Ji et al., 2022) and of progesterone (Ji et al., 2022) suggesting that **TPhP has also an estrogenic activity through an action on steroidogenesis**. Ji et al. (2022) shows that TPhP can promote cholesterol synthesis and its transfer through cellular membranes by a significant increase of HMGR and StAR gene expression levels respectively. TPhP also induces an increase of the expression of the CYP genes (CYP11A1, CYP11B2, CYP19A1, CYP19) and 17β-HSD1 gene, which are linked to steroidogenesis. TPhP up-regulated CYP11A1 gene expression, which then can result in an increased conversion of cholesterol to pregnenolone. In addition, the up-regulated CYP17 gene expression may cause more androstenedione synthesis, and up-regulated 3β-HSD2 will impact the next step of the steroid synthesis. The authors hypothesised that the increased progesterone levels noted in the study may result from the up-regulation of CYP11A1 and 3β-HSD2 gene expression. An increased androstenedione synthesis due to CYP17 up-regulation gene expression will then be followed by an increased conversion of testosterone and estrone to E2 *via* the up-regulated CYP19 and 17β-HSD1 gene expression, which ultimately resulted in an increase of E2 concentrations (mechanisms confirmed in two other studies on 5 phthalates (DEP, MEP, BBzP, MBzP and DiBP) (Sohn et al., 2016) and on the trichloroethylene (Tachachartvanich et al., 2018).

In Ji et al. (2022) a decrease of estrone is observed while CYP19 is upregulated and E2 level increases. The reason given by the authors is that the conversion of estrone to E2 by 17β-HSD1 is stronger than the activity of CYP19 to estrone synthesis. Lastly, inhibition of SULT2A1 expression observed in the two H295R assay may lead to more dehydroepiandrosterone (DHEA), participating in the production of E2 (Liu et al., 2012; Ji et al., 2022).

Conclusion: The two H295R assays performed with TPhP show that TPhP affects steroidogenesis by increasing estrogen levels (17ß-estradiol) and increasing expression of CYP19 and 3β-HSD2 genes involved in this pathway.

Other endocrine pathways

TPhP is shown to activate the transcription of human, mouse, zebrafish or Xenopus PPARγ (peroxisome proliferator-activated receptor) (Garoche et al., 2021). Transcriptional activation of human, mouse and fish PPARγ is also demonstrated in a multiplexed reporter assay (Medvedev et al. 2020; Houck et al., 2021). Kojima et al. (2013; 2016) also report agonist activity of TPhP on human PPARγ (COS-7 cells).

In Kojima et al. (2013; 2016) the authors investigated interactions with PXR receptor from COS-7 cell-based reporter gene assay (to detect the agonist activity of chemicals against PXR). TPhP showed PXR agonist activity (REC_{20} = 2.8 µM).

In vivo mechanistic data with regard to an endocrine activity

Table 11: Summary table of *in vivo* mechanistic data (chronologic order)

STUDIES INVESTIGATING ENDOCRINE DISRUPTION PROPERTIES OF TPhP			
Methodology	**Results (statistically significant change)**	**Remarks**	**Reference**
Adult Zebrafish (*Danio rerio*) – 4 months old Nominal concentration: 0.04 – 0.2 – 1 mg.l^{-1} No analytical measurement Solvent < 0.01% DMSO (v/v) Exposure of male and female fish separately Replicates: 3 with 3 fish for each replicate Exposure duration: 14 days	<u>Female Plasma sex hormone</u> 17β-estradiol (E2) ↑ at 1 mg.l^{-1} Testosterone (T) No change 11-ketotestosterone (11-KT) No change E2/T Ratio No change E2/11-KT Ratio ↑ at 1 mg.l^{-1} <u>Male Plasma sex hormone</u> 17β-estradiol (E2) ↑ at 1 mg.l^{-1} Testosterone (T) ↓ at 1 mg.l^{-1} 11-ketotestosterone (11-KT) ↓ at 1 mg.l^{-1} E2/T Ratio ↑ at 1 mg.l^{-1} E2/11-KT Ratio ↑ at 1 mg.l^{-1} <u>Female Transcriptional genes</u> *CYP17*[13] ↑ at 1 mg.l^{-1} *CYP19* ↑ at 1 mg.l^{-1} *VTG1*[14] ↓ at 1 mg.l^{-1} <u>Male Transcriptional genes</u> *CYP17* ↑ at 1 mg.l^{-1} *CYP19* ↑ at 1 mg.l^{-1} *VTG1* ↑ at 0.04; 0.2 and 1 mg.l^{-1}	2 (reliable with restriction) Test material: TPhP Purity unknown Non-guideline/ exploratory study Not GLP	Liu et al., 2012

[13] CYP17 and CYP19A were measured in gonad.
[14] *VTG 1* was measured in liver.

Photoperiod: 16h light – 8h dark Water temperature: 24°C ± 2°C Water pH: 7.2 to 7.8 Dissolved oxygen: > 7 $mg.l^{-1}$ Exposure medium: renewal every other day	<u>General toxicity</u> No significant mortality was observed (no details provided).		
Zebrafish embryos/larvae (*Danio rerio*) – 4hpf Nominal concentration: 0.02 - 0.2 - 2 $mg.l^{-1}$ Measured concentration: 48 hpf: 0.02 – 0.19 – 1.80 $mg.l^{-1}$ 120 hpf: 0 – 0 – 1.39 $mg.l^{-1}$ Solvent: 0.01% DMSO Replicates: Four separate wells with 20 eggs Exposure duration: up to 120 hpf Exposure medium: renewed at 48 h only.	Expression of mRNA for genes in six receptor (AhR, PPARα, ER1, TRα, G and MR) centered gene networks[15] *AHRRB* ↑ at 0.02 $mg.l^{-1}$only *CYP1A1* ↑ at 2 $mg.l^{-1}$ *NCOR2* ↑ at 2 $mg.l^{-1}$ *CYP1B1* ↑ at 2 $mg.l^{-1}$ *PPARα* ↑ at 2 $mg.l^{-1}$ *PPARGC1A* ↑ at 2 $mg.l^{-1}$ *LPL* ↑ at 2 $mg.l^{-1}$ *IL6* ↑ at 2 $mg.l^{-1}$ *PPARg* ↑ from 0.2 $mg.l^{-1}$ *ER2b* ↑ at 2 $mg.l^{-1}$ *TRα* ↑ at 2 $mg.l^{-1}$ *FUS* ↑ at 2 $mg.l^{-1}$ *GR* ↑ at 0.02 $mg.l^{-1}$ *RELA* ↑ at 2 $mg.l^{-1}$ *TGFb1* ↑ at 2 $mg.l^{-1}$ *HSP90AA1* ↑ at 2 $mg.l^{-1}$ *11βHSD* ↑ from 0.2 $mg.l^{-1}$ *EGFR* ↑ at 2 $mg.l^{-1}$ *MR* ↓ at 2 $mg.l^{-1}$ *HPSE* ↓ at 2 $mg.l^{-1}$ <u>General toxicity</u> Rates of hatching and survival were >90% and rate of malformation (spinal curvature, pericardial edema and yolk sac edema) was <5% in all exposure groups including controls (no details provided).	2 (reliable with restriction) Test material: TPhP Purity unknown Non-guideline/ exploratory study Not GLP	Liu et al., 2013a
Adult Zebrafish (*Danio rerio*) – 4/5 months old Nominal concentration: 0 – 0.04 – 0.2 – 1 $mg.l^{-1}$	<u>Female plasma sex hormone and VTG levels</u> E2 level ↑ at 1 $mg.l^{-1}$ E2/11-KT ratio level ↑ at 1 $mg.l^{-1}$ E2/T ratio level ↑ at 0.2 and 1 $mg.l^{-1}$ VTG ↑ at 0.2 and 1 $mg.l^{-1}$ Testosterone level ↓ at 1 $mg.l^{-1}$ 11-KT ↓ at 1 $mg.l^{-1}$ <u>Male plasma sex hormone and VTG levels</u> E2 level ↑ at 0.2 $mg.l^{-1}$	2 (reliable with restriction) Test material: TPhP	Liu et al., 2013b

[15] Only upregulated or downregulated gene are reported in this table.

Measured concentration: 0h: 0.03 – 0.14 – 0.89 mg.l^{-1} 48h: <LOD - <LOD – 0.38 mg.l^{-1} (LOD = 0.12 µg.l^{-1}) Solvent: DMSO (0.005%) Replicates: 6 replicates with six pair of fish Exposure duration: 21 days Photoperiod: 14h light – 10h dark Water temperature: 27 °C ± 1 °C Exposure medium: renewed in every 48h.	E2/11-KT level ↑ at 0.2 mg.l^{-1} VTG level ↑ at 1 mg.l^{-1} E2/T level ↓ at 0.04 and 0.2 mg.l^{-1} <u>Transcriptional genes in ovaries</u> *LHR* ↑ at 1 mg.l^{-1} *FSHR* ↑ at 1 mg.l^{-1} *HMGRA* ↑ at 1 mg.l^{-1} *STAR* ↑ at 0.2 mg.l^{-1} *17βHSD* ↑ at 0.04 mg.l^{-1} *CYP17A* ↑ at 1 mg.l^{-1} *CYP19A* ↑ at 1 mg.l^{-1} *HMGRB* ↓ at 1 mg.l^{-1} <u>Transcriptional genes in testis</u> *CYP11A* ↑ at 0.2 mg.l^{-1} *CYP17* ↑ at 1 mg.l^{-1} *CYP19A* ↑ at 1 mg.l^{-1} *LHR* ↓ at 0.04 mg.l^{-1} *HMGRA* ↓ at 1 mg.l^{-1} *STAR* ↓ at 1 mg.l^{-1} *17βHSD* ↓ at 0.2 mg.l^{-1} <u>Female transcriptional genes in brain</u> *GnRH2* ↓ at 1 mg.l^{-1} *GnRH3* ↓ at 0.2 mg.l^{-1} *GnRHR3* ↓ at 0.2 mg.l^{-1} *GnRHR4* ↑ at 1 mg.l^{-1} *LHβ* ↑ at 0.2 mg.l^{-1} *FSHβ* ↑ at 0.2 mg.l^{-1} *CYP19B* ↑ at 0.2 mg.l^{-1} *AR* ↑ at 0.2 mg.l^{-1} *Era* ↑ at 1 mg.l^{-1} *ER2β1* ↑ at 1 mg.l^{-1} <u>Male transcriptional genes in brain</u> *GnRH2* ↑ at 0.04 mg.l^{-1} *GnRH3* ↓ at 0.04 mg.l^{-1} *GnRHR2* ↑ at 0.04 mg.l^{-1} *GnRHR3* ↑ at 1 mg.l^{-1} *FSHβ* ↑ at 1 mg.l^{-1} *LHβ* ↓ at 0.04 mg.l^{-1} *CYP19B* ↑ at 1 mg.l^{-1} *AR* ↓ at 1 mg.l^{-1} *Era* ↑ at 1 mg.l^{-1} *ER2β1* ↑ at 0.2 mg.l^{-1} <u>General toxicity</u> No mortality was observed in any of the experimental concentrations during the exposure period (no details provided).	Purity: 99% Equivalent or similar to OECD TG 229 Not GLP	
Zebrafish embryos/larvae (*Danio rerio*) – 3hpf Nominal concentration: 0 – 40 – 200 – 500 µg.l^{-1}	<u>Hormone measurements in whole-body homogenate of 7 dpf Zebrafish larvae (100 larvae per replicate used)</u> T3 level ↑ at 40, 200 and 500 µg.l^{-1} T4 level ↑ at 40, 200 and 500 µg.l^{-1} <u>Gene expression in whole-body homogenate of 7 dpf Zebrafish larvae (20 larvae per replicate used)</u> *ttr* ↑ at 40, 200 and 500 µg.l^{-1} *tra* ↑ at 200 µg.l^{-1}	2 (reliable with restriction) Test material: TPhP	Kim et al., 2015

No analytical measurement Solvent: DMSO (0.005%) Replicates: 6 per treatment with 100 larvae per replicate Exposure duration: 7 days Photoperiod: 14h light – 10h dark Water temperature: 25°C ± 1°C Exposure medium: 50% renewed daily.	*dio1* ↑ at 500 µg.l^{-1} *nis* ↑ at 200 and 500 µg.l^{-1} *tg* ↑ at 200 and 500 µg.l^{-1} *ugt1ab* ↑ at 200 and 500 µg.l^{-1} *crh* ↓ at 500 µg.l^{-1} *trβ* ↓ at 500 µg.l^{-1} *tshβ* No change *tshr* No change *nkx2.1* No change *pax8* No change *Dio2* No change <u>General toxicity</u> Larval survival at 7d: 91.8% (control) – 92.5% (40 µg.l^{-1}) – 92% (200 µg.l^{-1}) – 88.7% (500 µg.l^{-1}). Body weight were not affected by the experimental concentrations.	Purity: 99% Non-guideline/ exploratory study Not GLP	
Larvae, juvenile and adult Zebrafish (*Dan io rerio*) – 4hpf Nominal concentration: 0 – 0.005 – 0.05 – 0.5 mg.l^{-1} Measured concentration: 24h: 0.0008 – 0.0055 – 0.131 mg.l^{-1} 48h: < LOD - < LOD – 0.011 mg.l^{-1} (LOD = 0.12 µg.l^{-1}) Solvent: DMSO (0.005%) Replicates:3 per treatment with 100 embryos per replicate Exposure duration: 120 days Photoperiod: 14h light – 10h dark	<u>Female plasma sex hormone</u> E2 level ↑ at 0.005 and 0.5 mg.l^{-1} Cortisol level ↑ at 0.5 mg.l^{-1} 11-KT level ↓ at 0.5 mg.l^{-1} E2/11-KT ratio level ↑ at 0.5 mg.l^{-1} T3 level ↑ at 0.5 mg.l^{-1} T4 level ↑ at 0.5 mg.l^{-1} <u>Male plasma sex hormone</u> E2 level ↑ at 0.005 mg.l^{-1} Cortisol level ↓ from 0.05 mg.l^{-1} 11-KT level ↓ from 0.005 mg.l^{-1} E2/11-KT level ↑ from 0.005 mg.l^{-1} T3 level No change T4 level No change <u>Female transcriptional genes in brain</u> *FSHβ* ↑ at 0.05 and 0.5 mg.l^{-1} *LHβ* ↑ at 0.005 and 0.5 mg.l^{-1} *GnRH3* ↑ at 0.005 and 0.5 mg.l^{-1} *Erα* ↑ at 0.5 mg.l^{-1} *pomc* ↑ at 0.5 mg.l^{-1} *mr* ↑ at 0.5 mg.l^{-1} *trhr2* ↑ at 0.5 mg.l^{-1} <u>Male transcriptional genes in brain</u> *LHβ* ↑ at 0.5 mg.l^{-1} *GnRH3* ↓ at 0.5 mg.l^{-1} *pomc* ↑ at 0.5 mg.l^{-1} trh ↓ at 0.5 mg.l^{-1} FSHβ No change Erα No change Transcriptional genes in ovaries	2 (reliable with restriction) Test material: TPhP Purity: 99% Non-guideline/ exploratory Not GLP	Liu et al., 2016

Water temperature: 27°C ± 1 °C Water pH: 7.2 to 7.8 Dissolved oxygen: > 7 $mg.l^{-1}$ Exposure medium: 50% renewed every 48h.	LHR ↑ at 0.5 $mg.l^{-1}$ STAR ↑ at 0.05 and 0.5 $mg.l^{-1}$ CYP19A ↑ at 0.5 $mg.l^{-1}$ CYP17 No change FSHR No change 3β-HSD No change 17β-HSD No change Transcriptional genes in testis STAR ↓ at 0.5 $mg.l^{-1}$ CYP17 ↓ at 0.5 $mg.l^{-1}$ FSHR ↑ at 0.005 $mg.l^{-1}$ LHR ↑ at 0.5 $mg.l^{-1}$ *3β-HSD* ↑ at 0.5 $mg.l^{-1}$ *17β-HSD* ↑ at 0.050 $mg.l^{-1}$ *CYP19A* No change <u>General toxicity</u> None of the test concentrations caused any distinct malformation, and there were no significant differences in mortality among treatment groups during the exposure duration (no details provided).		
Japanese medaka transgenic (*Oryzias latipes*) – o dph – Only male Nominal concentration: 1.6 – 8 – 40 $\mu g.l^{-1}$ Measured concentration: 0.134 – 0.299 – 1.43 $\mu g.l^{-1}$ Solvent: DMSO (0.001%) Replicates: 2 per treatment with 50 larvae per replicate Exposure duration: 100 days Photoperiod: 16h light – 8h dark Water temperature: 25°C ± 1 °C Water pH: 7.7 ± 0.2 Dissolved oxygen: > 7.8 ± 0.3 $mg.l^{-1}$	17β-E2 level ↑ at 0.29 and 1.43 $\mu g.l^{-1}$ T level ↓ at 1.43 $\mu g.l^{-1}$ 11-KT level ↓ at 1.43 $\mu g.l^{-1}$ <u>General toxicity</u> No data on systemic toxicity provided.	2 (reliable with restriction) Test material: TPhP Purity ≥ 95% Non-guideline/ exploratory Not GLP	Li et al., 2018

Number of tested fish: 16 males Exposure medium: flow-through system			
Japanese medaka transgenic (*Oryzias latipes*) – only females Solvent: DMSO (0.001%) Photoperiod: 16h light – 8h dark Water temperature: 25°C ± 1 °C Water pH: 7.7 ±0.2 Dissolved oxygen: > 7.8 ± 0.3 mg.l^{-1} Exposure medium: flow-through system **<u>Short-term exposure</u>:** adult exposed during 21 days Nominal concentration: 1.6 – 8 – 40 µg.l-1 Measured concentration: 243 – 589 – 1725ng.l^{-1} Replicates: 2 per treatment with 8 adult female per replicate **<u>Long-term exposure</u>:** larvae exposed during 100 days Nominal concentration: 1.6 – 8 – 40 µg.l-1	**Short-term exposure: 21 days** <u>Female plasma sex hormone</u> 17β-E2 level ↑ at 1725 ng.l^{-1} Testosterone level ↑ at 1725 ng.l^{-1} <u>Female transcriptional genes in liver</u> *VTG2* ↓ at 1725 ng.l^{-1} **Long-term exposure: 100 days** <u>Female plasma sex hormone</u> 17β-E2 level ↓ at 1773 ng.l^{-1} Testosterone level ↓ from 131 ng.l^{-1} 11-KT level ↓ from 131 ng.l^{-1} <u>Female transcriptional genes in liver</u> *VTG1* ↓ from 131 ng.l^{-1} *VTG2* ↓ from 363 ng.l^{-1} <u>General toxicity</u> No data on systemic toxicity provided.	2 (reliable with restriction) Test material: TPhP Purity ≥ 95% Non-guideline/ exploratory Not GLP	Li et al., 2019b

Measured concentration: 131 – 363 – 1773 ng.l^{-1} Replicates: 2 per treatment with 25 adult female per replicate			
Zebrafish adult (*Danio rerio*) Nominal Concentration: 0 – 0.04 – 0.2 – 1 mg.l^{-1} Measured concentration: 0h: 0.03 – 0.15 – 0.87 mg.l^{-1} 48h: < LOD - < LOD – 0.42 mg.l^{-1} (LOD = 0.12 µg.l^{-1}) Solvent: DMSO (0.005%) Replicates: For males: 3 replicates with 5 males per replicate. For females: 3 replicates with 5 females per replicate. exposure duration: 14 days Photoperiod: 14h light – 10h dark Water pH: 7.2-7.8 Dissolved oxygen: > 7 mg.l^{-1} Exposure medium: renewed at least three times per week	<u>Female plasma sex hormone</u> T4 level ↑ at 1 mg.l^{-1} T3 level ↑ at 1 mg.l^{-1} <u>Male plasma sex hormone</u> T4 level ↓ at 1 mg.l^{-1} T3 level ↓ at 1 mg.l^{-1} <u>Female transcriptional genes in brain</u> *crh* ↓ at 1 mg.l^{-1} *tshβ* ↓ at 1 mg.l^{-1} *tra* ↑ at 0.2 and 1 mg.l^{-1} *trβ* ↑ at 0.2 and 1 mg.l^{-1} <u>Male transcriptional genes in brain</u> *crh* ↑ at 0.2 and 1 mg.l^{-1} *tshβ* ↑ at 1 mg.l^{-1} *trβ* ↑ at 0.2 and 1 mg.l^{-1} *tra* No change <u>Female transcriptional genes in thyroid</u> *trβ* ↓ at 1 mg.l^{-1} *tra* ↑ at 1 mg.l^{-1} *dio2* ↑ at 1 mg.l^{-1} *tg* No change *tpo* No change *Dio1* No change <u>Male transcriptional genes in thyroid</u> *trβ* ↓ at 0.2 and 1 mg.l^{-1} *dio1* ↓ at 0.2 and 1 mg.l^{-1} *dio2* ↓ at 0.2 and 1 mg.l^{-1} *tra* No change *tg* No change *tpo* No change <u>General toxicity</u> No mortality was observed over the exposure period (no details provided).	2 (reliable with restriction) Test material: TPhP Purity 99% Non-guideline/ exploratory study Not GLP	Liu et al., 2019

Adult male rare minnows (*Gobiocypris rarus*) – 20-22 weeks old Nominal concentration: 0.012 – 0.12 $mg.l^{-1}$ Measured concentration: before renewal (T24h): 0.010 ± 0.0026 – 0.098 ± 0.031 $mg.l^{-1}$ Solvent: 0.01% acetone Exposure duration: 28 days Photoperiod: 14h light – 10h dark Water temperature: 25°C ± 1°C Exposure medium: renewal daily 30 fish per tank, 3 replicates per concentration	**Plasma steroid hormone levels** 11-KT levels: ↓ at 0.012 – 0.12 $mg.l^{-1}$ E2 levels: ↓ at 0.012 – 0.12 $mg.l^{-1}$ **Na^+/K^+ ATPase (NKA) activity** In testis: ↓ at 0.012 – 0.12 $mg.l^{-1}$ In sperm cells: ↓ at 0.012 – 0.12 $mg.l^{-1}$ <u>General toxicity</u> Survival rate (%): Control: 96.67 ± 2.89 TPHP - 0.012: 96.11 ± 5.36 TPHP - 0.12: 90.56 ± 5.18 Body weight: no change (no quantitative value provided in the publication) Total length: no change (no quantitative value provided in the publication)	2 (reliable with restriction) Test material: TPhP Purity ≥ 99% Non-guideline/ exploratory study Not GLP	Chen et al., 2020
Zebrafish adult (*Danio rerio*)*:* 5-month adult <u>Sub-chronic toxicity (10% of LC_{50})</u>	<u>Sex hormone and VTG levels in female whole-body homogenates</u> E2/T ratio level ↓ at 80 $\mu g.l^{-1}$ VTG level ↓ at 80 $\mu g.l^{-1}$ E2 No change T No change	2 (reliable with restriction) Test material: TPhP	He et al., 2021

Nominal concentration: 80 µg.l^{-1} No analytical measurement of TPhP Solvent: DMSO (0.01%) Replicates:10 males and 10 females exposed separately Exposure duration: 21 days Water temperature: 25°C ± 1 °C Photoperiod: 14h light – 10h dark Water pH: 7.2 ± 0.2 Dissolved oxygen: > 6.8 ± 0.2 mg.l^{-1} Exposure medium: renewed every day	<u>Sex hormone and VTG levels in male whole-body homogenates</u> E2/T ratio level: No change VTG: No change E2: No change T: No change <u>General toxicity</u> No data on systemic toxicity provided.	Purity 99% Non-guideline/ exploratory study Not GLP	
Japanese medaka (*Oryzias latipes*) – 16± 2 weeks old OECD 229 Nominal concentration (µg.l^{-1}): 20 – 64 – 200 – 640 Measured concentration (µg.l^{-1}): 2.13 – 7.19 – 17.1 – 44.9 (LOQ = 0.03 µg.l^{-1}) Replicates: 4 replicates per treatment with 3	<u>Female hepatic VTG level</u> VTG level: ↓$_1$ at 7.19, 17.1 and 44.9 µg.l^{-1} <u>Male phepatic VTG level</u> VTG level: No change <u>General toxicity</u> 4.2% of mortality at 44.9 µg.l^{-1} (1 female). No remarkable abnormal response (feeding activity, equilibrium, swimming…). No statistical analysis for length and weight parameters.	2 (reliable with restriction) Test material: TPhP Purity >97% According to OECD TG 229 Not GLP	Kawashima et al., 2021

males and 3 females per replicate Exposure duration: 21 days Water temperature: 25°C ± 2 °C Photoperiod: 16h light – 8h dark Water pH: 6.5-8.5 Dissolved oxygen: > than 60% of air saturation Exposure medium: Flow-through (5 times/day)			
Japanese medaka (*Oryzias latipes*) OECD TG 240 Age at start of exposure: F0: 12 weeks old F1: Embryo F2: Embryo Exposure duration: F0: 4 weeks F1: 15 weeks F2: 18 days (twice the median hatching date of the control) Nominal concentration ($\mu g.l^{-1}$): 0.5 – 1.6 – 5 – 16 - 50 Measured concentration ($\mu g.l^{-1}$): 0.501 – 1.62 – 4.54 – 15.2 – 48.4 Replicate: F0: 12 (control), 6 (tested concentrations)	<u>Female liver VTG level</u> F0 (adult): No change F1 (10 wpf, sub-adult): ↑ at 15.2 and 48.4 $\mu g.l^{-1}$ F1 (15 wpf, adult): ↓ at 0.501, 1.62, 4.54, 15.2 48.4 $\mu g.l^{-1}$ (dose response relationship) <u>Male liver VTG level</u> F0 (adult): <u>No change</u> F1 (10 wpf, sub-adult) <u>No change</u> F1 (15 wpf, adult) <u>No change</u> <u>General toxicity:</u> - Mortality F0 (adult): No change F1 (4 wpf, juveniles): ↑ at 48.4 $\mu g.l^{-1}$ (Mortality rate: 3%) F1 (8 wpf, sub-adult): No change F1 (15 wpf, adult): No change for the whole group (male + female) ↑ at 15.2 and 48.4 $\mu g.l^{-1}$ (for males only).	2 (reliable with restriction) Test material: TPhP Purity 99.9% According to OECD TG 240	MITI, 2021

F1 (1-10 weeks post fertilisation (wpf)): 12 (control), 6 (tested concentrations) F1 (10-15 wpf): 24 (control), 12 (tested concentrations) F2 (1-3 wpf): 12 (control), 6 (tested concentrations) Exposure medium: Flow-through (more 5 times/day) Water temperature: 25°C ± 1 °C Photoperiod: 16h light – 8h dark Water pH: 7.9 Dissolved oxygen: > than 60% of air saturation Mean fecundity of controls in F0 & F1: Greater than 20 eggs per pair per day Fertility (Fertilization rate) of all the eggs produced in F0 controls and F1 controls: Greater than 80% Hatchability of eggs in the F1 controls & F2 controls: ≥ 80% (average)			
Zebrafish embryos (*Danio rerio*) – 72hpf Nominal concentrations:	<u>Transcriptional genes</u> *tra* ↓ at 3.2 mg.l^{-1} *ttr* ↓ at 3.2 mg.l^{-1} *tshβ* ↓ at 9.8 mg.l^{-1} *dio1* ↓ at 3.2 mg.l^{-1} *dio2* ↑ at 3.2 mg.l^{-1} *cYP19A1b* No change	2 (reliable with restriction) Test material: TPhP	Lee et al., 2022

0.32 – 0.97 – 3.2 – 9.8 $mg.l^{-1}$ Solvent: DMSO (0.1% v/v) Replicates : 20 embryos per treatment Exposure duration : 24h	<u>General toxicity</u> Significant concentration-dependent decrease in body length at 0.97, 3.2 and 9.8 $mg.l^{-1}$ (n=10 embryos per treatment). Mortality > 50% at 9.8 $mg.l^{-1}$. No significant mortality observed for other concentrations (no details provided).	Purity > 98% Non-guideline/ exploratory study Not GLP	
OECD 234 (Fish sexual development test – FSDT) Zebrafish (*Danio rerio*) Nominal concentration: 1, 3.2, 10, 32, 100 $\mu g.l^{-1}$ Measured concentration: 1.11 – 3.01 – 7.76 – 33.3 – 76.8 $\mu g.l^{-1}$ (TPhP in 1L acetone) Replicates: 4 replicates per concentration with 30 fertilised eggs per replicate. Water temperature: 27°C ± 2 °C Photoperiod: 12h light – 12h dark Water pH: 7.6-7.8 Dissolved oxygen: > than 60% of air saturation Exposure duration: 73 days Exposure medium: flow-through system	<u>Sub-adult female plasma sex hormone and VTG level (10wpf)*</u> VTG level: ↑ at 76.8 $\mu g.l^{-1}$ 17β-E2 level: ↑ at 33.3 and 76.8 $\mu g.l^{-1}$ <u>Sub-adult male plasma sex hormone level (10 wpf)*</u> VTG level: No change 11-KT level: ↑ at 33.3 and 76.8 $\mu g.l^{-1}$ <u>General toxicity</u> Survival rates (post-hatching, 4-73 dpf): 88% (control group) - 84% (1.11 $\mu g.l^{-1}$); 80% (3.01 $\mu g.l^{-1}$); 77% (7.76 $\mu g.l^{-1}$); 63% (33.3 $\mu g.l^{-}1$) - 51% (76.8 $\mu g.l^{-1}$) Survival rates (post-hatching, 4-35 dpf): 89% (control group) - 87% (1.11 $\mu g.l^{-1}$); 84% (3.01 $\mu g.l^{-1}$); 80% (7.76 $\mu g.l^{-1}$); 67% (33.3 $\mu g.l^{-}1$) - 54% (76.8 $\mu g.l^{-1}$) Survival rates (post-hatching, 35-73 dpf): 98% (control group) - 97% (1.11 $\mu g.l^{-1}$); 96% (3.01 $\mu g.l^{-1}$); 96% (7.76 $\mu g.l^{-1}$); 95% (33.3 $\mu g.l^{-}1$) - 93% (76.8 $\mu g.l^{-1}$) *Statistical unit: all individual measurements per concentration (not the mean of replicates per concentration in contrast to analysis provided in the study report)..	2 (reliable with restriction) Test material: TPhP Purity > 99% OECD TG 234 GLP	Unpublished study report, 2021

Zebrafish embryo (*Danio rerio*) – (0-2 hpf) Nominal concentration: 0 – 20 – 50 – 100 – 500 and 1000 µg.l^{-1} No analytical measurement Solvent: DMSO (0.005% v/v) Water temperature: 28°C ± 1 °C Photoperiod: 14h light - 10h dark Replicates: 3 replicates per treatment with 40 embryos per replicate Exposure duration: 7 days Exposure medium: 90 % fresh exposure solution changed daily Measurement at 96 hpf and 7 dpf	<u>Transcriptional genes in whole body (96 hpf)</u> *BCL2A* ↓ at 20 µg.l^{-1} *CASP9* ↓ at 500 µg.l^{-1} *MBPA* ↓ at 1000 µg.l^{-1} *SYN2A* ↓ at 1000 µg.l^{-1} *FOXO3A* ↓ at 1000 µg.l^{-1} *PAX6A* ↓ at 500 and 1000 µg.l^{-1} *ALDH1A2* ↑ at 50 µg.l^{-1} *RDH1* ↓ at 1000 µg.l^{-1} *CRABP1A* ↓ at 1000 µg.l^{-1} *RBP2A* ↓ at 1000 µg.l^{-1} *RARAA* ↓ at 1000 µg.l^{-1} *CYP26A1* ↓ at 500 and 1000 µg.l^{-1} *RBP5* ↓ at 100, 500 and 1000 µg.l^{-1} <u>Transcriptional genes in whole body (7 dpf)</u> *ALDH1A2* ↑ at 100 and 500 µg.l^{-1} *MBPA* ↑ at 20 and 50 µg.l^{-1} *RDH1* ↑ at 500 µg.l^{-1} *RBP2A* ↑ at 20 µg.l^{-1} <u>General toxicity</u> From 120 hpf, the survival rate of zebrafish larvae was significantly decreased at 500 µg.l^{-1} and 1000 µg.l^{-1}. Mortality rate: 30% at 500 µg.l^{-1} and 70% at 1000 µg.l^{-1} 7 dpf.	2 (reliable with restriction) Test material: TPhP Purity > 98% Non-guideline/ exploratory study Not GLP	Zhang et al., 2023

Thyroid pathway

Clear sex-dependent alterations in plasma TH levels were observed in zebrafish with an increase of the plasmatic concentration of T3 and T4 in females (Liu et al., 2016; Liu et al., 2019) and a decrease or no change of the plasmatic concentration of T3 and T4 in males (Liu et al., 2016; 2019). Kim et al. (2015) also reported an increase of the whole body homogenate concentration of T3 and T4 in zebrafish embryos/larvae.

EAS modalities

The *in vivo* mechanistic effects described in the literature indicate that TPhP has EAS endocrine activity.

In Liu et al. (2012), the transcription of steroidogenic genes (CYP17 and CYP19A) was measured in adult Zebrafish gonads. Exposure of male and female to 1 mg.l^{-1} of TPhP significantly increased

CYP17 and CYP19A gene transcription. In Liu et al. (2013b), exposure to TPhP led to significant transcriptional changes in both fish testes and ovaries. In testes transcription levels of HMGRA, StAR, and 17β-HSD were significantly down-regulated, while transcriptions of CYP11A, CYP17, and CYP19A were significantly up-regulated. In ovaries, the major steroidogenic genes, including HMGRA, StAR, 17β-HSD, CYP17A, and CYP19A, were significantly up-regulated by exposure to TPhP. In Lee et al. (2022), no significant upregulation of the CYP19A1b expression level was observed for TPhP. However, a significant elevation of CYP19A1b was observed at the highest concentration with HO-*p*-TPhP, a metabolite of TPhP.

Several studies demonstrated a significant increase of plasmatic concentrations of E2 and E2/11-KT ratio in both sexes of zebrafish at the top dose (Liu et al., 2012; 2013b; 2016). An increase of E2 level was also noted in female zebrafish (Unpublished study report, 2021), male medaka (Li et al., 2018) and female medaka (Li et al., 2019b). Nevertheless, in one study (Li et al., 2019b) carried out on Japanese medaka, a decrease of E2 was noted after 100-day exposure of female larvae (age of fish at exposure initiation), while an increase of E2 was observed after 21-days exposure of adult females (age of fish at exposure initiation). Another study (Chen et al., 2020) showed a decrease of E2 in adult (20-22 weeks old at the start of the exposure) male rare minnows (*Gobiocypris rarus*) after 28 days of exposure. Finally in one study (He et al., 2021), no change in E2 level in male or female zebrafish whole body homogenates was noted.

Concerning testosterone, an increase in the plasma level could be observed after a short-term exposure in female medaka plasma, while a decrease was noted after a long-term exposure (Li et al., 2019b). In He et al. (2021), no change was recorded in female and male zebrafish fish homogenates. A decrease of testosterone was observed in male medaka and zebrafish (Li et al., 2018; Liu et al., 2012) and in female zebrafish (Liu et al., 2013b). Decreased levels of the hormone 11-ketotestosterone (11-KT) were recorded in female fish after exposure to TPhP (Liu et al., 2013b; Liu et al., 2016; Li et al., 2018). The variation in 11-KT for males also followed a decreasing trend (Liu et al., 2012; Liu et al., 2016; Liu et al., 2018; Chen et al., 2020), except in the Unpublished study report (2021), where the 11-KT increased in male fish. Moreover, E2/11-KT ratio increased in females and males (Liu et al., 2012; Liu et al., 2016; Li et al., 2018; Liu et al., 2013b). This trend could not be verified in the Unpublished study report (2021), as E2 levels were not measured in males and consequently the E2/11KT ratio could not be calculated.

In the same way, an activity on the E2/T ratio was noted, but the direction of variation was not established, with an increase of E2/T reported in some studies for males (Liu et al., 2012) or females (Liu et al., 2013b), and a decrease or no change reported in others for females (Liu et al., 2012) or males (Liu et al., 2013b) or both (He et al., 2021).

The observations of VTG concentrations also suggest an estrogenic activity of TPhP in female and male fish, with increased concentrations of VTG reported in Liu et al. (2013b), in the Unpublished study report (2021) (only for females) and in the MITI (2021) (only for female sub-adults). Studies are also reporting decreased or no changed concentrations in hepatic VTG of adult female medaka in Kawashima et al. (2021) and in MITI (2021) respectively or in whole-body homogenate of male and female zebrafish (He et al., 2021). Moreover, VTG hepatic levels were not modified in sub-adult or adult Medaka males in one assay (MITI, 2021). *VTG1* gene expression in liver is increased in adult male zebrafish in one assay (Liu et al., 2012), while the expression in liver of *VTG1* and/or *2* are decreased in adult females in other assays (Liu et al., 2012; Li et al., 2019b).

Vitellogenin is a key parameter to assess the potential endocrine disrupting properties and in particular estrogenicity. Nevertheless, the variability of VTG levels is known. This is why VTG is evaluated and correlated with other observations to conclude. Decrease in *vtg* gene expression was not consistent with observed increased levels of E2 in female zebrafish in Liu et al. (2012) and medaka exposed for 21 days in Li et al. (2019b). However, levels of VTG or *vtg* gene expression were correlated with E2 levels with consistent increase in these parameters observed in male zebrafish in Liu et al. (2012), in females in the FSDT (Unpublished study report, 2021)

and in both sexes in Liu et al. (2013b). Concurrent decreases in E2 and *vtg* gene expression were observed in medaka females exposed for 100 days in Li et al. (2019b).

Finally and specifically for females, the changes in VTG levels or *vtg* gene expression seem dependent on the life cycle of fish, the period of exposure and the reproductive status. An increase of VTG is observed following exposure to TPhP at the juvenile/sub-adult stage (Unpublished study report, 2021; MITI, 2021). In contrast, at adult stage, *vtg* expression or VTG levels were generally significantly decreased (He et al., 2021; MITI, 2021; Kawashima et al., 2021; Li et al., 2019b) as indicated in the figure below. This suggests an over estrogenic stimulation by TPhP at the juvenile stage which may have altered the maturation to functional gonads and their capacity to produce viable gonadocytes with consequence of altered fecundity and decreased ability to produce eggs at the mature stage.

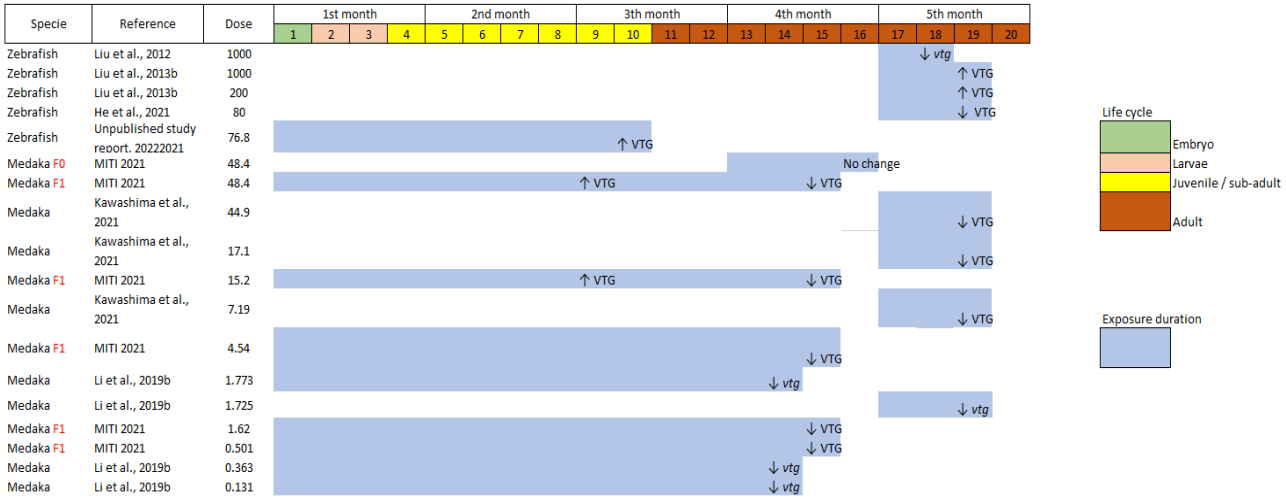


Figure 4: VTG levels or vtg gene expression in female fish related to the life cycle stage, their reproductive status (described in green to brown at the top of the figure) and the experimental duration of exposure (in blue violet in the main part of the figure) from studies in which changes were identified.

Conclusion: Transcription levels of key genes involved in the steroidogenesis were significantly upregulated or down regulated by exposure to TPhP in several studies. The available *in vivo* assays showed estrogenic as well as anti-estrogenic activity based on changes in sex hormone levels and VTG levels. Significant alteration of plasmatic concentrations of E2, E2/T ratio, and E2/11-KT ratio could result from this modification in the steroidogenesis pathway. These different perturbations of circulating steroid concentrations could depend on the fish developmental stage, species and tested concentrations. Change in E2 levels were observed in several (but not all) studies with concurrent modifications in VTG or *vtg* expression levels (Liu et al., 2012; Liu et al., 2013b; Li et al., 2019b; Chen et al., 2020; Unpublished study report, 2021). These studies provide support to the conclusion that TPhP exerts EAS activity.

Other endocrine pathways

In Zhang et al. (2023), the authors investigated the effects of TPhP exposure on transcriptional levels in zebrafish at different developmental stages namely 96 hpf and 7 dpf. The expression level of different genes were affected in zebrafish and 96 hpf larvae were shown to be more sensitive than 7 dpf larvae. The expression of apoptotic-related genes (*bcl2a* and *Casp9)* was down-regulated in 96 hpf zebrafish at the concentration of 20 $\mu g.l^{-1}$ and at 1000 $\mu g.l^{-1}$ respectively. The expression of neurodevelopmental-related genes (*pax6a* and *mbpa*, *syn2a*, *foxo3a)* was also down-regulated from 500 $\mu g.l^{-1}$ and at 1000 $\mu g.l^{-1}$ respectively.

Regarding the retinoic acid (RA) metabolic-related gene expression, at 96 hpf, the expression of *aldh1a2* was significantly up-regulated in 50 $\mu g.l^{-1}$ TPhP. Exposure to 1000 µg/L TPhP significantly downregulated the expressions of *rdh1*, *crabp1a*, *rbp2a* and *raraa*. Furthermore, the expression of *cyp26a1* was significantly decreased in the 500 and 1000 $\mu g.l^{-1}$ TPhP treated groups. The expression of *rbp5* was also decreased in 100, 500 and 1000 $\mu g.l^{-1}$ TPhP treated groups. Several genes in the RA metabolic pathway were also examined in zebrafish larvae at 7 dpf. The expression of *aldh1a2* was significantly up-regulated in 100 and 500 $\mu g.l^{-1}$ TPhP treated groups. And exposure to 500 $\mu g.l^{-1}$ TPhP significantly increased the expressions of *rdh1*. Exposure to 20, 50, 100 and 500 $\mu g.l^{-1}$ TPhP significantly up-regulated the transcriptions of *rbp2a*.

Although not extensively detailed here, it is also important to note that TPhP was previously shown to interact with the retinoic acid receptor (RAR). TPhP exhibits weak RARα antagonist activity (Jia et al., 2022; Isales et al., 2015), which could lead to ocular and cardiovascular malformations. The RAR signaling pathway is essential for reproduction and embryonic development. Indeed, genetic studies in zebrafish embryos that are deficient in RA-generating enzymes or RARs revealed that retinoic acid (RA) signalling regulates development of many organs and tissues, including the body axis, spinal cord, forelimb buds, skeleton, heart, eye, pancreas, lung and 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. The retinoid signaling pathway is an endocrine pathway considered to be susceptible to environmental endocrine disruption (OECD, 2021) and possibly related to endocrine effects on development.

In vivo adverse effect data with regard to an endocrine mode of action

Table 12: Summary table of *in vivo* adverse effect data (chronologic order)

STUDIES INVESTIGATING ENDOCRINE DISRUPTION PROPERTIES OF TPhP			
Methodology	**Results (statistically significant change)**	**Remarks**	**Reference**
Adult Zebrafish (*Danio rerio*) – 4/5 months old Nominal concentration : 0 – 0.04 – 0.2 – 1 $mg.l^{-1}$ No analytical measurement Measured concentration : 0h: 0.03 – 0.14 – 0.89 $mg.l^{-1}$ 48h: <LOD - <LOD – 0.38 $mg.l^{-1}$	<u>Female Zebrafish</u> Cumulative Egg number: ↓ at 0.2 and 1 $mg.l^{-1}$ Spawning event: ↓ at 0.2 and 1 $mg.l^{-1}$ Hatchability: ↓ at 0.2 and 1 $mg.l^{-1}$ Fertilisation success: No change Hepato-somatic index (HSI): No change Gonado-somatic index (GSI): No change <u>General toxicity</u> No mortality was observed in any of the experimental concentrations during the exposure period (no details provided).	2 (reliable with restriction) test material: TPhP Purity: 99% Equivalent or similar to OECD TG 229 Not GLP	Liu et al., 2013b

(LOD = 0.12 µg.l^{-1}) Solvent: DMSO (0.005%) Replicates: 6 replicates with six pair of fish Exposure duration: 21 days Photoperiod: 14h light - 10h dark Water temperature: 27 °C ± 1 °C Exposure medium: renewed in every 48h.			
Zebrafish embryos/larvae (*Danio rerio*) – 3hpf Nominal concentration: 0 – 40 – 200 – 500 µg.l^{-1} No analytical measurement Solvent: DMSO (0.005%) Replicates: 6 per treatment with 100 larvae per replicate Exposure duration: 7 days	Malformation rate ↑ at 500 µg.l^{-1} <u>General toxicity</u> Larval survival at 7d: 91.8% (control) – 92.5% (40 µg.l^{-1}) – 92% (200 µg.l^{-1}) – 88.7% (500 µg.l^{-1}). Bodyweight were not affected by the experimental concentrations.	2 (reliable with restriction) Test material: TPhP Purity: 99% Non-guideline/ exploratory Not GLP	Kim et al., 2015

Photoperiod: 14h light - 10h dark Water temperature: 25°C ± 1°C Exposure medium: 50% renewed daily.			
Larvae, juvenile and adult Zebrafish (*Danio rerio*) Nominal concentration: 0 – 0.005 – 0.05 – 0.5 $mg.l^{-1}$ Measured concentration: First 24h: 0.0008 - 0.0055 - 0.131 $mg.l^{-1}$ First 48h: < LOD - < LOD - 0.011 $mg.l^{-1}$ Solvent: DMSO (0.005%) Replicates:3 per treatment with 100 embryos per replicate Exposure duration: 120 days Photoperiod: 14h light - 10h dark Water temperature: 27°C ± 1 °C	<u>Female Zebrafish</u> GSI: ↓ at 0.005 and 0.5 $mg.l^{-1}$ HSI: No change Condition factor (CF): No change <u>Male Zebrafish</u> GSI: No change HSI: No change Condition factor (CF): ↓ at 0.5 $mg.l^{-1}$ <u>General toxicity</u> None of the test concentrations caused any distinct malformation, and there were no significant differences in mortality among treatment groups during the exposure duration (no details provided).	2 (reliable with restriction) Test material: TPhP Purity: 99% Non-guideline/ exploratory Not GLP	Liu et al., 2016

Water pH: 7.2 to 7.8 Dissolved oxygen: > 7 $mg.l^{-1}$ Exposure medium: 50% renewed every 48h.			
Japanese medaka transgenic (*Oryzias latipes*) – < 4hpf Nominal concentration: 5, 25, 125, 625 $\mu g.l^{-1}$ No analytical measurement Solvent: DMSO (0.01%) Replicates: 3 replicates per concentration Water temperature: 25°C ± 1 °C Photoperiod: 14h light - 10h dark Exposure duration: 14 d Exposure medium: renewed daily (at least 90%)	Hatchability (%) at 96h ↓ at 625 $\mu g.l^{-1}$ Time of hatching at 96h ↑ at 625 $\mu g.l^{-1}$ <u>General toxicity (14 d)</u> Heart rates ↓ at 125 and 625 $\mu g.l^{-1}$ Body length ↓ at 125 and 625 $\mu g.l^{-1}$ Gross abnormality ↑ at 625 $\mu g.l^{-1}$ Mortality No information given	2 (reliable with restriction) Test material: TPhP Purity > 99% Non-guideline/ exploratory Not GLP	Sun et al., 2016
Japanese medaka transgenic (*Oryzias latipes*) – 0 dph - Only male	<u>Male Medaka:</u> Gonadal intersex incidence ↑ at 1.43 $\mu g.l^{-1}$ Abnormal chasing behaviours ↑ at 0.29 and 1.43 $\mu g.l^{-1}$ Courtship behaviour ↑ at 0.29 $\mu g.l^{-1}$	2 (reliable with restriction) Test material: TPhP Purity ≥ 95%	Li et al., 2018

Nominal concentration : 1.6 – 8 – 40 µg.l^{-1} Measured concentration : 0.134 – 0.299 – 1.43 µg.l^{-1} Solvent: DMSO (0.001%) Replicates: 2 per treatment with 50 larvae per replicate Exposure duration: 100 days Photoperiod: 16h light - 8h dark Water temperature: 25°C ± 1 °C Water pH: 7.7 ± 0.2 Dissolved oxygen: > 7.8 ± 0.3 mg.l^{-1} Number of tested fish: 16 males Exposure medium: flow-through system	Chasing time ↑ at 0.29 µg.l^{-1} Successful mating rate ↓ at 1.43 µg.l^{-1} <u>General toxicity</u> No data on systemic toxicity provided.	Non-guideline/ exploratory Not GLP	
Japanese medaka transgenic (*Oryzias latipes*) – only females Solvent: DMSO (0.001%)	<u>Female medaka:</u> **Long-term exposure: 100 days** Number of mature oocyte ↓ at 0.13, 0.36 and 1.77 µg.l^{-1} Egg production ↓ at 0.36 and 1.77 µg.l^{-1}	2 (reliable with restriction) Test material: TPhP Purity ≥ 95% Non-guideline/ exploratory	Li et al., 2019b

Photoperiod: 16h light - 8h dark Water temperature: 25°C ± 1 °C Water pH: 7.7 ±0.2 Dissolved oxygen: > 7.8 ± 0.3 $mg.l^{-1}$ Exposure medium: flow-through system **Long-term exposure:** larvae exposed during 100 days Nominal concentration : 1.6 – 8 – 40 µg.l-1 Measured concentration : 131 – 363 – 1773 $ng.l^{-1}$ Replicates: 2 per treatment with 25 adult female per replicate	General toxicity No data on systemic toxicity provided	Not GLP	
Adult male rare minnows (*Gobiocypris rarus*) – 20-22 weeks old Nominal concentration : 0.012 – 0.12 $mg.l^{-1}$ Measured concentration : before renewal	HSI: no change (no quantitative value provided in the publication) GSI: no change (no quantitative value provided in the publication)	2 (reliable with restriction) Test material: TPhP Purity ≥ 99% Non-guideline/ exploratory study Not GLP	Chen et al., 2020

(T24h): 0.010 ± 0.0026 – 0.098 ± 0.031 mg.l^{-1} Solvent: 0.01% acetone Exposure duration: 28 days Photoperiod: 14h light – 10h dark Water temperature: 25°C ± 1°C Exposure medium: renewal daily 30 fish per tank, 3 replicates per concentration	<u>Sperm quality</u> Sperm concentration: no change Sperm vitality: no change Sperm velocity: ↓ at 0.012 – 0.12 mg.l^{-1} Sperm motility: ↓ at 0.012 – 0.12 mg.l^{-1} Sperm wobble: ↓ at 0.012 mg.l^{-1} Morphology abnormalities: short tails and bent tails at 0.012 – 0.12 mg.l^{-1} Apoptosis of testis: ↑ at 0.12 mg.l^{-1} <u>General toxicity</u> Survival rate (%): Control: 96.67 ± 2.89 TPHP - 0.012: 96.11 ± 5.36 TPHP - 0.12: 90.56 ± 5.18 Body weight: no change (no quantitative value provided in the publication) Total length: no change (no quantitative value provided in the publication)		
Japanese medaka (*Oryzias latipes*) – 16± 2 weeks old OECD 229 Nominal concentration (µg.l^{-1}): 20 – 64 – 200 – 640 Measured concentration (µg.l^{-1}): 2.13 – 7.19 – 17.1 – 44.9 Replicates: 4 replicates per treatment with 3 males and 3 females per replicate	Secondary sex characteristics: No change Egg number: ↓ at 44.9 µg.l^{-1} Fertilised egg number: ↓ at 44.9 µg.l^{-1} Fertility rate: No change HSI male: ↑ at 7.19, 17.1, 44.9 µg.l^{-1} HSI female: No change GSI male: No change GSI female: No change <u>General toxicity</u> 4.2% of mortality at 44.9 µg.l^{-1} (1 female). No remarkable abnormal response (feeding activity, equilibrium, swimming…). No statistical analysis for length and weight parameters	2 (reliable with restriction) Test material: TPhP Purity >97% According to OECD TG 229 Not GLP	Kawashima et al., 2021

Exposure duration: 21 days Water temperature: 25°C ± 2 °C Photoperiod: 16h light - 8h dark Water pH: 6.5-8.5 Dissolved oxygen: > than 60% of air saturation Exposure medium: Flow-through (5 times/day)			
Japanese medaka (*Oryzias latipes*) OECD TG 240 Age at start of exposure: F0: 12 weeks old F1: Embryo F2: Embryo Exposure duration: F0: 4 weeks F1: 15 weeks F2: 18 days (twice the median hatching date of the control) Nominal concentration ($\mu g.l^{-1}$): 0.5 – 1.6 – 5 – 16 - 50 Measured concentration	<u>Growth parameters</u> Total length male F0 (adult): No change F1 (10 wpf, sub-adult): ↑≠ at 0.501, 1.62, 4.54, 15.2 and 48.4 $\mu g.l^{-1}$ F1 (15wpf, adult): No change Total length female F0 (adult): ↓ at 15.2 and 48.4 $\mu g.l^{-1}$ F1 (10 wpf, sub-adult): ↑≠ at 0.501, 1.62, 4.54 $\mu g.l^{-1}$ F1 (15 wpf, adult): ↑≠ at 0.501, 1.62, 4.54 $\mu g.l^{-1}$ Total weight male F0 (adult): No change F1 (10 wpf, sub-adult): No change F1 (15 wpf, adult): No change Total weight female F0 (adult): ↓ at 0.501, 1.62, 4.54, 15.2 and 48.4 $\mu g.l^{-1}$	2 (reliable with restriction) Test material: TPhP Purity 99.9% According to OECD TG 240	MITI, 2021

($\mu g.l^{-1}$): 0.501 – 1.62 – 4.54 – 15.2 – 48.4 Replicate: F0: 12 (control), 6 (tested concentrations) F1 (1-10 wpf): 12 (control), 6 (tested concentrations) F1 (10-15 wpf): 24 (control), 12 (tested concentrations) F2 (1-3 wpf): 12 (control), 6 (tested concentrations) Water temperature: 25°C ± 1 °C Photoperiod: 16h light – 8h dark Water pH: 7.9 Dissolved oxygen: > than 60% of air saturation Mean fecundity of controls in F0 & F1: Greater than 20 eggs per pair per day Fertility (Fertilization rate) of all the eggs produced in F0 controls and F1 controls:	F1 (10 wpf, sub-adult) ↑ ≠ at 0.501, 4.54 $\mu g.l^{-1}$ F1 (15 wpf, adult) No change <u>Biological parameters</u> HSI male F0 (adult) No change F1 (10 wpf, sub-adult) ↓ ≠ at 0.501, 1.62, 4.54, 15.2 and 48.4 $\mu g.l^{-1}$ F1 (15 wpf, adult) No change HSI female F0 (adult) No change F1 (10 wpf, sub-adult) ↓ ≠ at 0.501, 1.62, 4.54 $\mu g.l^{-1}$ F1 (15 wpf, adult) No change GSI male F0 (adult) No change F1 (10 wpf, sub-adult) No change F1 (15 wpf, adult) ↑ at 48.4 $\mu g.l^{-1}$ GSI female F0 (adult) ↓ at 15.2 and 48.4 $\mu g.l^{-1}$ F1 (10 wpf, sub-adult) ↑ at 15.2 and 48.4 $\mu g.l^{-1}$ F1 (15 wpf, adult) ↑ at 4.54, 15.2 and 48.4 $\mu g.l^{-1}$ <u>Fecundity parameter: Total number of eggs laid (eggs/pair/day)</u> F0 Week 1 (Day1-7) No change Week 2 (Day 8-14) ↓ at 15.2 and 48.4 $\mu g.l^{-1}$ Week 3 (Day 15-21) ↓ at 15.2 and 48.4 $\mu g.l^{-1}$ 21 days ↓ at 48.4 $\mu g.l^{-1}$ <u>F1</u> Week 1 (Day 1-7) ↓ at 0.501, 1.62, 4.54, 15.2 and 48.4 $\mu g.l^{-1}$ Week 2 (Day 8-14) ↓ at 48.4 $\mu g.l^{-1}$		

Greater than 80% Hatchability of eggs in the F1 controls & F2 controls: ≥ 80% (average) Exposure medium: Flow-through (more 5 times/day)	Week 3 (Day 15-21) No change 21 days ↓ at 48.4 µg.l^{-1} <u>Fertility parameter: Number of eggs fertilized (eggs/day/female)</u> <u>F0</u> Week 1 (Day 1-7) No change Week 2 (Day 8-14) ↓ at 15.2 and 48.4 µg.l^{-1} Week 3 (Day 15-21) ↓ at 15.2 and 48.4 µg.l^{-1} 21 days ↓ at 48.4 µg.l^{-1} <u>F1</u> Week 1 (Day 1-7) ↓ at 0.501, 1.62, 4.54, 15.2 and 48.4 µg.l^{-1} Week 2 (Day 8-14) ↓ at 48.4 µg.l^{-1} Week 3 (Day 15-21) No change 21 days ↓ at 48.4 µg.l^{-1} <u>Fertility parameter: Fertilized rate (%)</u> <u>F0</u> Week 1 (Day 1-7) No change Week 2 (Day 8-14) No change Week 3 (Day 15-21) No change 21 days No change <u>F1</u> Week 1 (Day 1-7) No change Week 2 (Day 8-14) ↑≠ at 4.54 µg.l^{-1} Week 3 (Day 15-21) No change 21 days No change <u>Embryonic stage development parameter: Hatching (day)</u> <u>F1</u> ↓≠ at 0.501, 1.62, 4.54 and 15.2 µg.l^{-1} <u>F2</u> ↓≠at 0.501, 1.62, 4.54 and 48.4 µg.l^{-1} <u>Embryonic stage development parameter: Hatching rate (%)</u> <u>F1</u> ↓ at 4.54, 15.2 and 48.4 µg.l^{-1} <u>F2</u> No change		

	<u>Secondary sex characteristics: number of anal fin papillae per male</u> F0 (adult): No change F1 (10 wpf, sub-adult): ↑ ≠ at 0.501, 1.62, 4.54, 15.2 and 48.4 µg.l^{-1} F1 (15 wpf, adult): No change <u>Secondary sex characteristics: number of anal fin papillae per female</u> F0 (adult): No change F1 (10 wpf, sub-adult): No change F1 (15 wpf, adult): No change <u>Occurrence of intersexuality:</u> F0 (adult): No change F1 (10 wpf, sub-adult): No change F1 (15 wpf, adult): No change Simple sex-ratio based on external phenotypic sex (criteria not described in the study report) <u>calculated by DS:</u> F1 (10 wpf, sub-adult): No change <u>General toxicity:</u> <u>-Mortality</u> F0 (adult): No change F1 (4 wpf, juveniles): ↑ at 48.4 µg.l^{-1} (Mortality rate: 3%) F1 (8 wpf, sub-adult): No change F1 (15 wpf, adult): No change for the whole group (male + female) ↑ at 15.2 and 48.4 µg.l^{-1} (for males only)		
Zebrafish adult (*Danio rerio*): 5-month adult <u>Sub-chronic toxicity (10% of LC_{50})</u>	<u>Female Zebrafish:</u> HSI: ↑ at 80 µg.l^{-1} GSI: ↑ at 80 µg.l^{-1} Ovary maturation stages: ↓ at 80 µg.l^{-1} (oocytes less mature with more oocytes at the first two stages (perinuclear and corticolar alveolar oocytes). Structure of oocytes more irregular.	2 (reliable with restriction) Test material: TPhP Purity 99%	He et al., 2021

Nominal concentration : 80 µg.l^{-1} No analytical measurement Solvent: DMSO (0.01%) Replicates: 10 males and 10 females exposed separately Exposure duration: 21 days Water temperature: 25°C ± 1 °C Photoperiod: 14h light - 10h dark Water pH: 7.2 ± 0.2 Dissolved oxygen: > 6.8 ± 0.2 mg.l^{-1} Exposure medium: renewed every day TPhP		Inhibition of the ovarian development. <u>Male Zebrafish:</u> HSI: ↑ at 80 µg.l^{-1} GSI: ↓ at 80 µg.l^{-1} Spermatocyte maturation stages: ↓ at 80 µg.l^{-1} (more immature spermatocytes (spermatogonia and spermatocyte) and less mature spermatocytes (spermatids and spermatozoa). Fecundity/fertility: ↓ at 80 µg.l^{-1} of fertilisation rate, cumulative eggs, spawning, hatching rate. <u>General toxicity</u> No data on systemic toxicity provided.	Non-guideline/ exploratory Not GLP	
Amphibian tadpoles (*Rana zhenhaiensis*) Solvent: DMSO (0.01%) Nominal concentration : 0.02 and 0.1 mg.l^{-1}		Developmental stages (30 days): ↑ at 0.1 mg.l^{-1} (more advanced developmental stage) <u>General toxicity</u> No death occurred in any treatment groups throughout the exposure duration.	2 (reliable with restriction) Test material: TPhP Purity > 99% Non-guideline/ exploratory Not GLP	Lu et al., 2021

No analytical measurement Exposure duration: 30 days Replicates: 6 replicate aquaria, 60 tadpoles in each treatment group Stage GS26 (Gosner stage) Water temperature: 25°C ± 1°C Photoperiod: 12h light - 12h dark		Non-standard species	
Amphibians tadpoles (*Hoplobatrachus rugulosus*) Stage GS35 (Gosner stage) Solvent: DMSO (0.5 ‰ v/v) Nominal concentration: 0- solvent control - 10 – 50 – 100 – 200 µg.l^{-1} Exposure duration: 30 days Replicates: 3 replicates per treatment with 30 tadpoles per replicate	Survival (30d) ↓ at 50 µg.l^{-1} Metamorphosis rates (30d) ↓ at 50 µg.l^{-1} Metamorphosis time (30d) ↓ at 100 µg.l^{-1} <u>General toxicity</u> Survival rate: 87.8% (control group) – 87.8% (solvent control group) – 76.7% (10 µg.l^{-1}) – 66.7% (50 µg.l^{-1}) – 63.3% (100 µg.l^{-1}) – 61.1% (200 µg.l^{-1})	2 (reliable with restriction) Test material: TPhP Purity > 99% Non-guideline/ exploratory Not GLP	Chen et al., 2022

Water temperature: 29°C ± 2 °C Photoperiod: 14h light - 10h dark Water pH: 7.2 ± 0.2 Dissolved oxygen: > 5.78 ± 0.3 ppm Exposure medium: renewed daily			
OECD 234 (Fish sexual development test – FSDT) Zebrafish (*Danio rerio*) Nominal concentration: 1, 3.2, 10, 32, 100 µg.l^{-1} Measured concentration: 1.11 – 3.01 – 7.76 – 33.3 – 76.8 µg.l^{-1} (TPhP in 1L acetone) Replicates: 4 replicates per concentration with 30 fertilised eggs per replicate. Water temperature: 27°C ± 2 °C Photoperiod: 12h light - 12h dark Water pH: 7.6-7.8 Dissolved oxygen: > than 60% of air saturation	<u>Sex ratio</u> No statistically significant change % of females: 64.8% (control) – 52.6% (1.11 µg.l^{-1}) – 55.8% (3.01 µg.l^{-1}); 59.1% (7.76 µg.l^{-1}) – 67.5% (33.3 µg.l^{-1}) – 65.4% (76.8 µg.l^{-1}) Note: high proportion of females in the control group (64.8%) even if the acceptance criteria (30% males – 70% females) was fulfilled <u>Male Zebrafish:</u> Testis maturation stages ↑ at 76.8 µg.l^{-1} <u>General toxicity</u> Survival rates (post-hatching, 4-73 dpf): 88% (control group) - 84% (1.11 µg.l^{-1}); 80% (3.01 µg.l^{-1}); 77% (7.76 µg.l^{-1}); 63% (33.3 µg.l^{-}1) - 51% (76.8 µg.l^{-1}) Survival rates (post-hatching, 4-35 dpf): 89% (control group) - 87% (1.11 µg.l^{-1}); 84% (3.01 µg.l^{-1}); 80% (7.76 µg.l^{-1}); 67% (33.3 µg.l^{-}1) - 54% (76.8 µg.l^{-1}) Survival rates (post-hatching, 35-73 dpf): 98% (control group) - 97% (1.11 µg.l^{-1}); 96% (3.01 µg.l^{-1}); 96% (7.76 µg.l^{-1}); 95% (33.3 µg.l^{-}1) - 93% (76.8 µg.l^{-1})	2 (reliable with restriction) Test material: TPhP Purity > 99% OECD TG 234 GLP	Unpublished study report, 2021

Exposure duration: 73 days Exposure medium: flow-through system			
Zebrafish embryos/ larvae (*Danio rerio*) – 2hpf Nominal concentration : 8, 24, 72, 144 µg.l^{-1} No analytical measurement Replicates: 3 replicates per concentration Water temperature: 28°C ± 0.5 °C Photoperiod: 14h light - 10h dark Exposure duration: 120 h Exposure medium: renewed daily	Hatching rates ↓ at 72 and 144 µg.l^{-1} <u>General toxicity</u> Heart rates ↓ at 72 and 144 µg.l^{-1} Malformation rates ↑ at 72 and 144 µg.l^{-1} Survival rates ↓ at 144 µg.l^{-1} Body length ↓ at 144 µg.l^{-1} <u>Survival rates</u> 86.5% (control group) – 86% (8 µg.l^{-1}) - 84% (24 µg.l^{-1}) – 82% (72 µg.l^{-1}) – 80.5% (144 µg.l^{-1})	2 (reliable with restriction) Test material: TPhP Purity > 99% Non-guideline/ exploratory Not GLP	Fan et al., 2022
Zebrafish embryo (*Danio rerio*) – (0-2 hpf) Nominal concentration : 0 – 20 – 50 – 100 – 500 and 1000 µg.l^{-1}	Survival rate ↓ at 1000 µg.l^{-1} (6 dpf) ↓ at 500 µg.l^{-1} (7 dpf) Hatching rate ↓ at 50 µg.l^{-1} at 48 hpf ↓ at 500 µg.l^{-1} at 72 hpf Heart rate ↑ at 100 and 1000 µg.l^{-1} at 48 hpf ↓ at 500 µg.l^{-1} at 96 hpf <u>Behaviour changes</u>	2 (reliable with restriction) Test material: TPhP Purity > 98% Non-guideline/ exploratory Not GLP	Zhang et al., 2023

No analytical measurement Solvent: DMSO (0.005% v/v) Water temperature: 28°C ± 1 °C Photoperiod: 14h light - 10h dark Replicates: 3 replicates per treatment with 40 embryos per replicate Exposure duration: 7 days	Swimming distance ↑ at 20; 100; 500 and 1000 µg.l^{-1} Movement trajectory ↑ at 20; 100; 500 and 1000 µg.l^{-1} Number of apoptotic cells in the head, heart and intestine ↑ with the increase of TPhP concentration <u>General toxicity</u> From 120 hpf, the survival rate of zebrafish larvae was significantly decreased at 500 µg.l^{-1} and 1000 µg.l^{-1}.		

*: no concentration dependence

Thyroid pathway

Lu et al. (2021) studied exposure of amphibian tadpoles (Zhenhai brown frog, *Rana zhenhaiensis*) throughout the developmental period to assess physiological and metabolic impacts of TPhP on amphibian larvae. The exposure started with tadpoles at the Gosner stage (GS) 26. A total of 180 tadpoles were randomly selected and allocated to different aquaria, each containing the solvent control (DMSO), 0.02 and 0.1 mg TPhP.l^{-1} (6 replicate aquaria, 60 tadpoles in each treated group). After 30-days exposure the tadpoles were euthanised. Snout-vent length (SVL), tail length (TL), body weight (BW) and developmental stage were recorded. No death occurred in any treated groups throughout the exposure duration. Tadpoles from 0.1 mg.l^{-1}-treated group had more advanced developmental stages than those from the other two groups. However, there were no significant differences in other traits measured in this study.

Chen et al. (2022) studied the sub-chronic toxicity of TPhP to amphibian tadpoles (*Hoplobatrachus rugulosus*). GS35 tadpoles were divided in four TPhP-treated groups (three replicates/group, 30 tadpoles/replicate/30 L): 10, 50, 100, and 200 µg.l^{-1}. After two weeks of continuous TPhP exposure, the dechlorinated tap water culture was switched until the tadpoles completed metamorphosis (GS46). The endpoints were survival, metamorphosis, and development. All tadpoles were observed every 24 h until they underwent complete metamorphosis. The survival and metamorphosis rates were determined within 30 days of sub-chronic toxicity test, and the metamorphosis time was defined as the interval between the beginning of TPhP exposure and complete metamorphosis. When the tadpoles completed metamorphosis, their snout–vent lengths (SVLs) and body masses (BMs) were measured. The threshold concentration that affected survival and metamorphosis rate was 50 µg/L, while that affecting the metamorphosis time was 100 µg.l^{-1}. No significant differences were observed in the body size and condition of the tadpole after metamorphosis; however, BM and TPhP concentration were negatively correlated.

Conclusion: although indications of effects on thyroid exist, available information are scarce and possible effects of TPhP on the thyroid pathway were not further considered in the context of the SVHC identification.

EAS modalities

Based on the available data (Liu et al., 2013b; Li et al., 2019b; Kawashima et al., 2021; He et al., 2021; MITI, 2021; Zhang et al., 2023), it was considered that TPhP impairs fertility and fecundity in fish.

In Liu et al. (2013b) the effects of TPhP on reproductive endpoints were studied in adult zebrafish (*Danio rerio*) over 21 days. The study was performed with 12 fish per concentration, exposed to 0, 0.04, 0.2 and 1 $mg.l^{-1}$ nominal TPhP concentrations based on the results of a range-finding test. No mortality occurred at any concentration. There was a statistically significant decrease at 0.2 and 1 $mg.l^{-1}$ of the cumulative egg numbers, the number of spawning events and the hatchability.

Liu et al. (2016) studied the effects of TPhP on zebrafish (*Danio rerio*) during long-term exposure covering larval, juvenile and adult life stages. Embryos were collected with 4-h post fertilisation and exposed to 0, 0.005, 0.05 and 0.5 $mg.l^{-1}$ nominal concentrations of TPhP for 120 days post-fertilisation (dpf). There were 3 replicates per treatment, and each replicate contained 100 embryos and 300 ml of test solution. Half of the exposure solution was renewed every 48 h throughout exposure. Mortality, condition factor (CF), gonodasomatic index (GSI) and hepatosomatic index (HSI) were assessed at 120 dpf. No distinct malformations were observed at any concentration, and the authors concluded that there were no significant differences in mortality between treated groups (although the level of mortality observed, including controls, was not specified). The CF and GSI were unaffected up to 0.5 $mg.l^{-1}$ 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 0.5 $mg.l^{-1}$. The gonodasomatic index (GSI) in female zebrafish exposed for 120 days to TPhP was significantly decreased at 0.005 and 0.5 mg/L with no change in the hepatosomatic index (HSI), suggesting a negative impact on female gonad development.

In Sun et al. (2016), larvae (transgenic Japanese medaka) collected in the first hours post-hatching (< 4 hpf) were exposed to TPhP at nominal concentrations of 5, 25, 125, 625 $\mu g.l^{-1}$ (no analytical measurement during the test was reported) until 14 d. The media was renewed every 24 h (at least 90%). Embryos were observed 3 times daily, and the dead larvae were removed. At 60 h, their heart rates were monitored. At the end of the exposure duration, the body lengths of the larval medaka were measured. Exposure to TPhP significantly decreased hatchability, delayed time to hatch, and increased the occurrence of gross abnormalities at the highest exposure concentration (625 $\mu g.l^{-1}$) compared with controls. Moreover, embryo heart rate and body length were also decreased relatively to the controls in the TPhP exposure groups with concentrations over 125 $\mu g.l^{-1}$.

In Li et al. (2018), larvae (transgenic Japanese medaka, male only) collected on the first day post-hatching (0 dph) were exposed to TPhP at nominal concentrations of 1.6, 8, and 40 $\mu g.l^{-1}$ (0.134, 0.299 and 1.43 $\mu g.l^{-1}$ measured respectively) until 100 dph. A flow-through system with a 2-fold volume of water flowing through every 24 h was used. After exposure, 16 males were randomly selected and mated to non-exposed females in clean water for reproductive behaviours and success tests prior to observation of gonadal intersex. A significant gonadal intersex incidence of 26% in male medaka was observed at 1.43 $\mu g.l^{-1}$ (indicating a potentially feminisation risk for male fish). In this study chasing trajectory and time, courtship frequency and mating number were recorded to evaluate the effects of TPhP on reproductive behaviours. After exposure, the incidences of male fish with abnormal chasing behaviour indicated a dose-dependent increase. Additionally, significant decreases in the fertilisation rate after 1.43 $\mu g.l^{-1}$ TPhP exposure and in the hatching rate after 0.299 and 1.43 $\mu g.l^{-1}$ TPhP exposure were observed in the study.

Li et al. (2019b) studied the effects of TPhP on the reproduction of transgenic Japanese medaka by an exposure of 100 days following hatching (FSDT-like test). Medaka (50 individuals, duplicate experiments) were exposed to TPhP in a continuous flow system at 1.6, 8 and 40 µg.l^{-1} (measured concentrations 1.3, 3.6 and 17.7 µg.l^{-1}). The 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. After 100 days of exposure, oocytes underwent 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 females affected. The highest doses induced more substantial effects involving the absence of oocyte II (stage 3). 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). It should be noted that the concentration of TPhP was higher in the liver and the ovary than in muscle and brain.

These three previous studies (Sun et al., 2016; Li et al., 2018; 2019b) used transgenic fish, more sensitive than intact Medaka or zebrafish. These assays on genetically modified Medaka allow observation of effects on fecundity at very low concentrations (0.36 and 1.77 µg.l^{-1} for Li et al., 2019b) and includes other reproductive endpoints (e.g. courtship behavior at 0.29 µg.l^{-1} in Li et al., 2018). These data support the observations on fecundity made on intact zebrafish at concentrations of 0.2 and 1 mg.l^{-1} (Liu et al., 2013b) and in Medaka at 44.9 µg.l^{-1} (Kawashima et al., 2021) and at 48.4 µg.l^{-1} (MITI, 2021). The weight of evidence of all these studies supports the conclusion of an adverse effect on fecundity.

In Chen et al. (2020), sperm quality in adult male Chinese rare minnows after 28d of exposure was investigated. The age of fish at exposure initiation was 20-22 weeks old. Thirty fish per tank and three replicates per treatment were used. The medium was renewed daily. The nominal concentrations were 0 – 12 – 120 µg.l^{-1} (measured concentrations before renewal (T24h) were respectively 0 – 10 – 98 µg.l^{-1}). No significant difference in mortality rate was observed. No obvious effect on sperm concentration was detected whatever the tested concentration. Sperm velocity parameters and sperm motility were significantly decreased in all treatments. Sperm linearity was significantly decreased in the 12 µg.l^{-1} TPhP. The occurrence of the short tail morphology and the percentages of sperm with bent tails were significantly increased at every concentration tested. Finally, significant changes of apoptosis ratio were also found at 120 µg.l^{-1}.

In Kawashima et al. (2021), Japanese medaka (*Oryzias latipes*) were exposed to TPhP in a flow-through system for 21 days with 4 replicates per treatment. The age of fish at exposure initiation was 14 weeks old. Six fish (3 males and 3 females) were used per treatment. The endpoints measured were mortality, growth (length and weight), fecundity (number of eggs, number of fertile eggs, fertility rate), GSI, HSI and secondary sexual characteristics. To prevent excessive lethal effects, the highest test concentration was determined based on acute toxicity on Japanese medaka. The nominal concentrations were 0 – 20 – 64 – 200 – 640 µg.l^{-1} (measured concentration were respectively 0 – 2.13 – 7.19 – 17.1 – 44.9 µg.l^{-1}). Regarding the mortality, only one female fish died at the highest concentration (44.9 µg.l^{-1}), therefore it is considered that the validity criteria was met. The secondary sex characteristics (SSC) were not changed after exposure to TPhP. A statistically significant decrease in fecundity (number of total eggs produced) and in the number of total fertilized eggs was reported at 44.9 µg.l^{-1}. No significant effect on fertility rate (the ratio of the number of fertilized eggs to the number of total eggs) was observed.

As a follow-up of Kawashima et al. (2021) study, the ministry of the environment of Japan commissioned a MEOGRT study (MITI, 2021). This study aimed to evaluate the effects of TPhP over multiple generations under flow-through conditions starting with adult Japanese Medaka (parent generation, F0) up to the hatching of eggs of the third generation.

The selection of the range of the test concentrations was done based on the preliminary fish short-term reproduction assay (OECD TG229, Kawashima et al., 2021). In this previous study, 4.2% of mortality (no male mortality and 1/12 female mortality) was observed at the highest dose (44.9 µg.l^{-1}). A $LOEC_{TOX}$ > 44.9 µg.l^{-1} can be derived. Even if the OECD229 does not include

a reproduction phase and does not permit to take into account the toxicity of TPhP at earlier life stage, some uncertainty remains about the selection of the range of the test concentrations with a top concentration that should have been higher in the TG240. Most of the performance criteria and test validity requirements were met across the F0, F1, and F2 generations. However, histopathological analysis (F1, gonad, liver and kidney at 15 wpf) was not conducted in this study although this analysis has to be performed for endocrine disruptor testing purposes. In addition, the time to first spawn which normally has to be recorded after establishment of breeding pair, was not reported in the study report. Finally, the sex-ratio for subadult at 10wpf was not calculated by the CRO but by the Dossier Submitter (DS) in a second step based on the simple phenotypic sex. Another deviation is for F1 generation where exposure in the larval and subsequent stages require 12 fish per tank, whereas only 9 to 10 fish were present in the 15.2 µg.l^{-1} treatment group due to a very low hatching rate (49%). The nominal concentrations were 0 – 0.5 – 1.6 – 5 – 16 – 50 µg.l^{-1} (mean measured concentrations were within 20% of the nominal and were respectively 0 – 0.501 – 1.62 – 4.54 – 15.2 – 48.4 µg.l^{-1}).

- Survival:

  In the F0 generation, no significant mortality occured whatever the concentration tested. In the F1 generation although a significant decrease in survival was observed at 48.4 µg.l^{-1} in 4th wpf, no significant difference was observed in any concentrations in 8th wpf and 15th wpf for all fish (male and female). In 15th wpf and only for males, results showed significant mortality at 15.2 and 48.4 µg.l^{-1}.

- Development parameters:

  Length: in the F0 generation, a significant decrease of length of females was observed at 15.2 and 48.4 µg.l^{-1}. In the sub-adults of F1 generation (10 weeks old), a significant increase (but with no concentration dependence) was observed in male fish in all treatment groups and in female fish at 0.501, 1.62 and 4.54 µg.l^{-1}. In the adult F1 generation (15 weeks old), a significant increase in females only (no concentration dependence) was observed at 0.501, 1.62 and 4.54 µg.l^{-1}.

  Weight: whatever the generation, no significant difference was observed in male fish. On the other hand, female fish of F0 generation exhibited a significant decrease in weight at all concentrations (with concentration dependence). In the F1 generation, significant increase (but no concentration dependence) was observed for sub-adult female (10 weeks old) only and only at two concentrations (0.501 and 4.54 µg.l^{-1}).

Biological parameters:

HSI: in the F0 generation, no significant difference was observed whatever the concentration for both male and female fish. In the F1 generation, a significant decrease (no concentration dependence) was observed in sub-adult males at all concentrations and in sub-adult females at all concentrations except the two highest concentrations However, the decrease of the hepatosomatic index is transitory and is not seen in the adult stage (15 weeks old).

GSI: Significant differences were observed in female fish from 15.2 µg.l^{-1} with a decrease in the F0 generation (exposure at the adult stage), and an increase in the F1 generation. This significant increase was observed from 4.54 µg.l^{-1} in adult female fish (15 weeks old). For male, a significant increase was observed only in adult fish (15 weeks old) at the highest dose.

Reproduction:

Fecundity (production of eggs): the total number of produced eggs over the total period of observation was significantly decreased at 48.4 µg.l^{-1} in both F0 and F1 generations (over the 21 days).

In the F0 generation, when analysed week by week, there is no significant difference in the first week (Day 1-7), while a significant decrease in the following two weeks (Day 8-14 and Day 9-21), at 15.2 µg.l^{-1} and 48.4 µg.l^{-1}.

In the F1 generation, a significant decrease was observed at all tested concentrations in the first week (Day 1-7). In the second week, the significant decrease was observed only at the highest concentration and no difference in third week was exhibited.

Fertility parameter (number of eggs fertilized): The results for this parameter are correlated with the results for egg production. In the F0 and F1 generations (over the 21

days), the number of eggs fertilized over the total period of observation was significantly decreased at 48.4 µg.l^{-1}.

In the F0 generation, when analysed week by week, there is no significant difference in the first week (Day 1-7), while a significant decrease in the following two weeks (Day 8-14 and Day 9-21), at 15.2 µg.l^{-1} and 48.4 µg.l^{-1}.

In the F1 generation, a significant decrease was observed at all tested concentrations in the first week (Day 1-7). In the second week, the significant decrease was observed only at the highest concentration and no difference in third week was exhibited.

Fertility parameter (Fertilization rate): In the F0 and F1 generations, no significant difference was observed over the 21 days between the control group and any of the treatment groups, even if a significant transient increase (no concentration dependence) was observed during the second week in the F1 generation. Finally, a high variability (very high standard deviations) is noted at the highest dose of 48.4 µg.l^{-1} for the F1 generation. This high variability can be explained by a replicate (1/12) where average egg production was very low (5 eggs per day over 21 days instead of 17 eggs per day) and where no eggs were fertilised.

Embryonic stage development:

Hatching (Day):

The results of the F1 generation showed a significant decrease of the incubation period with no concentration dependence from 0.501 to 15.2 µg.l^{-1}. Results of the F2 generation (lifetime exposure) showed that the incubation period was significantly decreased (no concentration dependence) from 0.501 to 48.4 µg.l^{-1}, except for the 15.2 µg.l^{-1} treatment group.

Hatching rate (%):

Hatching rate was calculated at 18 dpf (two times the median hatching date of the control). Results of the F1 generation showed a significant decrease in the 4.54 to 48.4 µg.l^{-1} concentration groups. Results of the F2 generation (lifetime exposure), no significant difference was observed between control and treated groups.

Secondary sex characteristics (number of plates with papillae per fish):

In the F0 generation, male fish did not exhibit a significant difference whatever the tested concentration. Sub-adult male (F1 generation) fish exhibited a significant increase in all tested concentrations (no concentration dependence). This significant difference was not observed in adult male (F1 generation).

Female fish with anal fin papillae were not detected in any generation exposed and in any of the concentrations groups.

Occurrence of intersexuality:

In the F0 generation (adult), F1 (10wpf, sub-adult) and F1 (15wpf, adult), no change on occurrence of intersexuality was reported.

Simple sex-ratio (based on external phenotypic sex) calculated by DS:

In the F1 (10wpf, sub-adult), no significant change on the simple sex-ratio (based on external phenotypic sex) was observed.

This phenotypic sex as determined by the CRO was based on the external morphology of fish. However, no clear indication was given about the criteria used for the determination of the external phenotypic sex. Evaluation of the gonadal morphology and the genetic sex at 10 wpf were also reported. This simple phenotypic sex data were tentatively used to estimate the simple sex ratio that is mentioned in OECD 240. This simple sex ratio is however not expected to detect modest deviations because of the relatively small numbers of fish per replicate which will not provide sufficient statistical power. The phenotypic sex should also be determined in individual fish *via* histological examination of the gonads at 15wpf. Unfortunately, no histological evaluation of the gonads had been carried out preventing further characterisation of sex ratio.

During lifetime exposure in females, an improvement in development (weight, length) combined with an increase in GSI and VTG production were observed at the sub-adult stage. Despite the

increase in GSI and the assumed increase in ovary size (no histological analysis), egg production and VTG level were decreased at mature stage indicating a clear effect on egg production. Lastly the increase of VTG observed at the sub-adult stage is coherent with the estrogenic activity of TPhP and confirm the results observed in the other studies (estradiol levels and VTG levels).

He et al. (2021) studied acute toxicity with some endocrine indices and effects on reproduction of TPhP on adult zebrafish. The study was conducted with a single dose corresponding to less than 10% of the LC50 (based on a range-finding study), *i.e.*, 80 $\mu g.l^{-1}$. The test included groups of 10 separate male or female fish for 21-day exposure. Exposure to TPhP 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 were accompanied by histological changes. The testes and ovaries showed 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). Moreover, TPhP significantly reduced the egg production, the number of spawning, the fertilisation rate and the hatching rate. In conclusion, the study indicated that TPhP (80 $\mu g.l^{-1}$) altered gametogenesis in both sexes of adult zebrafish resulting in decreased fecundity.

Unpublished study report (2021) aimed to assess the potential endocrine activity and adverse effects of continuous exposure to TPhP for 73 days *i.e.,* on the early life stages and sexual differentiation of zebrafish (OECD 234). Thirty fertilised eggs were used for each test with 4 replicates per concentration. Endpoints were determined including hatching success and rates, mortalities during early life stage and juvenile growth, and maturation phase. Sex ratio was determined macroscopically and by histological examination of the gonads. Measurements were taken at the end of the study (73 dpf). Additionally, a histopathological examination was performed on the gonads and liver tissues. A maximum tolerated concentration (MTC) of 88.3 $\mu g.l^{-1}$ (where no effect on hatching success, but decrease of the post hatch survival rate were observed) has been defined by a range-finding study. In the main study, the exposure doses showed deviations (*i.e.,* 80% or 120%) from the nominal concentrations. Results were reported in mean measured concentrations of 0; 1.11; 3.01; 7.76; 33.3 and 76.8 $\mu g.l^{-1}$. According to the OECD 234 guidelines, all the validity criteria were met.

The post-hatching survival rates (4 dpf-73 dpf) observed were 88%; 84%; 80.0%; 77%; 63% and 51% (control group; 1.11; 3.01; 7.76; 33.3 and 76.8 $\mu g.l^{-1}$ respectively). The low survival rate observed at the two highest concentrations in the main study was not expected at this level of exposure in light of the range finding results (performed following the OECD guideline 2010 (96h, nom. concentrations: 1, 10 and 100 $\mu g.l^{-1}$, meas. concentrations: 0.93, 9.18, 88.3 $\mu g.l^{-1}$). Moreover, no acute or long-term zebrafish tests indicate the possibility of adverse effects below 88 $\mu g.l^{-1}$. If the survival rate is examined in detail, a relatively high mortality rate (>10%) is noted for the control even if it meets the validity criterion of 70% for control as indicated in the OECD TG 234. Fish are highly sensitive during the early life stages. This is confirmed when the survival rate is compared between 4dpf and 35dpf (89%; 87%; 84%; 80%; 67% and 54% (control group; 1.11; 3.01; 7.76; 33.3 and 76.8 $\mu g.l^{-1}$ respectively)) and between 35dpf and 73dpf (98%; 97%; 96%; 96%; 95% and 93% (control group; 1.11; 3.01; 7.76; 33.3 and 76.8 $\mu g.l^{-1}$ respectively)).

- Consequently, it is not possible to distinguish the toxic effect of TPhP from the variability of the intrinsic development during the early life stages of fish for the first three concentrations (1.11, 3.01 and 7.76 $\mu g.l^{-1}$);
- Only few cases of mortality between 35 dpf and test termination (73 dpf) were observed. TPhP shows no or very few mortality effects on zebrafish at the juvenile and adult stage, whatever the concentration.

It should be noted that according to the OECD 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 was not performed at the two highest concentrations where mortality occurred (across all replicates for each concentration). During

the early life stage, reduced growth in terms of total length was observed at the highest tested concentrations. This reduced size was likely due to the general systemic toxicity of TPhP. However, at 73 dpf, a subsequent exposure to TPhP led to the increase in mass of the exposed fish in a concentration-dependent manner with a statistically significant difference for the highest dose in males and the two highest concentrations in females. This mass 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 was not performed. The variability of the environmental conditions among the groups could impair the ability of the test to show an adverse effect in terms of population development or sex-ratio. Having the same density of fish in each tank can raise the weight of the tank factor in the statistical analysis. Fish density is extremely important for growth and development. Moreover, the high mortality rates can significantly reduce the number of fish per replicate and therefore reduce the statistical power.

Regarding the apical effect of the OECD 234, 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 in control group at termination of the test (30% males – 70% females) was fulfilled. The percentage of females for all the exposed groups increased from 52.6 to 65.4% and the percentage of males decreased from 46.3 to 33.3% in relation to increasing doses. The number of undifferentiated intersex was not significantly different (there was only one undifferentiated fish at the 1.11 and 76.8 $\mu g.l^{-1}$ concentrations). It is worth noting that the survival rate might 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 samples, a deficit of males was observed for these high doses, which might have altered the representativeness and therefore the interpretation of the measurements performed, in particular in males at these doses. It was 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 males only). The proportion of stage 2 (the most advanced stage found) increased from 55 to 90% for testes. Stage 4 in females increased from 15 (control) to 30% (highest dose). The acceleration in gonadal maturation is consistent with the elevated circulating steroid levels.

Concerning the histology of female gonads analysed at 3.33 and 76.8 $\mu g.l^{-1}$ and in the control group, a dose-dependent increase in all ovarian pathologies (oocyte atresia, egg debris, granulomatous inflammation) was 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 testis-ova was measured and was slightly decreased (not statistically significant) with combined acceleration of maturation of the gonad. However, the percentage of testis-ova males in the control was high (11%), which makes difficult the interpretation of the other doses. Moreover, although testis-ova will likely lead to a decrease in fertility or hatching success, the design of the test (FSDT, *i.e.,* OECD 234) precludes this type of examination since FSDT *per se* does 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 analysed while for the heart, only two groups were examined (control and 76.8 $\mu g.l^{-1}$). 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 males, 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. Cardiac lesions were also observed in treated

TPhP groups but no statistics could be performed due to the limited number of replicates and test concentrations.

In Fan et al. (2022) the authors explored the effects of TPhP alone and/or combined to nano-titanium dioxide (nano-TiO2) on the neurodevelopment of zebrafish larvae. Zebrafish embryos (within 2 h after fertilisation) were exposed to concentrations of 0, 8, 24, 72 and 144 $\mu g.l^{-1}$ (no analytical measurement) of TPhP alone for 120h. Zebrafish embryos/larvae were kept in an incubator at 28 ± 0.5 ◦C, with a light-dark ratio of 14 h/10 h. During the period of exposure, the solutions were renewed daily to keep the concentrations constant. No developmental toxicity was detected by treating with TPhP at 8 and 24 $\mu g.l^{-1}$, while higher concentrations of TPhP (72 $\mu g.l^{-1}$ and 144 $\mu g.l^{-1}$) significantly altered the hatching rate, the heart rate, the malformation rate, and the body length in zebrafish larvae (only at 144 $\mu g.l^{-1}$). Note that at the highest dose the inhibitions on the hatching rate, heartbeat and body length in zebrafish larvae were significantly promoted in the co-exposure groups where TiO2 was added, compared to TPhP alone.

In Zhang et al. (2023) the authors investigated the effects of TPhP exposure on developmental parameters, locomotor behaviour, oxidative stress, apoptosis and transcriptional levels in zebrafish at different developmental stages. Zebrafish embryos (0 – 2 hpf) were exposed to concentrations of 0, 20, 50, 100, 500 and 1000 $\mu g.l^{-1}$ of TPhP for 7 days (based on a range finding study with a 96h LC_{50} value of TPhP on zebrafish embryos of 5.13 $mg.l^{-1}$). The results showed that the survival rate decreased significantly compared to the control at the concentration of 500 µg/L from 120 hpf. In the same way, the hatching rate decreased at 50µg/L at 48 hpf and 500 µg/L at 72 hpf compared to the control. TPhP also significantly increased the heart rate of zebrafish larvae at 48 hpf (100 and 1000 $\mu g.l^{-1}$) and at 60 hpf (500 and 100 $\mu g.l^{-1}$) but significantly decreased at 96 hpf (500 $\mu g.l^{-1}$). Microscopic observations also showed that the exposure of TPhP led to several malformation symptoms such as yolk sac edema, spinal curvature, abnormal body pigmentation, pericardial edema, small eyes, etc. Concerning the behaviour changes, TPhP could affect the embryonic spontaneous movement. Regarding the locomotor behaviour and the neurotoxicity, all TPhP treated groups caused statistically significant increase in swimming distance and movement trajectory of zebrafish larvae except for the 50 $\mu g.l^{-1}$ groups. The authors also found that TPhP increased the number of apoptotic cells in the head, heart and intestine of zebrafish. In the head region, the number of apoptotic cells were localised in the eyeball of zebrafish larvae.

Conclusion: *in vivo* studies on fish reproduction show an alteration of gametogenesis in both sexes with a disruption of testis and ovary maturation followed by a decrease in reproductive success in terms of fecundity (decrease in egg production, spawning events) and fertility (impaired hatchability and fertilisation rate observed in most of the studies). *In vivo* studies on amphibian development show that TPhP could affect tadpole survival and metamorphosis.

Analysis of systemic toxicity in relation to the observation of EAS-related adverse effects

As recommended in the EDC guidance (ECHA/EFSA, 2018), adverse effects that are non-specific secondary consequences of other toxic effects shall not be considered for the identification of the substance as endocrine disruptor. The top concentration selected for the conduction of the ecotoxicological studies should provide information on substance toxicity at an exposure of the tested agent that should be tolerated without inducing significant chronic physiological dysfunctions, be compatible with animal survival and permits data interpretation in the context of the use of the study. It is recommended that a Maximum Tolerated Concentration (MTC) should be considered for the evaluation of changes which could be due to excessive systemic toxicity. In ecotoxicology, the MTC is defined as the highest test concentration of the chemical which results in less than 10% mortality.

For each study, information on mortality when available has been reported in **Table 11**, **Table 12**, **Table 13** and **Table 14**. Data do not systematically allow to determine a MTC value.

Japanese medaka (*Oryzias latipes*):

Five studies (Sun et al., 2016; Li et al., 2018; Li et al., 2019b; Kawashima et al., 2021; MITI, 2021) of interest are available. Two studies provide information on the survival rate of fish after exposure of TPhP: Kawashima et al. (2021) and MITI (2021).

Table 13: Summary table of survival data - Japanese medaka (*Oryzias latipes*)

Ref.	Age at study start	Duration of exposure	Sex	Media renewal	Nominal conc. ($\mu g.l^{-1}$)	Measured conc. ($\mu g.l^{-1}$)	Survival rate
Kawashima et al., 2021	16±2 weeks old	21d	All	Flow-through (5 times/day)	Control group	Control group	100%
					20	2.13	100%
					64	7.19	100%
					200	17.1	100%
					640	44.9	96%
MITI, 2021	F0 generation 12 weeks old F1: lifetime	F0: 23d	All	Flow-through (>5 times/day)	Control group	Control group	96%
					0.5	0.501	100%
					1.6	1.62	100%
					5	4.54	100%
					16	15.2	100%
					50	48.4	100%
		F1: 4wpf	All		Control group	Control group	100%
					0.5	0.501	100%
					1.6	1.62	100%
					5	4.54	100%
					16	15.2	100%
					50	48.4	97%
		F1: 8wpf	All		Control group	Control group	99%
					0.5	0.501	100%
					1.6	1.62	99%
					5	4.54	100%
					16	15.2	100%
					50	48.4	95%

		F1: 15wpf*	All		Control group	Control group	100%
					0.5	0.501	100%
					1.6	1.62	96%
					5	4.54	96%
					16	15.2	87%
					50	48.4	92%
			Male		Control group	Control group	100%
					0.5	0.501	100%
					1.6	1.62	100%
					5	4.54	92%
					16	15.2	83%
					50	48.4	92%
			Female		Control group	Control group	100%
					0.5	0.501	100%
					1.6	1.62	91%
					5	4.54	100%
					16	15.2	92%
					50	48.4	92%

* Number of post-pairing mortalities, (n of test animals = 12 per tested group)

In Kawashima et al. (2021), the lowest observed effect concentration (LOEC) on mortality from this study is above the highest tested concentration (44.9 $\mu g.l^{-1}$). The MTC was not exceeded in this study.

In MITI (2021) no significant mortality occurs in the F0 generation whatever the concentration tested in male, female and in the whole group. In the F1 generation although a statistical significant increase of mortality was observed at 48.4 $\mu g.l^{-1}$ in 4th wpf, no significant difference was observed in any concentrations in 8th wpf. In 15th wpf, the results showed significant mortality for males at 15.2 and 48.4 $\mu g.l^{-1}$. One dead in twelve male fish was observed at 4.54 and 48.4 $\mu g.l^{-1}$ and authors considered a significant difference at 48.4 but not at 4.54 $\mu g.l^{-1}$. Thus, the evaluation made by the study director on the mortality results must be treated with caution. For female fish and for the whole group (male + female), there was no significant difference whatever the concentration.

To conclude, it is considered that MTC was not exceeded in this MITI study.

Zebrafish (*Danio rerio*):

Eleven studies of interest are available : Liu et al., 2012; Liu et al., 2013a; Liu et al., 2013b ; Kim et al., 2015 ; Liu et al., 2016 ; Liu et al., 2019 ; He et al., 2021 ; Lee et al., 2022 ; Unpublished study report, 2021 ; Fan et al., 2022 ; Zhang et al., 2023. The information on mortality is summarised below.

Table 14: Summary table of survival data - Zebrafish (*Danio rerio*)

	Age at study start	Duration of exposure	Media renewal	Nominal conc. ($mg.l^{-1}$)	Measured conc. ($mg.l^{-1}$)	Survival rate
Liu et al., 2012	4 months	14d	Every other day	Control group		No significant mortality (no value provided)
				0.04	NAM	
				0.2		
				1		
No value for survival rate. MTC was not exceeded						
Liu et al., 2013b	4-5 months	21d	Every 48h	Control group		No mortality (no value provided)
				0.04	<LOD (48h)	
				0.2	<LOD (48h)	
				1	0.38 (48h)	
No mortality at 0.38 $mg.l^{-1}$. MTC was not exceeded						
Liu et al., 2019	4-5 months	14d	at least three times per week	Control group		No mortality (no value provided)
				0.04	0.03 (0h) <LOD (48h)	
				0.2	0.15 (0h) <LOD (48h)	
				1	0.87 (0h) 0.42 (48h)	
No mortality at 0.42 $mg.l^{-1}$. MTC was not exceeded						
He et al., 2021	Adult	21d	Every day	0.08	NAM	No information
No information. Impossible to determine a systemic toxicity threshold from survival rate						
Liu et al., 2013a	4hpf	120hpf	at 48h only	Control group		
				0.02	0 (120hpf)	> 90%
				0.2	0 (120hpf)	> 90%
				2	1.39 (120hpf)	> 90%
Mortality < 10% at 1.39 $mg.l^{-1}$. MTC was not exceeded.						
Kim et al., 2015	3hpf	7d	50% daily	Control group		91.8%
				0.04	NAM	92.5%
				0.2		92%
				0.5		88.7%
Mortality > 10% at 0.5 $mg.l^{-1}$ but not > 10% compared to mortality in the control group. MTC is not considered to have been exceeded.						
Liu et al., 2016	4hpf	120d	50% every 48h	Control group		
				0.005	0.0008 (24h) < LOD (48h)	No significant mortality (no value provided)
				0.05	0.0055 (24h) < LOD (48h)	
				0.5	0.131 (24h)	

					0.011 (48h)	
Impossible to determine a correct range of exposure concentrations. No value for survival rate. Impossible to determine a systemic toxicity threshold from survival rate. MTC was not exceeded.						
Lee et al., 2022	72hpf	24h	No information	Control group		
				0.32	NAM	No significant mortality (no value provided)
				0.97		
				3.2		
				9.8		< 50%
Low survival rate at 9.8 mg.l^{-1}. No value for survival rate. MTC may have been exceeded.						
Unpublished study report, 2021	<12hpf	73d	Flow-through system	Control group		88% (4-73 dpf) 89% (4-35 dpf) 98% (35-73 dpf)
				0.001	0.001	84% (4-73 dpf) 87% (4-35 dpf) 97% (35-73 dpf)
				0.0032	0.003	80% (4-73 dpf) 84% (4-35 dpf) 96% (35-73 dpf)
				0.01	0.008	77% (4-73 dpf) 80% (4-35 dpf) 96% (35-73 dpf)
				0.032	0.033	63% (4-73 dpf) 67% (4-35 dpf) 95% (35-73 dpf)
				0.1	0.077	51% (4-73dpf) 54% (4-35dpf) 93% (35-73 dpf)
Mortality in the control group > 10%. 0bservation of mortality during the early life stages in controls demonstrates that TPhP exposure is not the only driver for mortality, considering the high sensitivity of fish during these stages. During the juvenile growth and maturation phase (i.e. after day 35 dpf until test end), exposure to TPhP displays no or very few mortality effects on zebrafish, whatever the concentration.						
Fan et al., 2022	2hpf	120h	daily	Control group		87%
				0.008	NAM	86%
				0.024		84%
				0.072		82%
				0.144		81%
Mortality in the control group > 10% but not > 10% compared to mortality in the control group. Observation of mortality in controls demonstrate that TPhP exposure is not the only driver for mortality. MTC is not considered to have been exceeded.						
Zhang et al. 2023	2hpf	7d	90% daily	Control group		>80%
				0.02	NAM	90%
				0.05	NAM	90%
				0.1	NAM	90%
				0.5	NAM	30%
				1	NAM	20%
No exact value for the survival rate (observations from a graph). Mortality in the control group > 10%. Mortality > 10% compared to mortality in the control group above 0.5 mg/l. Observation of mortality in controls demonstrate that TPhP exposure is not the only driver for mortality.						

NAM: no analytical measurement

Data do not systematically allow to determine a MTC value. It is noted that due to some limitations in the studies (absence of measured concentrations) and differences in experimental design (stage and duration of exposure), it is impossible to determine a systemic toxicity threshold from survival rate for Zebrafish (*Danio rerio*).

Overall, mortality and its possible interference with EAS-adverse effects shall be interpreted in each study independently. Nevertheless, it should be highlighted that no or little mortality occur at the concentrations tested at the juvenile and adult stages at the tested concentrations.

Rare minnows (*Gobiocypris rarus*):

One study (Chen et al., 2020) on this species is available. The information on mortality is summarised below.

Table 15 : Summary table of survival data - Rare minnows (Gobiocypris rarus)

	Age at study start	Duration of exposure	Media renewal	Nominal conc. ($mg.l^{-1}$)	Measured conc. ($mg.l^{-1}$)	Survival rate
Chen et al., 2020	20-22 weeks	28d	Daily	Control group		96.67%
				0.012	0.010	96.11%
				0.12	0.098	90.56%

No significant difference in mortality rate was observed between the control and the treatment groups. The MTC was not exceeded in this study.

Conclusion regarding ED properties relevant for environment

Adverse effects relevant for ED identification

The available data indicate that exposure to TPhP damages fish reproduction by impacting processes and events during gametogenesis (oocyte growth, ovary and testis maturation), disrupting fish fecundity (eggs production and spawning) and fertility (fertilisation and hatching). Changes in fecundity and fertility are apical effects, which inform about potential adverse effects at fish population level.

A significant reduction in sperm velocity without changing sperm concentration or vitality observed in Chen et al. (2020) indicates a potential effect on sperm quality in Chinese rare minnows, even if an apoptosis in testis was also found at the highest tested concentration. The decrease in mature spermatocytes (He et al., 2021) suggests a potential delay in spermatogenesis. A significant gonadal intersex incidence of 26% in male medaka was observed at 1.43 $\mu g.l^{-1}$ (Li et al., 2018), indicating a potential feminisation risk for male fish. A disruption (concentration-dependent increase) of male-typical reproductive behaviour before spawning (chasing trajectory and time, courtship frequency and mating number) observed by Li et al. (2018) also supports the reproduction impairment of male fish after exposure to TPhP. This effect can explain the observed decrease in fertilisation rate.

Data show an inhibition of the ovarian development in female fish (He et al., 2021) and alterations of the ovaries (Li et al., 2019b; He et al., 2021). These alterations and development retardation can affect the fecundity of female fish with the observed decrease in egg production and spawning.

Despite the observation of some effects in the FSDT study, it should be noted that the quality of this study did not allow to evaluate properly EAS mediated parameters.

The decrease in egg production (Liu et al., 2013b; Li et al., 2019b; He et al., 2021; Kawashima et al., 2021; MITI, 2021) is a clear effect indicator of the impaired reproductive ability, reinforced by a decrease of spawning events (Liu et al., 2013b; He et al., 2021; MITI, 2021), an insufficient fertilisation rate (Li et al., 2018; He et al., 2021) and a decrease in hatchability (Liu et al., 2013b; Li et al., 2018; He et al., 2021; MITI, 2021 (only for F1 generation)), disrupting the generation of new individuals. This impact on fish reproduction could affect fish population stability and is considered as an adverse effect relevant at population level.

Moreover, it is important to note that several adverse effects on altered reproductive capacity of fish are observed in several studies at environmentally relevant levels without concurrent systemic effects. Indeed, no specific mention of mortality was reported in several studies (He et al., 2021; Li et al., 2018; Li et al., 2019b) although the absence of specific information on this parameter leaves some uncertainties. Most importantly, effects on hatching rate, on fecundity

and/or fertility were clearly observed without any concurrent mortality in Liu et al. (2013b), Kawashima et al. (2021) and MITI (2021).

TPhP shows adverse effects on fertility and fecundity of fish at environmentally relevant levels. The impact on fish reproduction could affect fish population stability and is considered as an adverse effect relevant at population level. These effects cannot be considered as a secondary non-specific consequence of general toxicity.

Data (*in vivo*) providing scientific evidence of an adverse effect of TPhP on the reproductive system are summarised in Table 16 below.

Table 16: Line of evidence in relation to TPhP sexual dysfunction in *in-vivo* studies

Endpoint	Biological model	Species	Exposure duration	Life cycle stage (start of the exposure) Sex (end of exposure)	Lowest significant concentration	Observed effects	Reference
Hatching rate	-	Zebrafish	21 days	Adult	0.2 $mg.l^{-1}$	↓	Liu et al., 2013b
Hatching rate	-	Japanese Medaka	100 days	Juvenile	0.299 $\mu g.l^{-1}$	↓#	Li et al., 2018
Hatching rate	-	Zebrafish	21 days	Adult	80 $\mu g.l^{-1}$	↓#	He et al., 2021
Sex ratio	-	Japanese Medaka	100 days	Juvenile	1.43 $\mu g.l^{-1}$	↑# intersex incidence in male fish	Li et al., 2018
Sex ratio	-	Zebrafish	73 days	Juvenile	-	##No change	Unpublished study report, 2021
Reproductive behaviour	-	Japanese Medaka	100 days	Juvenile	1.43 $\mu g.l^{-1}$	#Abnormal chasing behaviour in male fish	Li et al., 2018
Fecundity	-	Zebrafish	21 days	Adult	0.2 $mg.l^{-1}$	↓ egg production, spawning event	Liu et al., 2013b
Fecundity	-	Japanese Medaka	100 days	larvae	0.36 $\mu g.l^{-1}$	#↓ egg production	Li et al., 2019b
Fertility	-	Zebrafish	21 days	Adult	-	Fertility rate: no change	Liu et al., 2013b
Fertility	-	Japanese Medaka	100 days	Juvenile	1.43 $\mu g.l^{-1}$	↓# fertilisation rate	Li et al., 2018
Fecundity/fertility	-	Zebrafish	21 days	Adult	80 $\mu g.l^{-1}$	↓# fertilisation rate, cumulative eggs, spawning event	He et al., 2021
Fecundity/fertility	-	Japanese Medaka	21 days	Adult	44.9 $\mu g.l^{-1}$	↓ egg production and fertilised eggs*	Kawashima et al., 2021
Fecundity/fertility	-	Japanese Medaka	1-21 days	Adult (F0)	48.4 $\mu g.l^{-1}$	↓ egg production and fertilised eggs*	MITI, 2021
Fecundity/fertility	-	Japanese Medaka	12 weeks	Embryo (F1)	48.4 $\mu g.l^{-1}$	↓ egg production and fertilised eggs*	MITI, 2021
Histology	Ovaries	Japanese Medaka	100 days	larvae	0.13 $\mu g.l^{-1}$	↓# number of mature oocytes	Li et al., 2019b
Histology	Ovaries	Zebrafish	21 days	Female adult	80 $\mu g.l^{-1}$	↓# ovary maturation stages	He et al., 2021
Histology	Testes	Zebrafish	21 days	Male adult	80 $\mu g.l^{-1}$	↓# testis maturation stages	He et al., 2021
Histology	Testes	Zebrafish	73 days	Male juvenile	76.8 $\mu g.l^{-1}$	↑## testis maturation stages	Unpublished study report, 2021

*: however no significant effect on fertility rate (ratio)
No information on mortality

[##] Mortality > 10% observed, with mortality > 10% also in the control group
MTC not exceeded for the other studies

Endocrine activity

In vitro and *in vivo* studies are available supporting an **estrogenic activity** of TPhP, as summarised in Table 18 below.

Molecular Initiating events (MIEs)

- **Nuclear estrogen receptors**

The estrogenic activity is mainly mediated *via* nuclear estrogen receptors (nERs), which function as transcription factors upon ligand binding for genomic responses. In most vertebrates, including mammals, birds, and some lower vertebrates, two estrogen receptors exist: the nuclear intracellular receptors ERα and ERβ, considered to mediate the effect of E2 and VTG in liver of most fish species. Data show that TPhP has an agonist activity on human ERα and ERβ receptors as well as antagonist activity on ER (Liu et al., 2012; Kojima et al., 2013; Zhang et al., 2014; Kojima et al., 2016; Medvedev et al., 2020; Ji et al., 2022). The agonist activity is also reported for the medaka ER1 (Kawashima et al., 2021) and for the fish ER2β, frog ER1, turtle ER1 and chicken ER1 (Medvedev et al., 2020).

In some yeast two-hybrid assays with human and medaka ERα, an antagonist effect is shown indicating a competition between TPhP and E2 for agonist activity to ERα (Ji et al., 2020; Li et al., 2018). Zhang et al. (2014) demonstrated a binding affinity of TPhP to hERα with a docking approach. Moreover, TPhP can induce the ERE pathway demonstrated by a dose-response of the agonist effect in MVLN cells (transfected with the human ERE-luciferase reporter gene) (Ji et al., 2020).

- **GPER (membrane estrogen receptor)**

The estrogenic activity can also be mediated *via* membrane estrogen receptors as G protein-coupled membrane receptors (GPER). The role of GPER in oocyte maturation is well defined in zebrafish (Thomas, 2017; Chen et al., 2019; Wu et al., 2021). Activation of GPER induced Vtg genes expression while inhibition of GPER significantly attenuated the estrogenic effect on Vtg (Chen et al., 2019; Wu et al., 2021).

In Guan et al. (2022), the authors report that TPhP binds GPER with an IC_{50} of 4.2 µM. This is confirmed by E2-F displacement experiments. Combined transcriptome and proteome analysis show that TPhP can exert its estrogen-like proliferation-promoting effect through the interaction of the PI3K-Akt signalling pathway, MAPK signalling pathway, and EGFR signalling pathway, which are downstream pathways mediated by GPER. In Ji et al. (2022), the assay on SBRK3 cells shows that TPhP can interact with GPER and activate cAMP formation, which is one of the rapid signalling pathways induced by GPER activation. However, this activation is 10-times less efficient than the reaction induced by E2. Nevertheless, this activation *via* GPER is also confirmed by the results obtained with a TPhP co-exposure with the specific GPER30 inhibitor, G15, which inhibits the effects of TPhP in the assay.

The estrogenic activity of TPhP, mediated by ER and/or GPER, is supported by proliferation assays on MCF-7 cells that show TPhP promotes cell proliferation (Zhang et al., 2014; Krivoshiev et al., 2016; Ji et al., 2020).

- **Aromatase activity**

Aromatase (CYP19) catalyses the conversion of androgenic steroids to estrogenic steroids (Conley and Hinshelwood, 2001). The CYP19 plays a role in signaling pathways critical for sexual development and fertility. TPhP induced an increase of the expression of the CYP19 gene (Liu et al., 2012; Liu et al., 2013b; Liu et al. 2016; Ji et al., 2022) and 17β-HSD1 gene (Liu et al., 2013b; Liu et al., 2016; Ji et al., 2022), which is linked to the steroidogenic pathway. In Ji et al. (2022), a decrease of estrone was observed while CYP19 is upregulated and E2 level increased.

The reason given by the authors was that the conversion activity of estrone to E2 by 17β-HSD1 was stronger than the activity of CYP19 to estrone synthesis. TPhP affected steroidogenesis by increasing the estrogen levels (17ß-estradiol) *via* the up-regulation of CYP19.

Steroid hormone level and Vitellogenin

During medium-term exposure, TPhP induces an increase in circulating E2 concentrations in zebrafish and medaka (Liu et al., 2012; Liu et al., 2013b; Liu et al., 2016; Li et al., 2018; Li et al., 2019b; Unpublished study report, 2021) and a decrease in 11-KT (Liu et al., 2012; Liu et al., 2013b; Liu et al., 2016; Li et al., 2018; Li et al., 2019b). TPhP also induces an increase of VTG concentrations in Liu et al. (2013b) and Unpublished study report (2021), which support the estrogenic activity of TPhP.

Nevertheless, in one study (Li et al., 2019b) carried out on Japanese medaka, a decrease of E2 level was noted after 100-day exposure of female larvae (age of fish at exposure initiation), while an increase of E2 was observed after 21-day exposure of adult females (age of fish at exposure initiation).

The observations of VTG concentrations suggested an estrogenic activity of TPhP in female and male fish, with increased concentration of VTG (Liu et al., 2013b; MITI, 2021 (only for female sub-adults) and Unpublished study report (2021) (for females only)). Some results were also reported with decreased or unchanged concentration in hepatic VTG of adult female medaka in Kawashima et al. (2021) and MITI (2021), respectively, or in whole-body homogenates of male and female zebrafish (He et al., 2021). Moreover, VTG hepatic levels were not modified in Medaka males in one assay (MITI, 2021). *VTG1* gene expression in liver was increased in adult male zebrafish in one assay (Liu et al., 2012), while the expression in liver of vtg1 and/or 2 were decreased in adult females in other assays (Liu et al., 2012; Li et al., 2019b). These different perturbations of circulating steroid concentrations relate to the fish developmental stage, species and tested concentrations. VTG is a biomarker relevant for adult and juvenile stages for which toxicity is not observed at tested concentrations in the available studies with TPhP, including the FSDT study in which no or very little toxicity is observed after 35 dpf whatever the concentration. Thus, data available on VTG and analysed together with other relevant observations support that TPhP activates the estrogenic pathway and/or disturbs the steroidogenic pathway. Change in E2 levels were observed in several (but not all) studies with concurrent modifications in VTG or vtg expression levels (Liu et al., 2012; Unpublished study report, 2021; Liu et al., 2013b; Li et al., 2019b).

Finally, and specifically for female, the changes in VTG levels or *vtg* gene expression seem dependent on the life cycle of fish, the period of exposure and the reproductive status. An increase of VTG is observed following exposure to TPhP at the juvenile/sub-adult stage (Unpublished study report, 2021; MITI, 2021). In contrast, at adult stage, vtg expression or VTG levels were significantly decreased (He et al., 2021; MITI, 2021; Kawashima et al., 2021; Li et al., 2019b) as indicated in Figure 4 above. **This suggests an estrogenic over stimulation of TPhP at the juvenile stage which may have altered the maturation to functional gonads and their capacity to produce viable gonadocytes with consequence of altered fecundity and decreased ability to produce eggs at the mature stage.**

These studies provide support to the conclusion that TPhP exerts EAS activity.

Data therefore provide *in vitro* and *in vivo* evidence that TPhP has EAS activity. In absence of mortality reported in several studies, this activity cannot be considered as a secondary non-specific consequence of general toxicity.

Table 17: Line of evidence in relation to TPhP EAS activity

Assay category	Species/ Endpoint	Biological model	Exposure time	Life cycle stage (start of the exposure) Sex (end of exposure)	Parameter	Lowest significant concentration	Observed effects	Reference
In vitro endocrine activity								
Transactivation	hERα	CHO-K1 cells	24h	-	REC20	-	Weak agonist: 4.9 µM	Kojima et al., 2013
Transactivation	hERα	CHO-K1 cells	24h	-	REC20	-	Agonist: 0.27 µM	Zhang et al., 2014
Transactivation	hERα	Yeast cells	-	-	REC20	-	Agonist: 0.65 µM	Zhang et al., 2014
Transactivation	hERα	MCF-7 cells	60h	-	REC20	-	Agonist: 0.1 µM	Zhang et al., 2014
Transactivation	hERα	CHO-K1 cells	24h	-	REC20	-	Weak agonist: 4.6 µM	Kojima et al., 2016
Transactivation	hERß	CHO-K1 cells	24h	-	REC20	-	Weak agonist: 6.5 µM	Kojima et al., 2013
Transactivation	hERß	CHO-K1 cells	24h	-	REC20	-	Weak agonist: 7.3 µM	Kojima et al., 2016
Hormone measurement	Estradiol level	H295R cells	48h	Adult	b	1 $mg.l^{-1}$	↑	Liu et al., 2012
Hormone measurement	E2 level	H295R cells	48h	-	b	1 µM	↑	Ji et al., 2022
Hormone measurement	Testosterone level	H295R cells	48h	Adult	b	1 $mg.l^{-1}$	↑	Liu et al., 2012
Hormone measurement	Testosterone level	H295R cells	48h	-	b	1 µM	↓	Ji et al., 2022
Hormone measurement	E2/T ratio level	H295R cells	48h	Adult	b	0.1 $mg.l^{-1}$	↑	Liu et al., 2012
Hormone measurement	E2/T ratio	H295R cells	48h	-	b	1 µM	↑	Ji et al., 2022
Gene expression	CYP19A1	H295R cells	48h	Adult	b	1 $mg.l^{-1}$	↑	Liu et al., 2012

Gene expression	Cyp19	H295R cells	48h	-	b	1 µM	↑	Ji et al., 2022
Transactivation	ER	MCF-7 cells (MVLN cells)	72h	Adult	b	0.001 mg.l^{-1}	Antagonism	Liu et al., 2012
Transactivation	ER	MCF-7 cells (MVLN cells)	72h	-	EC50	EC50	Agonist activity (EC50 = 1.2 x 10-5 M)	Ji et al., 2022
Transactivation	mERα	MVLN cell	24h	-	b	IC50	Antagonist activity (IC 50 = 29 µM)	Li et al., 2018
Binding	-	SBRK3 cells	24h	-	b	-	Interaction with GPER via cAMP formation	Ji et al., 2022
Transactivation	mEsr1	-	-	-	b	-	Agonist activity	Kawashima et al., 2021
In vivo endocrine activity								
Hormone measurement	E2 level	Zebrafish plasma	120 days	Male embryo	b	5 µg.l^{-1}	↑	Liu et al., 2016
Hormone measurement	E2 level	Medaka plasma	100 days	Male embryo	b	0.299 µg.l^{-1}	↑$^{\#}$	Li et al., 2018
Hormone measurement	E2 level	Zebrafish plasma	14 days	Male adult	b	1 mg.l^{-1}	↑	Liu et al., 2012
Hormone measurement	E2 level	Zebrafish plasma	21 days	Male adult	b	0.2 mg.l^{-1}	↑	Liu et al., 2013b
Hormone measurement	E2 level	Zebrafish plasma	21 days	Male adult	b	-	No change	He et al., 2021
Hormone measurement	E2 level	Rare minnows plasma	28 days	Male adult	b	12 µg.l^{-1}	↓	Chen et al., 2020
Hormone measurement	E2 level	Zebrafish plasma	120 days	Female embryo	b	5 µg.l^{-1}	↑	Liu et al., 2016
Hormone measurement	E2 level	Medaka plasma	100 days	Female embryo	b	1.77 µg.l^{-1}	↓$^{\#}$	Li et al., 2019b
Hormone measurement	E2 level	Zebrafish plasma	73 days	Female embryo	b	33.3 µg.l^{-1}	↑$^{\#\#}$	Unpublished study report, 2021
Hormone measurement	E2 level	Zebrafish plasma	14 days	Female adult	b	1 mg.l^{-1}	↑	Liu et al., 2012

Hormone measurement	E2 level	Zebrafish plasma	21 days	Female adult	b	1 mg.l^{-1}	↑	Liu et al., 2013b
Hormone measurement	E2 level	Medaka plasma	21 days	Female adult	b	1.72 µg.l^{-1}	↑$^{\#}$	Li et al., 2019b
Hormone measurement	E2 level	Zebrafish plasma	21 days	Female adult	b	-	$^{\#}$No change	He et al., 2021
Hormone measurement	T level	Medaka plasma	100 days	Male embryo	b	1.43 µg.l^{-1}	↓$^{\#}$	Li et al., 2018
Hormone measurement	T level	Zebrafish plasma	14 days	Male adult	b	1 mg.l^{-1}	↓	Liu et al., 2012
Hormone measurement	T level	Zebrafish plasma	21 days	Male adult	b	-	$^{\#}$No change	He et al., 2021
Hormone measurement	T level	Medaka plasma	100 days	Female embryo	b	0.13 µg.l^{-1}	↓$^{\#}$	Li et al., 2019b
Hormone measurement	T level	Zebrafish plasma	14 days	Female adult	b	-	No change	Liu et al., 2012
Hormone measurement	T level	Zebrafish plasma	21 days	Female adult	b	1 mg.l^{-1}	↓	Liu et al., 2013b
Hormone measurement	T level	Medaka plasma	21 days	Female adult	b	1.72 µg.l^{-1}	↑$^{\#}$	Li et al., 2019b
Hormone measurement	T level	Zebrafish plasma	21 days	Female adult	b	-	$^{\#}$No change	He et al., 2021
Hormone measurement	E2/T level	Zebrafish plasma	14 days	Female adult	b	-	No change	Liu et al., 2012
Hormone measurement	E2/T level	Zebrafish plasma	21 days	Female adult	b	0.2 mg.l^{-1}	↑	Liu et al., 2013b
Hormone measurement	E2/T level	Zebrafish plasma	21 days	Female adult	b	80 µg.l^{-1}	↓$^{\#}$	He et al., 2021
Hormone measurement	E2/T level	Zebrafish plasma	14 days	Male adult	b	1 mg.l^{-1}	↑	Liu et al., 2012
Hormone measurement	E2/T level	Zebrafish plasma	21 days	Male adult	b	0.04 mg.l^{-1}	↓	Liu et al., 2013b

Hormone measurement	E2/T level	Zebrafish plasma	21 days	Male adult	b	-	#No change	He et al., 2021
Hormone measurement	11-KT level	Zebrafish plasma	120 days	Female embryo	b	500 µg.l^{-1}	↓	Liu et al., 2016
Hormone measurement	11-KT level	Medaka plasma	100 days	Female embryo	b	0.13 µg.l^{-1}	↓#	Li et al., 2019b
Hormone measurement	11-KT level	Zebrafish plasma	14 days	Female adult	b	-	No change	Liu et al., 2012
Hormone measurement	11-KT level	Zebrafish plasma	21 days	Female adult	b	1 mg.l^{-1}	↓	Liu et al., 2013b
Hormone measurement	11-KT level	Zebrafish plasma	120 days	Male embryo	b	5 µg.l^{-1}	↓	Liu et al., 2016
Hormone measurement	11-KT level	Medaka plasma	100 days	Male embryo	b	1.43 µg.l^{-1}	↓#	Li et al., 2018
Hormone measurement	11-KT level	Zebrafish plasma	73 days	Male embryo	b	33.3 µg.l^{-1}	↑##	Unpublished study report, 2021
Hormone measurement	11-KT level	Zebrafish plasma	14 days	Male adult	b	1 mg.l^{-1}	↓	Liu et al., 2012
Hormone measurement	11-KT level	Rare minnows plasma	28 days	Male adult	b	12 µg.l^{-1}	↓	Chen et al., 2020
Hormone measurement	E2/11-KT level	Zebrafish plasma	120 days	Female embryo	b	500 µg.l^{-1}	↑	Liu et al., 2016
Hormone measurement	E2/11-KT level	Zebrafish plasma	14 days	Female adult	b	1 mg.l^{-1}	↑	Liu et al., 2012
Hormone measurement	E2/11-KT level	Zebrafish plasma	21 days	Female adult	b	1 mg.l^{-1}	↑	Liu et al., 2013b
Hormone measurement	E2/11-KT level	Zebrafish plasma	120 days	Male embryo	b	5 µg.l^{-1}	↑	Liu et al., 2016
Hormone measurement	E2/11-KT level	Zebrafish plasma	14 days	Male adult	b	1 mg.l^{-1}	↑	Liu et al., 2012
Hormone measurement	E2/11-KT level	Zebrafish plasma	21 days	Male adult	b	0.2 mg.l^{-1}	↑	Liu et al., 2013b

Hormone measurement	VTG level	Zebrafish plasma	73 days	Female embryo	b	76.8 µg.l^{-1}	↑$^{\#\#}$	Unpublished study report, 2021
Hormone measurement	VTG level	Zebrafish plasma	21 days	Female adult	b	0.2 mg.l^{-1}	↑	Liu et al., 2013b
Hormone measurement	VTG level	Zebrafish plasma	21 days	Female adult	b	80 µg.l^{-1}	↓$^{\#}$	He et al., 2021
Hormone measurement	VTG level	Japanese Medaka plasma	21 days	Female adult	b	7.19 µg.l^{-1}	↓	Kawashima et al., 2021
Hormone measurement	VTG level	Japanese Medaka liver	4 weeks	Female adult (F0)	b	-	No change	MITI, 2021
Hormone measurement	VTG level	Japanese Medaka liver	10 weeks	Female adult (F1)	b	4.54 µg.l^{-1}	↑	MITI, 2021
Hormone measurement	VTG level	Japanese Medaka liver	15 weeks	Female adult (F1)	b	0.501 µg.l^{-1}	↓	MITI, 2021
Hormone measurement	VTG level	Zebrafish plasma	21 days	Male adult	b	-	$^{\#}$No change	He et al., 2021
Hormone measurement	VTG level	Japanese Medaka liver	4 weeks	Male adult (F0)	b	-	No change	MITI, 2021
Hormone measurement	VTG level	Japanese Medaka liver	10 weeks	Male adult (F1)	b	-	No change	MITI, 2021
Hormone measurement	VTG level	Japanese Medaka liver	15 weeks	Male adult (F1)	b	-	No change	MITI, 2021
Hormone measurement	VTG level	Zebrafish plasma	21 days	Male adult	b	1 mg.l^{-1}	↑	Liu et al., 2013b
Gene expression	vtg1	Medaka liver	100 days	Female embryo	b	0.13 µg.l^{-1}	↓$^{\#}$	Li et al., 2019b
Gene expression	vtg1	Zebrafish plasma	14 days	Female adult	b	1 mg.l^{-1}	↓	Liu et al., 2012
Gene expression	vtg1	Zebrafish plasma	14 days	Male adult	b	0.04 mg.l^{-1}	↑	Liu et al., 2012
Gene expression	vtg2	Medaka liver	100 days	Female embryo	b	0.36 µg.l^{-1}	↓$^{\#}$	Li et al., 2019b

Gene expression	vtg2	Medaka liver	21 days	Female adult	b	1.72 µg.l^{-1}	↓$^{\#}$	Li et al., 2019b
Gene expression	cyp19a mRNA	Zebrafish plasma	14 days	Female adult	b	1 mg.l^{-1}	↑	Liu et al., 2012
Gene expression	cyp19a mRNA	Zebrafish plasma	14 days	Male adult	b	1 mg.l^{-1}	↑	Liu et al., 2012
Gene expression	ER2b	Zebrafish	5 days	Embryos	b	2 mg.l^{-1}	↑	Liu et al., 2013a
Gene expression	CYP19A	Zebrafish – ovaries	21 days	Female adult	b	1 mg.l^{-1}	↑	Liu et al., 2013b
Gene expression	CYP19A	Zebrafish – ovaries	120 days	Female embryo	b	500 µg.l^{-1}	↑	Liu et al., 2016
Gene expression	CYP19b	Zebrafish – brain	21 days	Female adult	b	0.2 mg.l^{-1}	↑	Liu et al., 2013b
Gene expression	ERα	Zebrafish – brain	120 days	Female embryo	b	500 µg.l^{-1}	↑	Liu et al., 2016
Gene expression	ERα	Zebrafish – brain	21 days	Female adult	b	1 mg.l^{-1}	↑	Liu et al., 2013b
Gene expression	ER2β1	Zebrafish – brain	21 days	Female adult	b	1 mg.l^{-1}	↑	Liu et al., 2013b
Gene expression	CYP19A	Zebrafish – testis	21 days	Male adult	b	1 mg.l^{-1}	↑	Liu et al., 2013b
Gene expression	CYP19B	Zebrafish – brain	21 days	Male adult	b	1 mg.l^{-1}	↑	Liu et al., 2013b
Gene expression	ERα	Zebrafish – brain	21 days	Male adult	b	1 mg.l^{-1}	↑	Liu et al., 2013b
Gene expression	ER2β1	Zebrafish – brain	21 days	Male adult	b	0.2 mg.l^{-1}	↑	Liu et al., 2013b

b: qualitative assessment only, no parameter calculated.
$^{\#}$ No information on mortality
$^{\#\#}$ Mortality > 10% observed, with mortality > 10% also in the control group
MTC not exceeded for the other studies

Plausible link between adverse effects and endocrine activity

The available data indicate that an exposure of TPhP damages fish reproduction by impacting some processes and events during gametogenesis, disrupting fish fecundity (egg production and spawning) and fertility (hatching and fertilisation). Changes in fecundity and fertility are apical effects, which inform about potential adverse effects at the population level.

Although these reproduction parameters are sensitive to substances interfering with the 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.

These adverse effects are related to the disturbances in steroid synthesis and gametogenesis. In fact, all available *in vitro* and *in vivo* studies demonstrate that TPhP exerts an action on the endocrine hormone balance in fish. Depending on the developmental stage, exposure period, reproductive status, species and concentration, antagonist and agonist effects on nuclear and membrane estrogen receptors are observed in organisms as well as on transcription of genes involved in steroidogenesis, leading *in vivo* to perturbations of circulating steroid concentrations. Consistency between endocrine perturbation and adverse effect on reproduction is generally observed in the available studies. The data were analysed separately for males and females as the major steroid hormones in the regulation of fish gametogenesis is the estrogen E2 in females and the androgen 11KT in males.

Reproductive dysfunction in male fish

Based on the available information for males and lines of evidence for adversity and endocrine activity, the following potential sequence of causality linked events at different levels of biological organisation, that potentially lead to an adverse ecotoxicological effect (reproductive dysfunction in fish), is represented in the following figure.

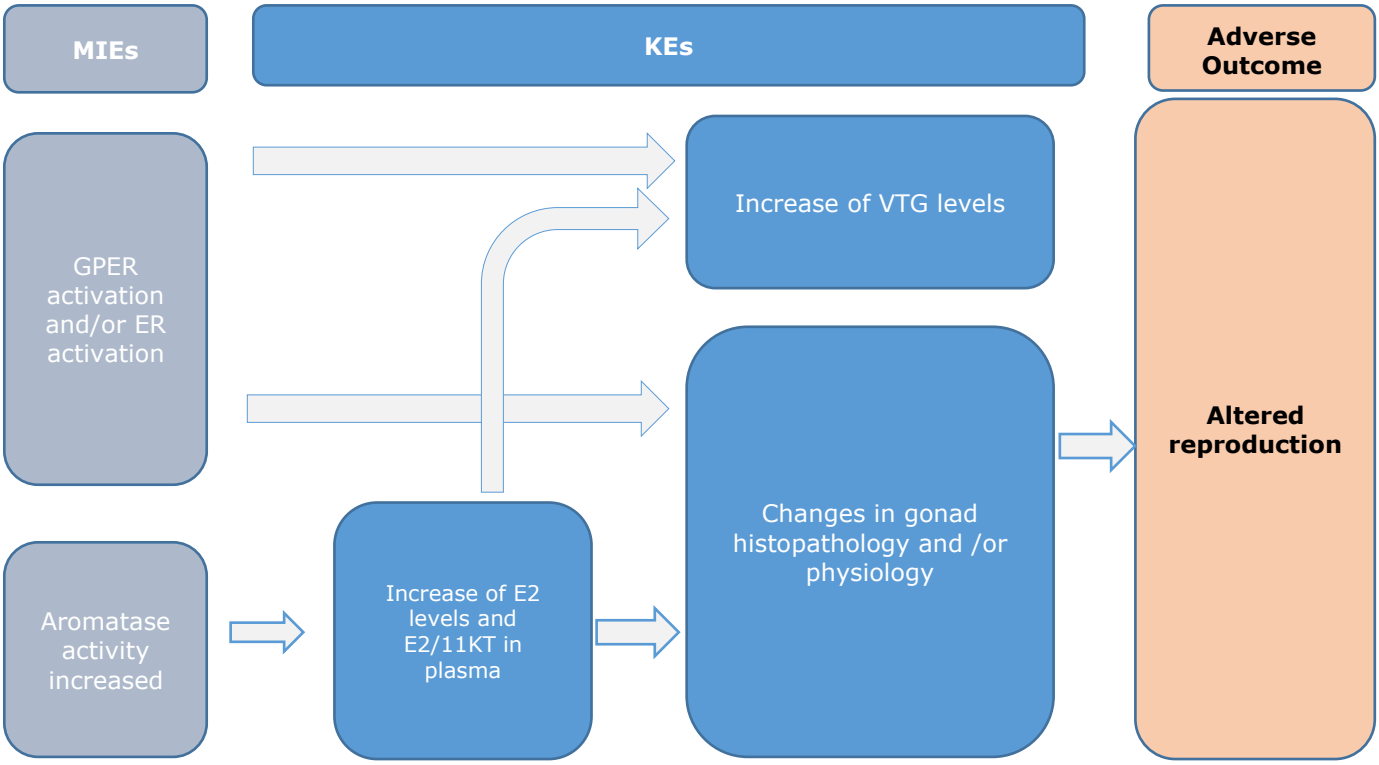


Figure 5: Potential sequences of linked events at different levels of biological organisation that potentially lead to an adverse ecotoxicological effect (reproductive dysfunction in male fish)

The decreased amount of mature spermatocyte cells (He et al., 2021) suggests a potential delay of spermatogenesis following the exposure to TPhP. A disruption (dose-dependent increase) of male-typical reproductive behaviours before spawning (chasing trajectory and time, courtship frequency and mating number) observed in Li et al. (2018) also supports the reproduction impairment of male fish by exposure to TPhP.

CYP19A and CYP19B were significantly upregulated in the testis and the brain respectively (Liu et al., 2012; 2013 b) and could increase conversion of T to E2. Moreover, estrogen receptors (ERα, ER2β1) were up-regulated in the brain (Liu et al., 2013b).

Generally, after TPhP exposure of male fish, plasma E2 increased significantly, plasma 11-KT decreased and E2/11-KT ratio increased (Liu et al., 2012; Liu et al., 2016; Li et al., 2018; Liu et al., 2013b). However, this trend could not be verified in the Unpublished study report (2021), as E2 levels were not measured in males and consequently the E2/11KT ratio could not be calculated. 11-KT plays an important role in the spermatogonial proliferation. A decreased 11-KT level could stop meiosis and germ cell maturation (Schulz et al., 2010). The decrease of 11-KT and the increase in E2 concentrations in plasma revealed by E2/11KT ratio (indicative of estrogenicity) can be associated with reduced semen production and density in male fish. Finally, the increase of the expression in liver of *VTG1* gene (Liu et al., 2012) and the increase of plasma VTG levels in male fish exposed to TPhP (Liu et al., 2013b; Unpublished study report, 2021) are consistent with the observed changes in sex hormones (increased E2 synthesis). These events support the reproduction impairment of male fish observed after exposure to TPhP. The following table highlights these relationships. It has to be noted that the reported concentrations are those for which the measured parameters are significant.

Table 18: Dose-response and temporal concordance between the key events for male fish

[Zebrafish – Japanese Medaka - male] potential dose–response and temporal concordance between the key events and AO							
Dose ($\mu g.l^{-1}$)	Estradiol level	11-KT level	E2/11KT ratio	VTG level	Testis histopathology (abnormalities)	Observed Adverse effect	Reference[###]
0.299[#]	↑ (100 days)					↓ hatching rate	Li et al., 2018
1.43[#]	↑ (100 days)	↓ (100 days)				↑ intersex incidence, abnormal chasing behaviour ↓ fertilisation rate.	Li et al., 2018
4.54						↓ Hatching rate (F1 only, not F2)	MITI, 2021
5	↑ (120 days)	↓ (120 days)	↑ (120 days)				Liu et al., 2016
12	↓ (28 days)	↓ (28 days)				↓ Sperm velocity ↓ Sperm motility ↓ Sperm wobble	Chen et al., 2020

15.2						↓ Hatching rate (F1 only, not F2)	MITI, 2021
33.3[##]		↑ (73 days)*					Unpublished study report, 2021
40				↑ (14 days)***			Liu et al., 2012
44.9						↓ Fertilised eggs**	Kawashima et al., 2021
48.4						↓ egg production (F0 & F1) ↓ fertilised eggs (F0 & F1) ↓ Hatching rate (F1 only, not F2)	MITI, 2021
50		↓ (120 days)	↑ (120 days)				Liu et al., 2016
76.8[##]		↑ (73 days)*			↑ testis maturation stage (73 days)		Unpublished study report, 2021
80[#]					↑ immature spermatocytes (spermatogonia and spermatocyte) ↓ mature spermatocytes (spermatids and spermatozoa)	↓ hatching rate ↓ fertilisation rate	He et al., 2021
120	↓ (28 days)	↓ (28 days)				↓ Sperm velocity ↓ Sperm motility ↓ Sperm wobble	Chen et al., 2020
200				↑ (14 days)***			Liu et al., 2012
200	↑ (21 days		↑ (21 days)			↓ hatching rate	Liu et al., 2013b
500		↓ (120 days)	↑ (120 days)				Liu et al., 2016
1000	↑ (14 days)	↓ (14 days)	↑ (14 days)	↑ (14 days)***			Liu et al., 2012
1000				↑ (21 days)		↓ hatching rate	Liu et al., 2013b

* Unpublished study report (2021), where E2 levels were not measured in males and consequently the E2/11KT ratio could not be calculated.
** However no significant effect on fertility rate (ratio).
*** Transcriptional gene *VTG1.*
[#] No information on mortality
[##] Mortality > 10% observed, with mortality > 10% also in the control group
MTC not exceeded for the other studies
Blank cells: parameter not investigated at this concentration.
###The key events reported in this table are from studies in which changes were identified.

Dose–response and temporal concordance between the key events and AO are often observed at concentration where systemic toxicity was not observed. It cannot be considered as a secondary non-specific consequence of general toxicity.

Reproductive dysfunction in female fish

Impairment of female fish fecundity is also a clear adverse effect observed after TPhP exposure with a decreased number of eggs and spawning events.

In fact, an inhibition of the ovarian development in female fish (He et al. 2021) and alterations of ovaries (Li et al., 2019b; He et al., 2021) were observed. Ovary maturation retardation affected the fecundity of female fish.

TPhP exposure subsequently leads to:
- a decrease in egg production (Liu et al., 2013b; Li et al., 2019b; He et al., 2021; Kawashima et al., 2021; MITI, 2021),
- a decrease in spawning events (Liu et al., 2013b; Li et al., 2018; He et al., 2021).

TPhP induced an increase in circulating E2 concentrations in female zebrafish and female medaka (Liu et al., 2012; Liu et al., 2013b; Liu et al., 2016; Li et al., 2019b; Unpublished study report, 2021). Nevertheless, no change was observed in one study (He et al., 2021). In another study (Li et al., 2019b) carried out on Japanese medaka, a decrease of E2 level was noted after 100-day exposure of female larvae (age of fish at exposure initiation), while an increase of E2 was observed after 21-day exposure of adult females (age of fish at exposure initiation). The authors hypothesised that anti-ER activity would retard ovary development, which would further decrease the E2 level in the long term. To support this hypothesis, the authors showed that the metabolite 4-OH-TPhP (but not TPhP) was able to inhibit E2 activity in the transgenic medaka model.

A perturbation in maturation-inducing steroids could cause impairment in oocyte maturation and quality thereby resulting in a decrease in cumulative fecundity.

VTG, which induces oocyte maturation in fish is synthesised in the liver in response to estradiol stimulation. TPhP induced a down regulation of the expression of *VTG1* and *VTG2* gene in liver (Liu et al., 2012; Li et al., 2019b) and perturbations of VTG concentrations (Liu et al., 2013b; He et al., 2021; Kawashima et al., 2021; Unpublished study report, 2021; MITI, 2021), associated with a concurrent perturbation of E2. Thus, TPhP activates the estrogenic pathway and/or perturbs the steroidogenic pathway, which can explain the synthesis perturbation of E2 and VTG after exposure to TPhP. Effects seem dependent on the developmental stage, exposure period, reproductive status, species and concentration.

The following table reinforces the hypothesis that TPhP disturbs steroid synthesis leading to impairment in oocyte maturation and subsequently in egg production and spawning by emphasising the consistency of the effects observed in each study. It has to be noted that all the measurements were performed at the adult stage and the reported concentrations are those for which the measured parameters are significant.

Table 19: Dose-response and temporal concordance between the key events and AO for female fish

[Zebrafish – Japanese Medaka - female] potential dose–response and temporal concordance between the key events and AO					
Dose (µg/L)	Estradiol level	VTG level	Gonad histopathology impairment	Observed Adverse effect	Reference[###]
0.131[#]		↓ (100 days)**	↓ number of mature oocyte (100 days)		Li et al., 2019b
0.299[#]				↓ hatching rate	Li et al., 2018
0.363[#]		↓ (100 days)***	↓ number of mature oocyte (100 days)	↓ egg production	Li et al., 2019b
0.501		↓ (15 week, F1)			MITI, 2021
1.43[#]				↓ fertilisation rate	Li et al., 2018
1.62		↓ (15 week, F1)			MITI, 2021
1.77[#]	↑ (21 days) ↓ (100 days)	↓ (21 days)***	↓ number of mature oocyte (100 days)	↓ egg production	Li et al., 2019b

4.54		↑ (10 week, F1) ↓ (15 week, F1)		↓ hatching rate (F1 only, not F2)	MITI, 2021
5	↑ (120 days)				Liu et al., 2016
7.19		↓ (21 days)			Kawashima et al., 2021
15.2		↑ (10 week, F1) ↓ (15 week, F1)		↓ hatching rate (F1 only, not F2)	MITI, 2021
17		↓ (21 days)			Kawashima et al., 2021
33.3##	↑ (73 days)				Unpublished study report, 2021
44.9		↓ (21 days)		↓ egg production ↓ fertilised eggs*	Kawashima et al., 2021
48.4		↑ (10 week, F1) ↓ (15 week, F1)		↓ egg production (F0 and F1) ↓ fertilised eggs (F0 and F1) ↓ hatching rate (F1 only, not F2)	MITI, 2021
76.8##	↑ (73 days)	↑ (73 days)			Unpublished study report, 2021
80#		↓ (21 days)	↓ number of mature oocyte (21 days)	↓ hatching rate ↓ fertilisation rate ↓ cumulative eggs ↓ spawning event	He et al., 2021
200		↑ (21 days)		↓ hatching rate ↓ egg production ↓ spawning event	Liu et al., 2013b
500	↑ (120 days)				Liu et al., 2016
1000	↑ (14 days)	↓ (14 days)**			Liu et al., 2012
1000	↑ (21 days)	↑ (21 days)		↓ hatching rate ↓ egg production ↓ spawning event	Liu et al., 2013b

* However no significant effect on fertility rate (ratio).
** Transcriptional gene *VTG1.*
*** Transcriptional gene *VTG2.*
# No information on mortality
Mortality > 10% observed, with mortality > 10% also in the control group
MTC not exceeded for the other studies
Blank cells: parameter not investigated at this concentration
###The key events reported in this table are from studies in which changes were identified.

Dose–response and temporal concordance between the key events and AO are often observed at concentration where systemic toxicity was not observed. It cannot be considered as a secondary non-specific consequence of general toxicity.

Conclusion

TPhP shows endocrine activity in fish with adverse effects on fecundity, fertility and subsequently to reproduction. These adverse effects can be related to the disturbances

in steroid hormone concentrations and impairment of gametogenesis. The resulting perturbation in maturation-inducing steroids could cause impairment in oocyte and testis maturation. Changes in fecundity and fertility are apical outcomes, which inform about potential adverse effects at the population level. Moreover, it is important to note that several adverse effects on altered reproductive capacity of fish are observed at environmentally relevant levels without concurrent systemic effect.

Overall, based on all available scientific evidence, it can be concluded that TPhP fulfils the WHO/IPCS definition of an endocrine disruptor:
- **It shows population relevant adverse effects on fertility and reproduction in fish.**
- **It has EAS activity as clearly shown both *in vitro* and *in vivo*.**
- **Consistency in the effect and EAS activity observed provides evidence that EAS modalities are plausibly biologically linked to the adverse effects.**

7. Conclusions on the SVHC Properties

CMR assessment

Not relevant for the identification of the substance as SVHC in accordance with Article 57 (a) to (f) of the REACH Regulation.

PBT and vPvB assessment

Not relevant for the identification of the substance as SVHC in accordance with Article 57 (a) to (f) of the REACH Regulation.

Assessment under Article 57(f)

Summary of the data on the intrinsic/hazardous properties (providing scientific evidence of probable serious effects to HH and/or ENV)

The available *in vitro* information demonstrates the capacity of TPhP to produce agonist activity on nuclear estrogen receptors ERα and ERβ of several vertebrate species including rat, mouse, fish, chicken, frog and turtle as evidenced by ER transactivation in reporter cell lines (Liu et al., 2012; Kojima et al., 2013; Zhang et al., 2014; Kojima et al., 2016; Li et al., 2018; Ji et al., 2020; Medvedev et al., 2020; Kawashima et al., 2021; Ji et al., 2022). In addition, TPhP can induce ER-regulated gene expression, and related physiological cell responses (e.g., increased cell proliferation in Zhang et al. (2014); Krivoshiev et al. (2016); Ji et al. (2020)). Two recent studies show that TPhP can also activate GPER (Guan et al., 2022; Ji et al., 2022). The available H295R assays (Liu et al., 2012; Ji et al., 2022) on human adrenal carcinoma cells show that TPhP affects steroidogenesis by increasing estrogen levels (17ß-estradiol) and by increasing expression of genes involved in this pathway like CYP19 and 3β-HSD2. *In vivo* fish studies indicate that CYP19A is significantly up-regulated by exposure to TPhP (Liu et al., 2016). Significant alteration of plasmatic concentrations of E2 and E2/T ratio (specifically in females) and E2/11-KT ratio (specifically in males) can result from this modification in the steroidogenesis pathway. The degree of perturbation of circulating steroid concentrations depends on the fish developmental stage, species and tested concentrations. The observations of VTG concentrations, that are consistent with perturbation of E2 concentrations, suggest an EAS

activity of TPhP in female and male zebrafish, with altered concentration of VTG (Liu et al., 2013b; Unpublished study report, 2021). Therefore, TPhP exerts an effect on the endocrine balance in fish. It has EAS activity as clearly shown both *in vitro* and *in vivo*.

In vivo studies on fish reproduction show an alteration of gametogenesis in both sexes with a disruption of testis (He et al., 2021; Unpublished study report, 2021) and ovary maturation (Li et al., 2019b; He et al., 2021) followed by a decrease in reproductive success in terms of fecundity: decrease in egg production (Li et al., 2019b; Kawashima et al., 2021; MITI 2021), spawning events (He et al., 2021; Liu et al., 2013b), fertility (impaired hatchability in Liu et al. (2013b); Li et al. (2018); He et al., (2021)), and fertilisation rate (Li et al., 2018; He et al., 2021). Therefore, TPhP shows adverse effects on fertility and reproduction in fish, observed in several studies at levels without concurrent systemic effects.

The consistency between the observed adverse effects and EAS activity provides evidence that EAS modalities are plausibly biologically linked to the adverse effects. Depending on the developmental stage, exposure period, reproductive status, species and concentration, antagonist and agonist effects are observed in organisms, leading *in vivo* to perturbations of circulating steroid concentrations in most of the analysed studies.

The effect observed on reproduction in fish (fertility and fecundity) can affect population stability and is considered as an adverse effect relevant at population level.

Based on all available scientific evidence, it can be concluded that TPhP fulfils the WHO/IPCS definition of an endocrine disruptor.

Equivalent level of concern assessment

Environment

Triphenyl phosphate shall be identified as a substance of very high concern in accordance with Article 57(f) of Regulation (EC) 1907/2006 (REACH) because of its endocrine disrupting properties for which there is scientific evidence of probable serious effects to environment, which gives rise to an equivalent level of concern to those substances listed in points (a) to (e) of Article 57 REACH.

In order to assess equivalent level of concern (ELoC), some of the factors identified in ECHA (2012) to evaluate ELoC for sensitisers, which are considered relevant for the present case, are also included in the list below:

- Characteristics of the effects:
    - *Probable adverse environmental effects related to:*
        - *Severity, irreversibility and delayed effects.*
    - *Broad environmental relevance.*
    - *Other factors:*
        - *Wide occurrence and environmental distribution.*
        - *Concern related to co-exposure and combined effects.*
        - *Societal concern.*
        - *Is derivation of 'safe concentration' possible?*

The following description includes the elements relevant to the analysis in an environmental context.

Probable serious ecotoxicological effects

Severity, irreversibility and delayed effects

TPhP induces adverse effects on reproductive organs and fecundity, and affects the reproductive ability of fish. Changes in fecundity and fertility are apical outcomes, which inform about potential adverse effects at the population level, which can lead to serious effects on ecosystems. Moreover, it is important to note that several adverse effects on altered reproductive capacity of fish are observed at environmentally relevant levels without concurrent systemic effect. Exposure during the juvenile period of fish may cause gonad histopathological changes, leading to delayed effects on survival and growth of offspring. There is evidence that a short time exposure may be sufficient to provoke long-term effects.

Broad environmental relevance

Effects on sexual development and reproductive ability *via* endocrine EAS MoA has a broad environmental relevance. As data on only a small proportion of the existing species are available, mainly in fish, potential effects on other organisms remain unknown. Adverse effects are thus not expected to be restricted to certain taxonomic groups or species. As the main endocrine systems are known to be largely conserved among vertebrate species in various environments, it is likely that a wide range of wildlife species with different functions in ecosystems could be affected.

Wide occurrence and environmental distribution

Several literature studies demonstrate that TPhP can be found in the environment and TPhP has a widespread use, which leads to a ubiquitous occurrence. TPhP may enter the environment via emissions from various sources, which is supported by occurrence and monitoring studies (water, sediment, sewage sludge, indoor dust/air). Therefore, environmental species including predators and vulnerable species (Giulivo et al., 2017; Garcia-Garin et al., 2020; Sala et al., 2021; Sala et al., 2022) are exposed to TPhP. As long as emissions of TPhP do not cease, exposure of species from the environment cannot be avoided. In addition, human biomonitoring data demonstrate also the presence of TPhP or its metabolites in human milk (Sundkvist et al., 2010; Kim et al., 2014; Zheng et al., 2021), placenta (Ding et al., 2016; Zhao et al., 2017) and urine (Carignan et al., 2016; Li et al., 2019a) confirming human exposure to this substance from the very early stages of life. Moreover, TPhP has also frequently been detected in indoor house dust samples in Europe, including Belgium and Sweden, and outside Europe, including Japan, Philippines and USA (Kanazawa et al., 2010; Kim et al., 2013; Marklund et al., 2003; Stapleton et al., 2009; Van den Eede et al., 2011). Thus, it can be concluded that TPhP reaches diverse environmental compartments and biota of remote areas including humans.

Concern related to co-exposure and combined effects

TPhP is a constituent or an impurity of several registered substances[16]. Thus, mixture effects, where substances act additively or with synergistic effects, cannot be excluded and this might impact the threshold of toxicity. Besides, environmental occurrence and human biomonitoring data (see section 3.2) show that TPhPis detected in the environment, in environmental species as well as in human fluids together with other organophosphate flame retardants. Typical examples are sewage plant effluents and human fluids where TPhPoccurs jointly with organophosphate flame retardants (Carignan et al., 2016; Ding et al., 2016; Kim et al., 2014; Li et al., 2019; Sundkvist et al., 2010; Ya et al., 2019; Zhao et al., 2016; Zhao et al., 2017; Zheng et al., 2021).

Societal concern

In relation to the environment, the impairment of fertility can be an issue regarding species survival. There is an increasing concern related to the preservation of biodiversity and increasing evidence that it is threatened due to various causes including global warming and excessive pressure due to human activities (Jenssen, 2006). EDCs may also contribute to the pressure on the survival of endangered species (Tubbs and McDonough, 2018). Preserving and restoring ecosystems and biodiversity is one of the key aims of the European Green Deal (European Commission, 2019) that is an integral part of the European Commission's strategy to implement the United Nation's 2030 Agenda and the sustainable development goals[17].

[16] https://echa.europa.eu/documents/10162/c7a3c8b0-3fd3-3a4a-e791-2b4ae6a696d5
[17] https://sustainabledevelopment.un.org/post2015/transformingourworld

Is derivation of 'safe concentration' possible?

Endocrine regulation, which is set up during critical life stages in vertebrates, is a complex feedback process. Any disturbance of this regulation during transient but vulnerable life stages can lead to irreversible effects for the entire lifetime or even in the following generations which will also be dependent on the organism group. Moreover, based on the available ED specific test guidelines, it is difficult to assess the latency of the effects. Therefore, prediction of future effects and derivation of safe concentration for the environment is associated with large uncertainties. Thus, effects may be overlooked, not expressed or equivocal. For TPhP, for example, no data is available for most of the trophic levels, which makes it difficult to derive a safe exposure level in the environment. Another reason may be that low effect concentrations are difficult to determine definitively, as effects may only be observed in certain life stages or time windows. Additionally, seasonal effects may lead to difficulty in predicting the impact on the development of different groups of organisms.

The ELoC elements are summarised in Table 20 below.

Table 20: ELoC summary reporting

	Adverse effects on reproduction	Overall conclusion
Probable serious effects?*	▪ Adverse effects on reproductive organs of fish exposed to TPhP. ▪ Adverse effect on fecundity and reproductive ability in fish exposed to TPhP. ▪ Several adverse effects on altered reproductive capacity of fish observed at environmentally relevant levels without concurrent systemic effect.	YES ▪ Pattern of ED-related effects associated with serious dysfunction in fish (fecundity, reproductive ability, gonad histopathological effects).
▪ Delayed and irreversibility of effects?	▪ Exposure during juvenile period of fish may cause gonad histopathological changes, leading to delayed effects on survival and growth of offspring. ▪ There is evidence that a short time TPhP exposure may be sufficient to provoke long-term effects on survival and growth of offspring. ▪ Irreversible effects during the entire lifetime or even in the following generations are expected following disruption of endocrine regulation during transient but vulnerable life stages.	YES ▪ Exposure during the juvenile period of fish has been shown to have consequences in relation to ED-related effects later in life. ▪ Short time TPhP exposure may be sufficient to provoke long-term effects on survival and growth of offspring. ▪ Due to conservation of the reproductive endocrine system in a wide range of taxa in different ecosystems, other species or taxa can be impacted as well.
▪ Broad environmental relevance	▪ Broad environmental relevance due to the effects observed on sexual development and reproductive ability *via* endocrine EAS MoA. ▪ As data on only a small proportion of the existing species are available, mainly fish, potential effects on other organisms remain unknown. ▪ It is very likely that a wide range of wildlife species with different functions in ecosystems could be affected.	YES ▪ ED MoA with broad environmental relevance.

Wide occurrence and environmental distribution	▪ Widespread use of TPhP, which leads to an ubiquitous occurrence. ▪ Environmental species including predators and vulnerable species (fin whale) can be exposed more or less continuously to TPhP and exposure can thus not be avoided. ▪ Concern supported by occurrence and environmental monitoring data (water, sediment, sewage sludge, indoor dust/air) and human studies demonstrating the presence of TPhP or its metabolites in human milk, placenta and urine.	YES ▪ Wide occurrence and environmental distribution.
Potential to cause combined effects (co-exposure)	▪ TPhP is a constituent or an impurity of several registered substances[18]. ▪ Mixture effects cannot be excluded and may impact the threshold of toxicity. TPhP and organophosphate flame retardant co-exposure have been documented in sewage plant effluents and in human fluids.	YES ▪ Combined exposure cannot be avoided and thus combined effects cannot be ruled out.
Societal concern?	▪ Fertility impairment can be an issue regarding environmental species survival. ▪ Increasing concern about biodiversity preservation. ▪ EDC may also contribute to the challenge of survival of endangered species.	YES ▪ Major societal environmental concerns.
Uncertainties in deriving safe concentration limits	▪ Endocrine regulation, which is set up during critical life stages in vertebrates, is a very complex feedback process. ▪ This complex ED feedback process prevents to predict potential future effects and thus safe exposure levels for the environment. ▪ For TPhP, data is not available for all trophic levels, which makes it difficult to derive a safe exposure level in the environment.	YES ▪ Derivation of safe concentration associated with large uncertainties.

**This factor is intended to discuss the severity of the effects and not their probability*

[18] https://echa.europa.eu/documents/10162/c7a3c8b0-3fd3-3a4a-e791-2b4ae6a696d5

Summary of the ELoC assessment

Triphenylphosphate (TPhP) fulfils the WHO/IPCS definition of an endocrine disruptor relevant for the environment. The very high concern raised by this property is substantiated by the severity and irreversibility of the effects observed on organisms. These effects are relevant at the population level and may have long term consequences. A large variety of species may be adversely affected and there are difficulties to quantify a safe level of exposure with regard to the endocrine mediated effects.

In conclusion, there is scientific evidence that TPhP causes probable serious effects to the environment due to its endocrine disrupting properties, which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of the REACH Regulation.

TPhP is identified as a substance of very high concern in accordance with Article 57(f) of Regulation (EC) 1907/2006 (REACH) because it is a substance with endocrine disrupting properties for which there is scientific evidence of probable serious effects to the environment, which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 REACH.

Conclusion on the Article 57(f) assessment

TPhP is identified as a substance of very high concern in accordance with Article 57(f) of Regulation (EC) 1907/2006 (REACH) because it is a substance with endocrine disrupting properties for which there is scientific evidence of probable serious effects to the environment, which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 REACH.

Endocrine activity

The available *in vitro* information demonstrates the capacity of TPhP to produce agonist activity on nuclear estrogen receptors ERα and ERβ of several vertebrate species including rat, mouse, fish, chicken, frog and turtle as evidenced by ER transactivation in reporter cell lines (Liu et al., 2012; Kojima et al., 2013; Zhang et al., 2014; Kojima et al., 2016; Li et al., 2018; Ji et al., 2020; Medvedev et al., 2020; Kawashima et al., 2021; Ji et al., 2022). In addition, TPhP can induce ER-regulated gene expression, and related physiological cell responses (e.g., increased cell proliferation in Zhang et al., 2014; Krivoshiev et al., 2016; Ji et al., 2020). Two recent studies show that TPhP can also activate GPER (Guan et al., 2022; Ji et al., 2022). The available H295R assays (Liu et al., 2012; Ji et al., 2022) on human adrenal carcinoma cells show that TPhP affects steroidogenesis by increasing estrogen levels (17ß-estradiol) and by increasing expression of genes involved in this pathway like CYP19 and 3β-HSD2. *In vivo* fish studies indicate that CYP19A is significantly up-regulated by exposure to TPhP (Liu et al., 2016). Significant alteration of plasmatic concentrations of E2 and E2/T ratio and E2/11-KT ratio can result from this modification in the steroidogenesis pathway. The degree of perturbation of circulating steroid concentrations depends on the fish developmental stage, species and tested concentrations. The observations of VTG concentrations, that are consistent with perturbation of E2 concentrations, suggest an EAS activity of TPhP in female and male Zebrafish, with altered plasmatic concentration of VTG (Liu et al., 2013b; Unpublished study report, 2021). Therefore, TPhP exerts an effect on the endocrine balance in fish. It has EAS activity as clearly shown both *in vitro* and *in vivo*.

Adverse effects

In vivo studies on fish reproduction show an alteration of gametogenesis in both sex with a disruption of testis (He et al., 2021; Unpublished study report, 2021) and ovary maturation (Li

et al., 2019b; He et al., 2021) followed by a decrease in reproductive success in terms of fecundity : decrease in egg production (Li et al., 2019b; Kawashima et al., 2021; MITI, 2021), spawning events (He et al., 2021; Liu et al., 2013b), fertility (impaired hatchability in Liu et al., 2013b; Li et al., 2018; He et al., 2021), and fertilisation rate (Li et al., 2018; He et al., 2021). Therefore, TPhP shows adverse effects on fertility and reproduction in fish, observed in several studies at levels without concurrent systemic effects.

Plausible link between adverse effects and endocrine activity

The consistency between the observed adverse effects and EAS activity provides evidence that EAS modalities are plausibly biologically linked to the adverse effects. Depending on the developmental stage, exposure period, reproductive status, species and concentration, antagonist and agonist effects are observed in organisms, leading *in vivo* to perturbations of circulating steroid concentrations in most of the analysed studies.

The effect observed on reproduction in fish (fertility and fecundity) can affect population stability and is considered as an adverse effect relevant at population level.

Based on all available scientific evidence, it can be concluded that TPhP fulfils the WHO/IPCS definition of an endocrine disruptor.

Equivalent level of concern

The very high concern raised by this property is substantiated by the severity and irreversibility of the effects on organisms and populations that may have long term consequences, the large variety of species that may be adversely affected and the difficulties to quantify a safe level of exposure with regard to the endocrine mediated effects.

Conclusion

In conclusion, there is scientific evidence that TPhP causes probable serious effects to the environment due to its endocrine disrupting properties, which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) of Article 57 of the REACH Regulation.

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