Helsinki, 10 August 2023

Addressees
Registrants of 1-[4-(1,1-dimethylethyl)phenyl]-3-(4-methoxyphenyl)propane-1,3-dione listed in the last Appendix of this decision.

Registered substance subject to this decision (the Substance)
Substance name: 1-[4-(1,1-dimethylethyl)phenyl]-3-(4-methoxyphenyl)propane-1,3-dione
EC number: 274-581-6
CAS number: 70356-09-1

Decision number: Please refer to the REACH-IT message which delivered this communication (in format SEV-D-XXXXXXXXXX-XX-XX/F)

DECISION ON SUBSTANCE EVALUATION

Under Article 46 of Regulation (EC) No 1907/2006 (REACH), you must submit the information listed below:

1. **Information required to clarify the potential risk related to Endocrine disruption in the environment**

   An amphibian metamorphosis assay (AMA); test method: OECD TG 231, using the Substance and the following specifications:

   - The test material must be representative for the Substance as put on the market, in particular with respect to the concentrations of impurities.
   - For the preparation of the test solutions, you must consider the approaches described in the OECD Guidance Document 23. You must demonstrate that the approach used to prepare test solutions was adequate to maximise the concentration and the stability of the Substance in the test solutions. You must monitor the test concentrations of the Substance throughout the exposure duration and report the results. If it is not possible to demonstrate the stability of exposure concentrations (i.e., measured concentration(s) not within 80-120% of the nominal concentration(s)), you must express the effect concentration based on measured values. In all cases, the selected approach must be justified, documented, and communicated to the MSCA before starting the *in vivo* phase of this request.
   - A dose range-finding test must be performed.
   - At least four concentration levels of the Substance with four replicates must be tested.
   - At day 21, liver histopathology and assessment of the hepatosomatic index must be performed.

Deadlines

The information must be submitted by ***16 February 2026***.

Conditions to comply with the information requested

To comply with this decision, you must submit the information in an updated registration dossier, by the deadlines indicated above. The information must comply with the IUCLID robust study summary format. You must also attach the full study report for the corresponding study/ies in the corresponding endpoint of IUCLID.

You must update the chemical safety report, where relevant, including any changes to classification and labelling, based on the newly generated information.

You will find the justifications for the requests in this decision in the Appendix/ces entitled 'Reasons to request information to clarify the potential risk.'

You will find the procedural steps followed to reach the adopted decision and some technical guidance detailed in further Appendices.

Appeal

This decision may be appealed to the Board of Appeal of ECHA within three months of its notification to you. Please refer to http://echa.europa.eu/regulations/appeals for further information.

Failure to comply

If you do not comply with the information required by this decision by the deadline indicated above, ECHA will notify the enforcement authorities of your Member State.

Authorised[1] under the authority of Mike Rasenberg, Director of Hazard Assessment

[1] As this is an electronic document, it is not physically signed. This communication has been approved according to ECHA's internal decision-approval process.

Basis for substance evaluation

The objective of substance evaluation under REACH is to allow for the generation of further information on substances suspected of posing a risk to human health or the environment ('potential risk').

ECHA has concluded that further information on the Substance is necessary to enable the evaluating Member State Competent Authority (MSCA) to clarify a potential risk and whether regulatory risk management is required to ensure the safe use of the Substance.

The ECHA decision requesting further information is based on the following:

(1) There is a potential risk to human health or the environment, based on a combination of hazard and exposure information.
(2) Information is necessary to clarify the potential risk identified; and
(3) There is a realistic possibility that the information requested would allow improved risk management measures to be taken.

The Appendices entitled 'Reasons to request information' describe why the requested information are necessary and appropriate.

Appendix A – Reasons to request information to clarify the potential risk related to Endocrine disruption in the environment

Introduction/background

The Substance was evaluated in 2015 based on the following concerns: Suspected PBT/vPvB, consumer use, exposure of environment, high (aggregated) tonnage, wide dispersive use. A substance evaluation decision number SEV-D-2114357832-44-01/F was taken on 23rd March 2017 requesting data on i) Aerobic mineralisation in surface water – simulation biodegradation test, EU C.25 (OECD TG 309, "pelagic test"), ii) Aerobic and anaerobic transformation in aquatic sediment systems, EU C.24 (OECD TG 308), iii) Long-term toxicity testing on aquatic invertebrates (test method: Daphnia magna reproduction test, EU C.20./OECD TG 211), iv) Long-term toxicity testing on fish (test method: Fish, early- life stage (FELS) toxicity test, OECD TG 210).

The requested data was submitted in 2021 and assessed by the evaluating MSCA. Based on the available information, the evaluating MSCA considers that the Substance is potentially PBT as the substance is considered to fulfil the P and B criteria; information on T is currently considered inconclusive despite the availability of standard long-term studies on aquatic organisms. During the follow-up of the first substance evaluation, an additional concern for endocrine disrupting properties in the environment was identified based on new information made available in academic literature (See section 1.1). This decision aims to clarify the new concern for endocrine disrupting properties in the environment.

1. Potential risk

1.1 Potential hazard of the Substance in the environment

Following its assessment of the available relevant information on the Substance, the evaluating MSCA and ECHA have identified a potential hazard of endocrine disrupting (ED) properties for the environment, more specifically related to thyroidal and anti-androgenic activity of the Substance, which must be clarified.

According to the amended CLP Regulation (Commission Delegated Regulation (EU) 2023/707 of 19 December 2022), *"An endocrine disruptor is an substance or mixture that alters one or more functions of the endocrine system and consequently causes adverse effects in an intact organism, its progeny, populations or subpopulations".* This also meets the IPCS/WHO (2002) definition of endocrine disruptors.

Based on this definition, the substance is an endocrine disruptor (ED) if all the following conditions are met:

a) it shows endocrine activity, i.e., has the potential to alter one or more functions of the endocrine system.
b) it shows an adverse effect in an intact organism or its offspring or future generations; and
c) there is a biologically plausible link between the endocrine activity and the adverse effect, i.e., there is correlation between an endocrine activity and an adverse effect.

1.1.1 Evidence based on Mammalian data

An uterotrophic assay (immature rats; exposure via diet from post-natal days 21 to 25) testing the Substance revealed no estrogenic activity in the applied doses (421 and 636 mg/kg bw/d), whereas the positive control ethinylestradiol (significant at ≥ 0.342 µg/kg bw/d) induced the expected increase in uterus weight (Schlumpf et al., 2001).

Data of repeated-dose toxicity studies in rats (comparable to OECD TG 408; oral (feed); tested up to 1000 mg/kg bw/d) and rabbits (comparable to OECD TG 410; dermal; tested up to 360 mg/kg bw/d) are available in the registration dossier. Toxicological findings comprise slight liver toxicity in the rat, but no findings indicating endocrine disruptive properties or related adversity are reported in the two available repeated-dose toxicity studies. Thyroid histology was performed in the rat study and no treatment-related changes are reported.

Furthermore, the registration dossier contains a developmental toxicity study in rats (oral (gavage) from gestation day 6 to 17 tested up to 1000 mg/kg bw/d) based on OECD TG 414 with deviations (this investigation included the rearing of a subset of the offspring until weaning). No toxicity in dams or offspring is reported. The study is very limited regarding the ability to detect endocrine disruptive properties of the test substance (e.g., neither histology of organs in dams nor determination of ano-genital distance or nipple retention in offspring were performed).

In conclusion, the available data provide no evidence for endocrine disrupting effects in mammals, although the database is very limited. Beside the negative mammalian *in vivo* data, further *in vitro* and *in vivo* fish data are available which trigger the concern that the Substance might act as an endocrine disrupter in the environment. Thus, the evaluating MSCA does not consider the limited mammalian data available as sufficient to conclude on the absence of the endocrine properties of the Substance.

1.1.2 Evidence based on *in vitro* studies

1.1.2.a Thyroidal activity

(Klopcic and Dolenc, 2017)

In vitro cell-based reporter gene assays were conducted in a study performed by Klopcic and Dolenc (2017) to examine thyroid and anti-thyroid as well as anti-androgen activity of the Substance. The thyroid reporter gene assay used a GH3.TRE-Luc cell line and luciferase activity as readout for receptor activation. DMSO was used as vehicle (<0.5%). The antagonistic thyroid assay was conducted using the same experimental set up but with co-exposure to T3 (0.25 nM). Bisphenol A was chosen as positive control for the antagonistic assay. Cytotoxicity was assessed using a resazurin assay with an exposure duration of 24 h for the GH3.TRE-Luc cells. The study was assigned a Klimisch score of 2 by the evaluating MSCA.

Results:

- Thyroidal activity:
  The Substance was shown to exhibit thyroidal activity in the luciferase reporter gene assay with an EC_{50} value of 1 nM and a maximum induction of 2.19-fold at 10 µM compared to the vehicle control. T3 as positive control had an EC_{50} of 150 nM and showed a maximum induction of 13.28-fold over vehicle control at 100 µM.

- Anti-thyroidal activity:
  Anti-thyroidal activity of the Substance in the used cell line was shown at 10 and 25 µM in a co-exposure treatment of the cells using T3 (0.25 nM) as a natural agonist. Maximum inhibition down to 19% compared to T3-treated cells was observed at 25 µM of the Substance. However, this concentration of the Substance was in the concentration range where cytotoxicity appeared in a co-treatment setting with T3 (see below).

- Cytotoxicity:
  In the GH3.TRE-Luc cells the Substance did not show cytotoxicity up to 10 µM in the resazurin assay. Significant reduction in the metabolic capacity of the cells compared to solely T3-treated cells was observed at 25 µM of the Substance when co-exposed to 0.25 nM T3 (no information at 10 µM of the Substance and co-exposure to T3 was given).

In your comments on the draft decision, you considered the results of the study performed by Klopcic and Dolenc (2017) as not plausible and unreliable (including by using ToxRTool) based on the following arguments:

- The reported EC_{50} for T3 activity, used as a positive control, was too high in comparison to T3 activity values in reference studies (Freitas *et al*. 2011 and 2014).

- You regarded the thyroid results of Klopcic and Dolenc (2017) as not plausible, thereby referring to three 'general' chemicals including the Substance, that have higher potency than the positive control T3 itself.

- The thyroid antagonistic assay could be considered unreliable due to the lack of responsiveness to T3 in the agonistic assay and results are confounded by cytotoxicity.

- The concentration of DMSO in the agonistic and antagonistic thyroid assays had a concentration of <0.5% v/v and not 0.1% v/v, as prescribed in OECD TG 455 and 458.

ECHA notes that T3 activity in the agonistic assay performed by Klopcic and Dolenc (2017) is lower compared to the cited reference studies. This might be because the specific cells used by Klopcic and Dolenc (2017) are less sensitive than the cells used by Freitas 2011 and 2014. However, this does not disqualify the whole assay as unreliable as the expected activity of T3 is observed.

With respect to the relative potency of T3 compared to the three test chemicals ECHA notes that T3 shows a higher EC_{50} value (0.16 µM) than the Substance (0.001 µM), 2MR (2-methylresorcinol) (0.03 µM) and BHA (Butylated hydroxyanisole) (0.0001 µM) but exhibits a much higher induction compared to the three test chemicals. Thus, compared to T3 the test chemicals are less potent to induce TR responses, which is plausible. The lower calculated EC_{50} values for the test chemicals might be due to specific binding to the TR that does not lead to full receptor activation but shows an interference with the receptor protein. Hence, these results cannot be claimed to be unplausible and do not render the whole assay as unreliable.

Regarding the reliability and plausibility of the antagonistic set-up ECHA considers that there is T3 responsiveness in the agonistic assay, and that the activity of the positive control used in the antagonistic set-up, the known thyroid antagonist BPA was in a similar range as reported by Freitas et al. 2011. This shows that the antagonistic assay set up used in the study by Klopcic and Dolenc is adequate to show antagonistic effects and hence shows the reliability of the assay with respect to this endpoint. Figure 3E of the publication shows that inhibition by the Substance starts at 10 µM and is significant at 25 µM. At 10 µM, about 35 % inhibition was seen (read and calculated from the concentration-response curve). At 25 µM of the Substance, inhibition was about 81 %. However, at 25 µM of the Substance, slight cytotoxicity was seen in GH3.TRE-Luc cells co-exposed to T3 (0.25 nM), whereas at 10 µM of the Substance, no information on cytotoxicity is available (see also below).

ECHA notes that the assay performed by Klopcic and Dolenc (2017) was not performed

according to OECD TG 455 and 458. Additionally, the solvent concentration is not a validity criterion. Finally, the study authors stated that cytotoxicity was not observed at DMSO concentrations < 0.5% (v/v).

US EPA screening data, ToxCast™ assays

- You highlighted various thyroid assays from the US EPA screening data, ToxCast™ assays (Friedmann et al. 2017) that were negative (i.e., thyrotropin releasing hormone receptor reporter gene assay, thyroid stimulating hormone receptor reporter gene assay and a target background cAMP generation in cells lacking TSHR), and a thyroid receptor α and β reporter gene assay, that was positive at a concentration above concentrations where cytotoxicity appeared.

  ECHA and the evaluating MSCA considered these results. The assays are high-throughput screening assays with limited conclusiveness. However, these negative assays cannot override the indications obtained from the studies described in this decision and a conclusion that the Substance has no endocrine activity cannot be drawn solely from the set of the CompTox data.

1.1.2.b Androgenic and anti-androgenic activity

(Klopcic and Dolenc, 2017)
For the anti-androgen reporter gene assay an MDA-kb2 cell line expressing endogenous androgen receptors (AR) as well as glucocorticoid receptor proteins (GR) and containing a stably transfected plasmid with a luciferase reporter construct was used. As vehicle, DMSO was used (0.1 % in medium). To measure the anti-androgenic activity of substances the cells were co-treated with 0.5 nM dihydrotestosterone (DHT). Inhibition of luciferase was measured and compared to the vehicle control of DHT (0.5 nM). Known androgen antagonist flutamide was used as positive control.

Furthermore, competitive AR binding was investigated using the PolarScreen AR Competitor Assay (Invitrogen). After 2 h incubation with the Substance, changes in fluorescence polarization compared to the control was measured. DHT was used as positive control.

The viability of the MDA-kb2 cells after 24 h was tested using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay, MTS.

- Anti-androgen activity

The androgen inhibitory activity of the Substance was measured in the reporter gene assay by co-exposure of MDA-kb2 cells to the Substance and 0.5 nM DHT as a natural agonist. The Substance showed a maximum inhibition at 0.01 µM with a reduction in luciferase induction to 0.83-fold compared to the control. The positive control flutamide showed a maximum inhibition at 10 µM with a reduction to 0.65-fold induction compared to the DHT control. The assay is assessed with Klimisch 2.

- Cytotoxicity:

The Substance did not show cytotoxicity to MDA-kb2 cells (used for the anti-androgen assay) up to 1 µM.

- AR binding potential:

The analysis of the PolarScreen AR Competitor Assay data showed that the Substance can competitively bind to the AR protein in this assay. An IC_{50} of 1.2 µM was calculated for the Substance. DHT, which was used as a reference substance, yielded an IC_{50} of 19.5 nM in the same experimental set up.

(Schreurs *et al*., 2005)
Reporter gene assays were conducted to assess the interaction of the Substance and other substances with the estrogen-, androgen- and progesterone-receptor proteins for the agonistic and antagonistic mode of action.

To assess androgenic and anti-androgenic activity a CALUX assay was conducted using U2-OS cells. A DHT concentration of 0.1 nM (EC_{50}) was used for the measurement of anti-androgenicity. For the anti-androgenic assay concentrations of the Substance ranged from 0.1 to 10 µM.

The study was assigned a Klimisch score of 2 by the evaluating MSCA.

Results:
The Substance exerted no androgenic effect. There was a slight anti-androgenic effect with an IC_{50} of 11 µM (extrapolation outside the tested dose range, as the highest concentration of 10 µM showed an inhibition of slightly less than 50%). The positive controls for inhibition of hAR were flutamide and vinclozolin with IC_{50} values of 0.5 and 0.1 µM, respectively.

(Ma *et al*., 2003)
Androgen and anti-androgen reporter gene assays with the Substance were conducted using cell line MDA-kb2. As solvent for the Substance, ethanol was used (1% in medium). DHT (10 nM) was used as positive control for the agonistic assay.
In the antagonistic assay, the cells were co-exposed to 0.1 or 0.5 nM DHT. Flutamide and bicalutamide served as positive controls for the anti-androgenic assay.

The study was assigned a Klimisch score of 2 by the evaluating MSCA.

Results:
There was no androgen agonistic activity of the Substance. The Substance was also inactive in the anti-androgenic assay up to 10 µM and co-exposure to 0.5 nM DHT. The positive controls flutamide and bicalutamide had IC_{50} values of 3.62 and 0.083 µM respectively by co-exposure to 0.5 nM DHT in the anti-androgenic assay.

In the outcome of your ToxRTool evaluation (Annex 1 to the comments), you dispute the anti-androgenic activity of the Substance observed in Klopcic and Dolenc, 2017 and Schreurs et al., 2005 based on the following:

- The effects of the Substance are observed in the presence of cytotoxicity or are of low quality/lack plausibility. Furthermore, you state that the acceptability range for the positive control flutamide is not reported.

  To ECHA it is unclear what is meant by "low quality" and the "acceptability range for the positive control." There are two *in vitro* studies showing anti-androgenic activity of the Substance (Klopcic and Dolenc, 2017 and Schreurs *et al*. 2005) and one study which was negative regarding anti-androgenic activity (Ma *et al*. 2003). Even if it remains unclear why the positive control flutamide is significantly less sensitive than the Substance in the study performed by Klopcic and Dolenc (2017), the effects of the Substance cannot be disregarded. It might be that the Substance is much more active and the concern for an anti-androgenic activity remains. Klopcic and Dolenc (2017) examined cytotoxicity. The authors stated that there was no cytotoxicity up to 1 µM to MDA-kb2 cells with coexposure to 0.5 nM DHT. Antiandrogen activity of the Substance was seen below this concentration (IC_{50} is 0.02 nM). The cause for the stepwise-looking curve in Klopcic and Dolenc (2017) is most probably a very steep dose-response curve, which would be more obvious using another fitting algorithm. Regarding acceptability range for the positive control flutamide, there was no

cytotoxicity seen. Ma et al. (Ma et al., 2003) determined an IC_{50} of 3.62 µM for flutamide with coexposure to 0.5 nM DHT in a reporter gene assay with the same cell line as used by Klopcic and Dolenc (2017). Since this value is in the same range as the IC_{50} value of 4.57 µM determined by Klopcic and Dolenc (2017) it is confirmed that the assay was properly conducted, and the results are reliable.

Further, the result from Klopcic and Dolenc (2017) is supported by the result from Schreurs *et al*. (2005), who conducted an AR CALUX bioassay and observed anti-androgenicity of the Substance. The extrapolated IC_{50} was slightly above the highest measured test concentration of 10 µM (IC_{50}: 11 µM). However, cytotoxicity was not assessed and reported.

- In your comments you note that the competitive binding in the PolarScreen™ assay by Klopcic and Dolenc (2017) is not reliable (Klimisch score 3) due to an incorrect product number given in the publication, a too short incubation duration of 2 h, and an inconsistency between the concentration response curve reported in Figure 5 and the specification of the IC_{50} of DHT. Therefore, you conclude that the assay was not performed correctly.

  ECHA considers that the incorrect product number of the PolarScreen AR competitor assay is not a valid reason to invalidate the test as it is most probably a copy-paste-error from the authors, as the same number was noted for the GR and AR assays.

  The PolarScreen™ Nuclear Receptor Competitor Assay user guide[2] specifies as incubation time for the Nuclear Receptor Competitor Assay at least 2 h, which was the study duration used by Klopcic and Dolenc (2017). It is an instruction manual for several nuclear receptors (AR, ER, GR, PR) and is therefore rather unspecific. Moreover, it seems to be older, since a product number is specified that is not more produced[3]. ECHA agrees that the instruction manual (PolarScreen™ AR Competitor Assay, Green)[4] that you cited, specified at least 4h incubation time. Nevertheless, the instruction manuals are not contradicting, as the Nuclear Receptor Competitor Assay user guide stated that the duration time is at least 2 h, and not absolutely. ECHA does not agree that a study using 2 h incubation is less reliable compared to a 4h incubation, since even with the shorter incubation time, a competitive binding of the Substance to the AR was seen, which may have been even more pronounced with a prolonged incubation time. Following a careful analysis of the curves in the graph depicting binding to the androgen receptor (Fig. 5, Klopcic, and Dolenc, 2017), the derived values for DHT and the Substance are probably correct. The curved graph fits the DHT data, whereas the graph that shows a steep drop are values obtained with the Substance. The drop of the graph marked with triangles near log 0 µM fits to the IC_{50} of 1.2 µM for the Substance, whereas the curved graph marked with asterisks fits to the IC_{50} of circa 2E-8 M for DHT (exact IC_{50} = 19.47 nM). Consequently, ECHA does not consider the performed PolarScreen assay to be unreliable or of low relevance.

  Regarding the US-EPA CompTox Dashboard screening data that you cited and detailed as Annex 4 to your comments, ECHA notes that one result from the screening assays (MDA-kb2 cells with coexposure of 0.5 nM R1881) is also positive for anti-androgen activity, albeit at a concentration where cytotoxicity was observed. There is another

[2] https://assets.thermofisher.com/TFS-Assets/LSG/manuals/polarscreen_nr_competitor_assay_universal_man.pdf
[3] https://www.thermofisher.com/order/catalog/product/A15897
[4] https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FPolarScreen_AR_Competitor_Assay_Green_PI.pdf

anti-androgen assay with the MDA-kb2 cell line with co-exposure to 10 nM DHT, which is negative. For the androgen antagonistic assay with HEK 293T cells the coexposure is not defined.

1.1.2.c Estrogenic and anti-estrogenic activity

(Schlumpf *et al.*, 2001)
The Substance was tested using the E-screen with MCF-7 cells. The cells were exposed to the Substance at concentrations from 0.1 to 100 µM. Estradiol (E2) at the concentrations 0.1 pM to 0.01 µM served as positive control. The stock solutions were prepared using ethanol. The final concentration of ethanol in the medium were between 1.0 and 0.001 %. The study was assigned a Klimisch score of 2 by the evaluating MSCA.

Result: The Substance did not cause significant cell proliferation compared to the control and hence did not show estrogenic effects in this assay. The positive control E2 showed significant cell proliferation at 1 pM up to 10 nM.

(Schreurs *et al.*, 2002)
Reporter gene assays were conducted to determine estrogenic and anti-estrogenic activity of the Substance by Schreurs et al. 2002 using stable hERα and hERβ transfectants of HEK293 cells. Ethanol was used as solvent. Exposure of the cells lasted for 24 h. E2 at 10 nM served as positive control. Anti-estrogenic activity was tested by co-exposure to a submaximal concentration of E2 (5 pM for ERα and 50 pM for ERβ).
The study was assigned a Klimisch score of 2 by the evaluating MSCA.

Results from tests with the Substance at concentrations of 0.1 µM to 100 µM are discussed. Significantly increased estrogenic activity was seen at 10 and 100 µM on the hERα, and at 100 µM on the hERβ. Anti-estrogenic activity was only seen at 0.1 and 1 µM on hERα with very slightly but significantly decreased activity to approx. 80% compared to E2 (100%). No anti-estrogenic effect was seen at 10 and 100 µM on hERα, and on hERβ at 0.1 to 100 µM.

(Schreurs *et al.*, 2005)
Reporter gene assays were conducted to assess the interaction of the Substance and other substances with the estrogen-, androgen- and progesterone-receptor proteins for the agonistic and antagonistic mode of action.

Estrogenic and anti-estrogenic activity was tested using 293HEK cells. Anti-estrogenicity was tested by co-exposure to E2 of 3 and 100 pM for hERα and hERβ, respectively.

The study was assigned a Klimisch score of 2 by the evaluating MSCA.

Results:
The estrogenic activation of hERα by the Substance reached its plateau level at 37% (no EC_{50} was estimated) compared to the E2 control. No estrogenic effect on hERβ was observed. As positive control E2 was used (EC_{50} for hERα 2.1 pM, for hERβ 83 pM). As it was not mentioned that the Substance exerts an anti-estrogenic effect, it is concluded that it was not anti-estrogenic in this study.

1.1.2.d Summary of *in vitro* effects:
The Substance showed thyroidal agonistic activity in a reporter gene assay with an EC_{50} of 1 nM (Klopcic and Dolenc, 2017) and antagonistic activity (however at the high concentration of 25 µM cytotoxicity was observed, whereas at the concentration 10 µM also antagonistic activity was seen, but no information regarding cytotoxicity was given).

The thyroid in vitro assays by Klopcic and Dolenc (2017) have some deficiencies (see above), but altogether the quality of the assays is sufficient to give indication for thyroid activity of the Substance.

Weak AR-mediated antagonistic effects were shown by two studies (Klopcic and Dolenc, 2017 and Schreurs *et al*., 2005), while the study performed by Ma *et al*. (2003) indicated no anti-androgenic activity of the Substance. Androgenic effects were found in none of the studies investigating this endpoint.

With regard to estrogenic activity, two studies (Schreurs *et al*., 2002), (Schreurs *et al*., 2005) report on weak ER agonism while one study (Schreurs *et al*., 2002) describes a weak anti-estrogenic activity of the Substance. The E-screen assay performed by Schlumpf *et al*. (2001) did not show estrogenic effects of the Substance.

1.1.3 *in vivo* evidence related to the Thyroid modality in fish

(Ka and Ji, 2022)
The study investigated the effect of the Substance on wild-type (AB strain) and thrαa−/− (thyroid hormone receptor alpha a knockout fish) zebrafish embryos exposed for 120 h in 96 well culture plates. 30 embryos in each of three replicates were used per concentration. The endpoints mortality, embryo coagulation, hatching, malformation, length, and weight were examined. The exposure began within 2 h post fertilisation and lasted for 120 h. The concentrations tested were 0.3, 1, 3, 10, 30 µM (93.1, 310.4, 931, 3104, 9310 µg/L) nominal with a control and solvent control (DMSO, 0.01 % in the stock solution). The effects in treatments were compared to solvent control only. The temperature was 26±1 °C.

For thyroid hormone measurements, only wild-type zebrafish embryos were exposed in three replicate groups of 250 embryos each to the Substance for 120 h. 150 larvae per replicate were collected after the exposure was terminated. The validity criteria of OECD TG 236 (Fish Embryo Acute Toxicity (FET) Test) for hatchability in the control (80%) was met. The validity criteria of the FET for survival in the control is also met, however the tests are not comparable, as FET has a duration of 96 h, whereas here the exposure duration was 120 h.

The study was assigned a Klimisch score of 2 by the evaluating MSCA.

Results:

- Biological effects in wild-type embryos (data on thrαa knockout fish are not considered due to uncertainties raised by your comments to the DD and the independent review by Duis and Coors (2022) (Annex 3 to your comments). In this decision only effects on wild-type zebrafish observed in the Ka and Ji (2022) study were used as a basis for the potential endocrine disruption hazard, as there were several shortcomings connected with the transgenic zebrafish):

As described by the authors (Ka and Ji, 2022), the embryo coagulation was slightly above 10% at 3 µM. Hatchability reflects the results for embryo coagulation. At 3 µM, the hatchability was slightly below 90%. Larvae survival reflects both endpoints above. At 3 µM, the survival was roughly 85%. Survival in the control was about 92% (values read from graph). Embryo coagulation, hatchability and larvae survival were significantly decreased at 3 µM and higher concentrations, showing dose-dependency.

The time to hatch was significantly increased at the highest concentration tested, i.e., 30 µM. There was no effect on larvae length, but the larvae weight was significantly decreased at 10 and 30 µM. The malformation rate increased significantly at 10 and 30 µM.

The above effects may reflect thyroidal and/or systemic effects.

- T3/T4 hormone content at 120 hpf (hours post fetch):
  - The T3 level was significantly increased at 30 µM and a dose-dependency existed.
  - The T4 content was significantly decreased at 10 and 30 µM, also here a dose-dependency beginning at 3 µM was observed.
  - The ratio of T3/T4 (normalized to the solvent control embryo) was significantly increased at 30 µM with a dose-dependent increase seen beginning at 0.3 µM.
  - The effect values for T4 and T3 were at concentrations where also systemic toxicity was seen. It is unclear whether the effects were mediated by thyroidal activity of the Substance or caused by systemic toxicity. This uncertainty will be clarified by the information requested in the present decision.

- Gene expression at 120 hpf:
  - TRH (thyreotropin releasing hormone) was significantly upregulated at 1 µM and higher, dose-dependency was observed.
  - TSHβ (thyroid stimulating hormone beta) was also dose-dependently upregulated, upregulation was significant at 30 µM. It is expressed in the thyrotropic cells of the pituitary, and it is a biomarker for changes on the HPT axis.
  - TSHR (thyroid stimulating hormone receptor) was significantly upregulated at 10 and 30 µM.
  - TRαa (thyroid receptor alpha a) was significantly downregulated at 3, 10 and 30 µM; TRβ (thyroid receptor beta) was significantly downregulated at 10 and 30 µM.
  - DEIO2 (thyronine deiodinase 2) was significantly upregulated at 30 µM. The thyroid receptors and DEIO2 are important components of the thyroidal action, changes of them may indicate endocrine effects in any tissue. TG (thyroglobulin) was significantly upregulated at 30 µM. TG is specifically expressed in the thyroid. It is upregulated by TSH, therefore fits the parallel upregulation of TG to the upregulation of TSHβ.
  - NIS (sodium/iodide symporter) and DEIO1 gene expression: no effects.
  - TPO (thrombopoietin; not thyroid peroxidase, as named in the publication, because the NCBI Accession Number is from thrombopoietin) was significantly downregulated at 30 µM. Hence, this endpoint is irrelevant for assessing thyroid activity of the Substance.
  - The effect values were partly significant at concentrations where also systemic toxicity was seen.

- Antioxidant enzyme activities:
  - SOD (superoxide dismutase) activity was significantly increased at 12 h and 144 h. CAT (catalase) activity was significantly increased at 72 h and 144 h. After recovery CAT activity returned to normal level, but SOD activity was still significantly higher.

In your comments on the draft decision (also independently assessed by Duis and Coors, 2022, Annex 3), you concluded that the results of Ka and Ji (2022) are neither relevant nor reliable for hazard/risk assessment purposes, based on the following:

- You commented that the IPCS/WHO (2002) definition is used to define an endocrine disruptor (ED) substance. The quality and consistency of the data, and evidence of

confounding effects of excessive toxicity (i.e., mortality, cytotoxicity) should be considered when assessing the evidence for endocrine effects.

Regarding this also see the ECHA response to your comment: 'Effects were reported at concentrations causing systemic toxicity' below. In short there are some main reasons why the study nevertheless can be used:
- The study is used to illustrate the concern for ED properties which necessitates further information for clarification, not to conclude on ED properties of the Substance. This must be considered when assessing the quality of the study.
- The study is a short-term study which is unsensitive due to short term design, and not very specific to detect thyroid substances. Therefore, the concentrations where thyroid effects appear are near to concentrations with systemic toxicity or even overlapping.
- The validity criteria of OECD 236 are valid for a 96-hour exposure, but not for 120-h exposure with additional suboptimal exposure conditions like small cavities.
- The thyroidal effects seen give a picture of correlations, connected by the thyroidal control circuit that shows that the organisms tried to retain normal thyroid hormone levels (see also our response to correlation analysis of effects (Spearman correlation) below).

- The solvent concentration of ≤100 µL/L could not increase the maximum achievable dissolved concentrations of poorly water-soluble substances and you referred to Weymann, *et al*. (2012) and OECD (2019).

  ECHA notes that the recommendation to use a solvent at 100 µL/L for poorly soluble substances is included in the OECD guidelines. With respect to Ka and Ji (2022) it is assumed that the Substance was at least partly dissolved with the help of the solvent. The authors did not note the appearance of undissolved test material. Furthermore, even if the Substance was not fully dissolved in this study, effects are observed suggesting that the Substance was taken up by the animals and that effect concentrations might be even lower than calculated.

- Fewer fish were subjected to analysis for the treatments showing high mortality and thereby rendering observed effects unreliable.

  ECHA agrees that at concentrations where mortality appeared fewer fish were examined. Mortality shows that endocrine sensitive effects observed and identified as statistically significant, even with a lower number of fish also lowering the statistical power in these groups, might be overlayed with systemic toxicity or a non-specific consequence of this systemic toxicity. To clarify this and hence to finally conclude whether the Substance fulfills the WHO/IPCS criteria or not, a long-term endocrine sensitive test is needed as requested in this decision.

- Regarding correlation analysis of effects (Spearman correlation) you consider that the conclusion by Ka and Ji (that the thyroid disruption activity of the Substance induced decreased larval weight and delayed hatching) and the selected concentrations, were not current state of science and did not meet the current guidance for ED evaluation.

  ECHA agrees that the study data do not allow for the conclusion that the observed adverse effects are mediated via a thyroid mode of action. However, a connection between delayed hatching and decreased larval weight to thyroid activity of the Substance cannot be excluded since thyroid disruption can affect development and growth. To conclude on thyroid activity and related adverse effects of the Substance

a long-term amphibian study is necessary. Regarding the noted high concentrations, these are nominal concentrations, and the real measured concentrations are expected to be lower, keeping in mind the high adsorption potential of the Substance to e.g., the test vessel.

Regarding the observed thyroid effects and their relation to the overall effects observed in the study, ECHA notes that:

- The T4 level decreased in a dose-response related manner from 3 to 30 µM, being significant at 10 and 30 µM.
- The authors stated that significant decrease of T4 level with upregulation of trh, tshβ, and tshr genes indicates feedback in the hypothalamus and pituitary gland to maintain hormonal homeostasis. Further, they assume that the Substance may affect trh and tsh directly or indirectly by negative feedback responses to produce more T4.
- Ka and Ji (2022) further assumed that the upregulation of the deiodinase type II gene in larvae is connected to a low T4 level since T4 is converted to T3 by deiodinase type I and II through outer ring deiodination. The T3 hormone level was dose-dependently increased and significantly elevated at 30 µM, which fit to the picture of a feedback regulation trying to compensate for the decrease in T4. Whether the decrease in T4 is mediated via an endocrine mode of action or is secondary to unspecific systemic toxicity must be investigated with the requested long-term amphibian study.

With respect to the overall effects observed in the study, ECHA notes that effects on thyroidal endpoints were partly seen at concentrations where systemic toxicity appeared. This may be due to the low sensitivity of this short-term test to thyroid specific effects that need longer time frames to transform into significant adverse effects. Therefore, the concentrations where thyroid effects were seen are rather high and hence presumably overlapping with systemic toxicity.

- OECD test guidelines for mechanistic toxicity studies stipulate that tests should be conducted in the absence of systemic toxicity. You referred to a citation from the RADAR assay (OECD TG 251) "*The maximum test concentration should be set by the solubility limit of the test chemical in the test medium, the MTC, or the maximum concentration inducing more than 10% combined mortality and/ or malformations in eleutheroembryos, or a maximum concentration of 100 mg/L, whichever is lowest*". You further noted that the RADAR assay is a mechanistic study similar to the study described by Ka and Ji (2022) but focusing on the AR rather than the thyroid. You further noted that endocrine effects observed at concentrations higher than the maximum tolerated concentration shall generally not be considered indicative of endocrine disruption.

  ECHA notes that the RADAR assay utilises transgenic Japanese medaka and is therefore considered very sensitive. Hence, specific endocrine activities can be observed at comparably lower concentrations than in a less sensitive assay like this by Ka and Ji. In this decision only effects on wild-type zebrafish observed by Ka and Ji were used as basis for the potential endocrine disruption hazard, as there were several shortcomings connected with the thrαa knockout zebrafish.

  However, the effects on the HPT axis seen by Ka and Ji (2022) cannot be neglected even when there is an overlay of systemic toxicity. As stated above, to clarify whether the effects are thyroid mediated or caused by systemic toxicity, a thyroid sensitive long-term test is necessary and requested in this decision. ECHA further notes that for regulatory purposes, testing up to systemic toxicity (i.e., recording a full dose-

response curve) is necessary to clearly discriminate ED specific effects and systemic toxicity as well as to cover e.g., long-term NOEC or LOEC values for further regulatory measures.

- Regarding the correlation analysis made by Ka and Ji (2022), you stated that "*the conclusion that changes in hormone levels and gene expression led to effects at the apical level […] is not supported by the presented data.*" You further referred to Dang et al (2012) and OECD (2018) and stated that thyroid sensitive endpoints could be affected by general toxicity. Further, that effects apart from the expression were seen at concentrations causing significant effects on survival and that these effects are not suitable to derive conclusion on endocrine disruption.

  ECHA did not draw conclusions on apical effects from the study by Ka and Ji (2022). However, it is stated that effects on HPT axis related endpoints (e.g. T3 and T4 levels as well as specific gene expression) are affected and that it cannot be excluded that apical endpoints will be adversely affected via this interference with the HPT axis and hence this potential hazard must be clarified in a long-term and thyroid sensitive test as requested in this decision.

- The study has fundamental shortcomings regarding the experimental setup, reporting and analysis of results.

  ECHA agrees that the study has some shortcomings (the single points are discussed further below), nevertheless the study gives indications for a possible thyroid activity of the Substance that fit to the *in vitro* effects observed. The available data from the discussed *in vitro* and *in vivo* studies are judged to be of sufficient relevance and reliability to indicate a potential hazard that must be further investigated. ECHA agrees that the data available are not sufficient to conclude on the potential ED properties of the Substance, but the data also cannot be used to conclude that there is no potential hazard regarding endocrine disrupting properties of the Substance in the environment.

- An independent review of the study by Duis and Coor (2022) explains why the study is neither relevant nor reliable for hazard/risk assessment, based on the following:
  - No details regarding the source, health, or origin of the parental fish were reported. It is unknown if the parental zebrafish were from a reliable source, of an optimal age for spawning, were in good health, or if the fish had been acclimated to husbandry environmental conditions for a minimum of 14 days prior to the collection of embryos.

    ECHA notes that the zebrafish were defined as wild type (AB strain) by the authors. As one of the authors is professor at the [redacted] and has experience with zebrafish studies, it is assumed that they obtained the fish from an institute or trustworthy fish breeder or from the university itself. Furthermore, ECHA notes that "*This study was approved by the Institutional Animal Care and Use Committee of* [redacted]." It was stated by Ka and Ji (2022) that only healthy zebrafish embryos were selected and the control survival in the test was in a valid range, therefore it is assumed that the fish were healthy.

  - A lack of reported details on the number of females from which embryos were collected and that, to avoid genetic bias, eggs should be collected from a minimum of three breeding groups, mixed and randomly selected (OECD TG 234, 2013). The overall fertilisation rate of the collected eggs was also not reported to determine the health status of the eggs.

ECHA considers that eggs from a minimum of three breeding groups were taken since a single female spawn at least 50 to 80 eggs per day (see OECD 236, p. 15), and for the study 3 replicate groups of 250 eggs per concentration (for 5 concentrations + control + solvent control) were needed. Although the fertilisation rate was not assessed, ECHA does not consider this a valid reason for a lower reliability and relevance with respect to the thyroid endpoints measured.

- No details on the exposure media or parameters (only temperature and photoperiod) were reported. No information was reported on the dissolved oxygen concentration or pH to determine if the conditions were suitable for the culture of zebrafish. You also noted that dissolved oxygen concentrations are typically one of the validity criteria in fish studies (e.g., OECD TG 236).

  ECHA notes that the control larvae did not show signs of elevated toxicity, and therefore it is assumed that the oxygen content and pH were suitable for culture of the fish. Moreover, thyroid endpoints would not be impacted by these parameters, and it is not uncommon that this information is not available in a study from a publication.

- No details were provided on the preliminary range-finding test with regards to the concentrations tested and toxicity observed to determine whether the concentration range was appropriate. This is important since acute toxicity effects were observed in the definitive studies.

  ECHA has addressed the issue of systemic toxicity and specific ED effects was discussed above. Furthermore, it cannot be concluded that the thyroid specific effects observed are solely a secondary consequence of systemic toxicity and hence further long-term testing is requested to clarify this issue.

- No information on preparation of test solutions (beyond use of a solvent, dimethyl sulfonate at 0.01%), or test substance renewal (study run as static, semi static system). This is particularly important because the test solutions were above water solubility limits. There is no information on whether the test solutions were clear or whether there was evidence for undissolved test material. If the system was also run as a static exposure, without the dissolved oxygen concentrations being measured it is not possible to determine if these concentrations decreased over the study resulting in a negative effect on the larvae.

  ECHA notes that it is assumed that the exposure regime was static, as it was conducted in 96 well plates. This is suitable, as it was a short-term exposure of 120 h, and the Substance is not a biodegradable substance. Moreover, in the control no enhanced toxicity was seen, hence it is assumed that physical effects and suffering from decreased oxygen content did not occur. Therefore, the information provided is considered sufficient, keeping also in mind that the study was from a publication.

- An analytical confirmation of the actual exposure concentrations of the zebrafish was not conducted to determine if nominal concentrations were achieved and concentrations were stable over the exposure duration.

  ECHA considers that the real test concentrations decreased with increasing test duration and hence were lower than the nominal test concentrations. This is mostly the case with poorly soluble substances. The decrease of the concentration

in the medium may also be related to photodegradation or adsorption to vessel or organic matter. However, as the aim of the study was not to provide effect concentrations the measurement of test concentrations was not necessary in this special case.

- No dilution water control data was reported to compare with the solvent control, so a solvent effect cannot be discounted.

  ECHA considers that, although results were only reported for the solvent control, the comparison of treatments to the solvent control is more important as a comparison to only the water control would be. Moreover, as the study is not used to identify the Substance as an ED and only indications from the study are derived, this shortage can be accepted, and the study is useable.

- Larvae were pooled for wet weight, hormone measurements and changes in gene expression. Changes in wet weight, hormone measurements and gene expression could not be related to individual fish. An "n" of three for the pooled data was also considered insufficient to be statistically robust.

  ECHA notes that the gene expression was examined using ten larvae with three replicates to measure changes of gene transcription. For hormone measurements "*three replicate groups of 250 wild-type embryos per concentration group were exposed to the test substance for 120 h, and 150 larvae per each replicate were collected after the exposure was terminated. Homogenized larvae samples were used for hormone measurement.*". For hormone and gene expression, examination of four replicates would have been better and for gene expression a higher number of larvae. In this case, the results could have had less deviations and hence were perhaps significant at lower concentrations. However, the lower number of replicates/larvae is no reason for assigning Klimisch 3, also having in mind that there is no guidance available for those examinations. For length and weight "*body length (ten larvae per replicate, n=30) and wet weight (ten larvae pooling per replicate, n=3) were measured.*" Measurement of growth (weight and length) is not an endpoint in OECD TG 236 and therefore there is no guidance about the necessary number of fish larvae or replicates for such a test. Also here, a higher number than 3 replicates would have been beneficial, in order to have a more robust statistical calculation. However, this disadvantage is no reason for lower reliability.

- The assumptions for the statistical methods were not checked (i.e., normality and homogeneity of variance) to determine if the data met these parameters and therefore the appropriate statistical methods were chosen.

  ECHA notes that: "*For the endpoints observed at the organism, hormonal, and genetic level from wild-type zebrafish embryo/larvae and the significance of differences between solvent control and treatment groups was assessed by one-way analysis of variance using SPSS software (version 27, [redacted]). Correlation between various endpoints were assessed using Spearman correlation analysis from the [redacted]).* ". ECHA considers that the statistical evaluation is sufficient to see indications for thyroidal activity.

- Mistakes in the reporting (for example it is unclear whether expression of the gene for thyroid peroxidase or thrombopoietin was measured) which reduces confidence in the reporting of other endpoints.

ECHA has amended the decision to clarify this point, as the accession number for TPO was wrong. It was the number for thrombopoietin and not for thyroid peroxidase, a mistake with the abbreviation TPO. The other accession numbers were checked and confirmed. Therefore, the other endpoints are considered as reliable.

- Tested concentrations were greater than the water solubility for the Substance, the tested concentration range was also considerably higher than the reported environmental concentrations, by a factor of over 1000.

  ECHA agrees that the nominal concentrations were much higher than the water solubility of the Substance. However, it is assumed that the real concentrations were lower than the nominal concentrations. Moreover, this test only gives indications for thyroid action and therefore serves to justify the concern for ED properties rather than concluding on the ED status of the Substance.

  The tested concentrations were higher than the reported concentrations in the environment, however there might be very sensitive environmental animals and exposure durations in the environment are much longer, perhaps life-long. Hence, for endocrine substances no safe effect level can be estimated. Moreover, there are a lot of other endocrine acting substances in the environment, therefore mixture effects are always possible. In addition, hydrophobic substances may be adsorbed to suspended matter in the water (Fagervold et al., 2019). The Substance in Fagervold et al. had a high experimentally examined solubility of 19 mg/L in fresh water and of 47 mg/L in sea water, the authors stated that this was caused by adsorption to very small, suspended particles (<1.6µm) and most likely do not represent the freely dissolved fraction of the Substance.

- Effects were reported at concentrations causing systemic toxicity.

  ECHA refers to the argumentation regarding the correlation analysis of effects above and response regarding MTC (maximum tolerated concentration) above. Moreover, the test was only a short-term test, hence not really sensitive. Therefore, the concentrations were rather high, where thyroid effects were seen at all, and are presumably near to concentrations causing systemic toxicity. ECHA also notes that the high concentrations are nominal concentrations.

  Regarding the comparison of effects to validity criteria of OECD 236 (the exposure duration in OECD 236 is 96 h and in this study 120 h), ECHA notes the following:

  Hatchability: The hatching rate at 3 µM (circa 88%, all values read from graph) was higher than the validity criteria of OECD 236, and at 10 µM hatchability was only very slightly lower compared to the validity criteria (circa 78%). The validity criteria in OECD TG 236 for hatching rate is 80%. Compared to control, the values at 3, 10 and 30 µM were significantly decreased, however at 3 µM the difference to the control is very small.

  Survival: At 3 µM in wild-type embryos survival (circa 85%, all values read from graph) was not much lower compared to the validity criteria for the control of OECD TG 236 (validity criteria for survival: 90% after 96 h exposure). The value for survival at 10 µM was around 73 % and hence effects at this concentration are used with care. Compared to control, the values for survival at 3, 10 and 30 µM were significantly decreased, albeit at 3 µM the difference to the control is very small. However, regarding survival, the exposure duration was 120 h in the

test, and not 96 h as specified in OECD TG 236, and other test conditions were not optimal, e.g., the small volume of test cavities. Hence there were some stressors to the larvae, and lower survival could be expected in this study and the validity criteria for survival from OECD TG 236 cannot entirely be assigned to the study here, keeping also in mind that this study is from a publication. Moreover, this test is a unsensitive short-term test, with a presumably short gap between concentrations showing endocrine effects and systemic toxicity. In a more specific sensitive long term test lower concentrations can be used and cause endocrine specific response below the level of systemic toxicity.

1.1.4. Further in vivo fish studies available for the Substance

OECD TG 210, Fish early-life stage toxicity test (registration dossier, 2021):
The assay was conducted as limit test according to OECD TG 210 with *Danio rerio* using a flow-through design. The nominal concentration tested of the Substance was 6 µg/L. The solvent *N,N*-dimethylformamide (DMF) was used with a final concentration in the test medium of 50 µL/L. The mean measured concentrations of BMDM were in the range of 55 to 77% of the nominal value. The arithmetic mean measured concentration of the Substance in the test solution was 4 µg/L.

Effects:
No effects on hatching success, survival, body length and weight were observed. The NOEC is ≥4 µg/L. There were no visible abnormalities observed; all fish were healthy and showed normal behaviour during the test. The validity criteria of OECD TG 210 were fulfilled.

The study is assigned a Klimisch score of 1, however the authors of the study stated that it was not possible to solve a higher concentration than 4 µg/L (measured) of the Substance in the test medium. In the registration dossier a water solubility of 27 µg/L is reported. The OECD Guidance Document No. 23 recommends a maximum concentration for the solvent of 100 µL/L. It is unclear why in this study the final solvent concentration in the test medium was only 50 µL/L.

In your comments on the draft decision, you confirmed that you made efforts to maximise the amount of the Substance in solution. Up to this concentration no effects were seen. ECHA takes note of this.

1.1.5 Discussion on the available *in vitro* and *in vivo* studies:

***In vitro*:**
There were effects seen on thyroidal/antithyroidal and anti-androgenic activity in (Klopcic and Dolenc, 2017) and anti-androgenic activity in (Schreurs et al., 2005). (Ma et al., 2003) did not observe anti-androgenic and androgenic activity. Schreurs *et al*. (2005) did not observe androgenic activity. (Schreurs et al., 2005) and (Schreurs et al., 2002) observed weak estrogenicity, but no estrogenicity was seen in (Schlumpf et al., 2001). In conclusion, weak anti-androgenic and thyroidal and anti-thyroidal activity was observed for the Substance.

***In vivo*:**
The data on T3 and T4 level as well as the observed changes in the thyroid related gene expression pattern in the study by Ka and Ji (2022) show that the Substance can interfere with the hypothalamic-pituitary-thyroid (HPT) axis in fish and feedback mechanisms are activated to compensate for this interference. This mode of action fits to the observed thyroidal activity of the Substance in the available *in vitro* study.

Walter *et al*. (2019) described the normal level of thyroid hormones (TH) in developing zebrafish from 6 hpf to 120 hpf. At 24 h the concentration of T3 was the highest of the thyroid hormones measured, reflecting the high maternal contribution of T3 in eggs. Then T3 decreased over the first five days of development. In comparison, the T4 concentration in embryos at 24 hpf was low and increased approximately 10-fold by 72 hpf and 50-fold by 120 hpf. Walter *et al*. further explained that the increase of T4 is the result of the development of thyroid follicles that begin to produce T4 at around 48 to 72 hpf.

In contrast, in Ka and Ji (2022), zebrafish embryos exposed to the Substance (at 120 hpf) show a dose-dependent increase of the T3 concentration and a dose-dependent decrease of the concentration of T4 in comparison to unexposed embryos. This could be due to an impairment of thyroid follicles leading to T4 deficiency.

T3 and T4 are essential in promoting embryonic development and growth (Walter *et al*., 2019). Therefore, it might be possible that the effects on weight, embryo coagulation, hatching and survival relate to the effects of the Substance on the thyroid hormone level.

Several genes relevant for thyroid signaling were up- or down-regulated, e.g.:
- The thyreotropin releasing hormone (trh, sign. from 1 µM), the thyroid stimulating hormone beta (tshβ, sign. from 30 µM) and the thyroid stimulating hormone receptor (tshr, sign. from 10 µM) were dose-dependently upregulated.
  Ka and Ji stated that "Significant decrease of T4 level with upregulation of trh, tshβ, and tshr genes indicates feedback in the hypothalamus and pituitary gland to maintain hormonal homeostasis." Further they assume that the Substance "may affect trh and tsh directly or indirectly by negative feedback responses to produce more T4."
- The thyroid stimulating hormone beta (tshβ) which was significantly up regulated at 30 µM (high standard deviation at lower concentrations) is expressed in the thyrotropic cells of the pituitary, and therefore a biomarker for changes on the HPT axis.
- Thyroglobulin (tg), specifically expressed in the thyroid, was significantly upregulated at 30 µM. It is upregulated by TSH, therefore fits the parallel upregulation of tg to the upregulation of tshβ.
- Both thyroid hormone receptors alpha and beta (Trαa and TRβ) were significantly downregulated, TRαa from 3 and TRβ from 10 µM.
- DEIO2 (thyronine deiodinase 2) was significantly upregulated at 30 µM. Ka and Ji noted that the upregulation of the deiodinase type II gene in larvae is connected to a low T4 level since T4 is converted to T3 by deiodinase type I and II through outer ring deiodination; this may further contribute to the observed significant increase in the T3/T4 ratio.

As these genes are important components of the thyroidal action, changes in their expression indicate endocrine thyroidal effects. The dose-dependent and distinct changes in these thyroidal relevant genes indicate effects on the feedback mechanism of the hypothalamus, pituitary gland, and the thyroid to maintain hormonal homoeostasis.
As explained above, the effects on thyroidal endpoints which already appeared in the range of systemic toxicity might be due to the short-term test design with low sensitivity.

Having also considered the additional information provided in your comments on the draft decision, ECHA and the evaluating MSCA disagree with your conclusion that there is no convincing *in vitro* or *in vivo* evidence for the potential hazard of the Substance. The observed *in vivo* effects on gene expression and changes in T3/T4 levels in zebrafish embryos raise a concern that the Substance can act as an endocrine disruptor in the environment via interference with the HPT axis in vertebrates. The thyroidal and antithyroidal *in vitro* effects provide further indication for thyroidal activity of the Substance. Furthermore, the available *in vitro* data point to a possible anti-androgenic

activity of the Substance. However, given the *in vivo* evidence in fish, the thyroidal effects of the Substance will be followed-up first within this decision to conclude on the endocrine disrupting properties of the Substance.

In conclusion, the available and current information is not sufficient to conclude on the potential hazard. The available relevant data for the Substance demonstrate endocrine activity of the Substance but do not allow to conclude on adversity and population relevance. Hence, further information is needed on endocrine disrupting properties for the environment.

1.2 Potential exposure

According to the information you submitted in all chemical safety reports and registration dossiers, the aggregated tonnage of the Substance manufactured or imported in the EU is in the range of 1,000 – 10,000 tonnes per year.

Furthermore, you reported that the Substance is used as an UV filter in cosmetics and personal care products.

Due to the use in cosmetics, the Substance can enter the aquatic compartment via wastewater or direct discharges. Therefore, exposure to the environment cannot be excluded and can be even demonstrated via the following literature data. Several studies detected the Substance in surface water bodies ((Poiger et al., 2004); (Remberger, 2011); (Vila et al., 2016); (Labille *et al*., 2020); (Sánchez Rodríguez et al., 2015), in waste water treatment sludge ((Rodil et al., 2009); (Tsui et al., 2014)) and in sediments (Kaiser *et al*., 2012a; Kaiser *et al*., 2012b), (Tsui et al., 2015)). Concentrations found usually were in the ng/L range and once even in the µg/L range (Vila *et al*., 2016). Fagervold et al. found the Substance with a much higher content in the water surface microlayer (in an oily film on the surface of a lake) than in general surface water (14.7 ng/L in surface water, 531 ng/L in the water surface microlayer)), (Fagervold et al., 2019). The presence of the Substance well above the solubility limit reported by Fagervold et al. (2019) indicates that it is dissolved via binding to small particulate matter which is relevant for filter feeders.

1.3 Identification of the potential risk to be clarified

Based on information from the published literature, there is sufficient evidence to justify that the Substance may be an endocrine disruptor in the environment.

The information you provided on manufacture and uses demonstrates a potential for exposure of the environment.

Based on this hazard and exposure information the substance poses a potential risk to the environment.

As explained in Section 1.1 above, the available information is not sufficient to conclude on the potential hazard of endocrine disruption in the environment. Consequently, further data is needed to clarify the potential risk related to endocrine disrupting properties.

1.4 Further risk management measures

If the endocrine disrupting properties in the environment of the Substance are confirmed, the evaluating MSCA will analyse the options to manage the risk(s). New regulatory risk management measures could be identification as substance of very high concern as an endocrine disrupter to the environment under Article 57(f) of REACH and authorisation or restrictions of the use of the Substance based on the environmental endocrine disrupting

properties. These regulatory measures would result in stricter risk management measures, such as improved measures at manufacturing sites, better waste management and revised instructions on safe use, if appropriate.

ECHA notes that the Commission Delegated Regulation (EU) 2023/707 has introduced new hazard classes for endocrine disruptors about human health and the environment to the CLP Regulation 1272/2008. Therefore, harmonised classification as an endocrine disruptor for the environment is an additional regulatory risk management measure which could be taken for the Substance.

2. How to clarify the potential risk

2.1 Amphibian metamorphosis assay (OECD TG 231) including liver histopathology and assessment of the hepatosomatic index

a) Aim of the study

As detailed in Section 1.1, information is required to conclude on the potential ED properties of the Substance in the environment. The available *in vitro* and fish *in vivo* data indicate that the thyroid system is a target of an endocrine activity of the Substance.

The requested amphibian metamorphosis assay (AMA, (OECD, 2009)) will provide basic mechanistic information on the interaction of the Substance with the thyroid system of vertebrates.

As further detailed in Section 2.1.c, the requested study is concluded to be the most appropriate assay, since it yields data which will be essential to further clarify the environmental ED concern. Furthermore, if adverse effects on metamorphosis are observed, they may be used to conclude on population relevance and hence whether the Substance fulfils the WHO/IPCS definition of an ED in the environment as well as the newly established criteria under the CLP framework for EDs in the environment based on thyroid disrupting properties without the need for an additional *in vivo* assay. The WHO/IPCS definition is the basis for a classification as ED according to the new hazard classes for ED introduced to CLP.

The AMA is recognised as a critical assay of the OECD Conceptual Framework (CF) (OECD, 2018) because amphibian metamorphosis provides a well-studied, thyroid-dependent process which responds to substances active within the HPT axis (OECD TG 231, 2009). The EFSA/ECHA guidance on Endocrine Disruptors (ECHA et al., 2018) states that "*in the case of amphibians, changes in thyroid histopathology should be considered adverse at the population level only when observed together with effects on development (i.e. delay or acceleration). This is due to the fact that thyroid histopathology often represents compensation to thyroid insufficiency (Marty et al., 2017). Nevertheless, changes in development in amphibians, even if observed in the absence of investigation of thyroid histopathology, are considered population relevant effects. However, the degree of delay or acceleration in the development that can be considered adverse at population level is uncertain (Marty et al., 2017)".*

The evaluating MSCA considers the AMA as more than a screening test based on the potentially observed effects on metamorphic development which can be regarded to be of population relevance unless available information demonstrates the contrary. Thus, the requested AMA study can potentially be conclusive with respect to the ED properties of the Substance for the environment.

In case the effects on metamorphosis remain inconclusive regarding population relevance,

but the mechanistic data obtained from the requested AMA strengthen the concern for environmental ED properties by showing interference of the Substance with the HPT axis, this concern may need to be followed up by further testing, e.g., by a Larval Amphibian Growth and Development Assay (LAGDA) according to OECD TG 241.

The need for further information to clarify the remaining concern will be considered during the evaluating MSCA's follow-up evaluation of the information requested in the present decision. Any subsequent requests for information to clarify the concern will be made in a new draft decision after the follow-up evaluation is completed.

b) Specification of the requested study

Test material and concentration

The test material must be representative for the Substance as put on the market, in particular with respect to the concentrations of impurities.

For the preparation of the test solutions, you must consider the approaches described in the OECD Guidance Document 23. You must demonstrate that the approach used to prepare test solutions was adequate to maximise the concentration and the stability of the Substance in the test solutions. You must monitor the test concentrations of the Substance throughout the exposure duration and report the results. If it is not possible to demonstrate the stability of exposure concentrations (i.e., measured concentration(s) not within 80-120% of the nominal concentration(s)), you must express the effect concentration based on measured values. In all cases, the selected approach must be justified and documented. You must contact the evaluating MSCA before starting the in vivo assay. A dose range-finding test must be performed.

A dose range-finding test must be performed to reduce technical challenges and increase the robustness and quality of the data obtained in the main study. The range finding test shall be used to determine the test concentration setting for the main test. The highest test concentration must be set at a concentration where a systemic toxic effect occurs. At least four concentration levels with four replicates must be tested in the main study to obtain a full dose-response relationship to derive a sound LOEC/NOEC and to distinguish ED mediated effects from systemic toxicity.

Route of exposure/ solvent

The Substance must be dissolved in the test media and the animals are exposed via media.
Further specifications

In addition to thyroid histopathology (already required as a standard investigation in OECD TG 231), the liver histology at day 21 (study termination) must be investigated in the randomly chosen tadpoles for thyroid histopathology (5 tadpoles per replicate tank).

Tissue collection, fixation and analysis must be performed as explained in OECD series on testing and assessment No. 228: Guidance Document on Histopathology Techniques and Evaluation (OECD TG 2015) for the LAGDA. The assessment of treatment groups achieving developmental stage 60 and above is covered in Annex 3 of OECD TG 231 and must be performed accordingly.

Histopathology of the liver and assessment of the hepatosomatic index must be included in the test protocol to enhance the conclusiveness of the requested study, i.e., to clarify whether possible effects on the HPT axis in amphibians are mediated via an endocrine mode of action or should be seen as an indirect consequence of possible liver toxicity.

To address the missing information identified above, the OECD TG 231 study will allow to identify information on adverse thyroidal effects, which are required to conclude on the endocrine disrupting properties, and to confirm whether the observed thyroidal mode of action is a potential risk posed by the Substance.

Request for the full study report

You must submit the full study report which includes:

- a complete rationale of test design and
- interpretation of the results
- access to all information available in the full study report, such as implemented method, raw data collected, interpretations and calculations, consideration of uncertainties, argumentation, etc.

This will enable the evaluating MSCA to fully and independently assess all the information provided, including the statistical analysis, and to efficiently clarify the potential hazard for the Endocrine disruption of the Substance.

In your comments on the draft decision, you propose the following specifications to ensure that the requested study is conducted using the most appropriate study design for the test item:

- A lower amount of solvent should be used (20 µL/L) or another method to bring the Substance in solution, including saturator columns, with the method selection confirmed based on preliminary solubility trials and results from the range finder.

  ECHA considers that the maximum solvent concentration recommended in the test guideline must be used to maximise the dissolved fraction of the Substance.

- The final number of test concentrations, with a minimum of three concentrations, should be based on the range finding test, as specified in OECD TG 231. You further disagree with the request that the highest test concentration must be set at a concentration where systemic toxicity occurs, thereby referring to OECD TG 231.

  ECHA considers that for regulatory purposes at least four test concentrations in the final test are necessary to derive a full dose response curve and to distinguish between endocrine effects and systemic toxicity.

ECHA and the evaluating MSCA consider that the requested study design is the most appropriate for obtaining the information necessary to clarify the potential hazard of the Substance.

c) Alternative approaches and how the request is appropriate to meet its objective

The request is:

- appropriate, because it will provide information to further clarify whether the Substance shows endocrine activity and related adverse effects in the environment via an interaction with the thyroid system. This will enable the evaluating MSCA to either conclude on potential ED properties regarding a thyroidal mode of action of the Substance or to decide whether and which further testing may be necessary to conclude regarding environmental ED effects.

- the least onerous measure because there is no equally suitable alternative method available to obtain the information that would clarify the potential hazard. Possible

alternatives would be a Xenopus Eleutheroembryonic Thyroid Assay (XETA, OECD TG 248) assay or a level 4 test of the OECD Conceptual Framework (CF) (OECD, 2018) such as a LAGDA (OECD TG 241).

The XETA is an aquatic screening test and may provide some mechanistic information. However, the available data clearly point to an interaction of the Substance with the HPT axis, but the underlying mode of action is currently unclear.

In accordance with the ECHA/EFSA guidance, in this case an AMA (OECD TG 231) is more appropriate since it covers a broader range of pathways and endpoints. Additionally, the AMA can provide more sound information on adverse effects on metamorphosis and hence reduces the likeliness of follow-up testing, which must be done in case of a positive XETA to conclude on the ED properties.

A LAGDA assay could also clarify the proposed thyroidal activity of the Substance. However, the LAGDA test would require more vertebrate animals and would be more resource intensive. A LAGDA assay can be a suitable follow-up test if the requested AMA study remains inconclusive and/or provides hints that adverse effects on metamorphosis might be observed at the metamorphic climax state that cannot be covered by the AMA design. Level 3 testing of the OECD CF with fish, e.g. according to Fish Short-Term Reproduction Assay (OECD TG 229) or the 21-day fish assay (OECD TG 230) and higher tier fish testing like a Fish Sexual Development Test (OECD TG 234) or the Medaka Extended One Generation Reproduction Test (OECD TG 240) are concluded to be less appropriate at this stage compared to an amphibian study since the available data point to thyroidal activity of the Substance. Validated thyroidal endpoints are not yet covered by the available OECD fish test guidelines.

Furthermore, with respect to the concern raised, there is no other experimental study available at this stage that will generate the necessary information and does not require the testing of vertebrate animals.

In your comments on the draft decision, you conclude that further testing of the Substance in amphibians is not justified and is inconsistent with ECHA's commitments under REACH to minimise animal testing and undertake studies with animals only as a last resort.

ECHA and the evaluating MSCA disagree with your conclusion for the following reasons:
(1) There is sufficient evidence to justify that the Substance may be an endocrine disruptor in the environment. There is a potential risk to the environment, based on a combination of hazard and exposure information.
(2) The available information is not sufficient to conclude on the potential hazard. Consequently, further data is necessary to clarify the potential risk related to endocrine disrupting properties.
(3) There is a realistic possibility that the information requested would allow improved risk management measures to be taken.

Consequently, the request is necessary, and is the most suitable, and least onerous measure with respect to testing of vertebrate animals.

2.2 References relevant to the requests (which are not included in the registration dossier)

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Appendix B: Procedure

This decision does not imply that the information you submitted in your registration dossier(s) are in compliance with the REACH requirements. ECHA may still initiate a compliance check on your dossiers.

12-month evaluation

Due to initial grounds of concern for PBT/vPvB and for exposure of the environment, the Member State Committee agreed to include the Substance in the Community rolling action plan (CoRAP) to be evaluated in 2015. Germany is the competent authority ('the evaluating MSCA') appointed to carry out the evaluation.

In accordance with Article 45(4) of REACH, the evaluating MSCA carried out its evaluation based on the information in the registration dossier(s) you submitted on the Substance subsequent to a decision dated 23 March 2017 and on other relevant and available information.

In the course of the 'follow up' evaluation, the evaluating MSCA identified additional concerns for the potential risk related to endocrine disruption.

The evaluating MSCA completed its 'follow up' evaluation considering that further information is required to clarify the following concerns: endocrine disruption.

Therefore, it submitted a draft decision (Article 46(1) of REACH) to ECHA.

Decision-making

ECHA notified you of the draft decision and invited you to provide comments.

(i.) Registrant(s) commenting phase

ECHA received your comments and forwarded them to the evaluating MSCA.

The evaluating MSCA took your comments into account. The request was not amended but the deadline was amended.

Amendment of the deadline:

ECHA has exceptionally extended the standard deadline by 12 months to consider currently longer lead times in contract research organisations.

(ii.) Notification to MSCAs

The evaluating MSCA notified the draft decision to the competent authorities of the other Member States and ECHA for proposal(s) for amendment.

As no amendments were proposed, ECHA took the decision according to Articles 52(2) and 51(3) of REACH.

(iii.) Follow-up evaluation

After the deadline set in this decision has passed, the evaluating MSCA will review the information you will have submitted and will evaluate whether further information is still needed to clarify the potential risk, according to Article 46(3) of REACH. Therefore, a subsequent evaluation of the Substance may still be initiated after the present substance evaluation is concluded.

Appendix C: Technical Guidance to follow when conducting new tests for REACH purposes

Test methods, GLP requirements and reporting

Under Article 13(3) of REACH, all new data generated as a result of this decision must be conducted according to the test methods laid down in a European Commission Regulation or to international test methods recognised by the Commission or ECHA as being appropriate.

Under Article 13(4) of REACH, ecotoxicological and toxicological tests and analyses must be carried out according to the GLP principles (Directive 2004/10/EC) or other international standards recognised by the Commission or ECHA.

Under Article 10(a)(vi) and (vii) of REACH, all new data generated as a result of this decision must be reported as study summaries, or as robust study summaries, if required under Annex I of REACH. See ECHA Practical Guide on How to report robust study summaries[5].

Test material

Before generating new data, you must agree within the joint submission on the chemical composition of the material to be tested (Test Material) which must be relevant for all the registrants of the Substance.

1. *Selection of the Test material(s)*

   The Test Material used to generate the new data must be selected taking into account the following:
   - the variation in compositions reported by all members of the joint submission,
   - the boundary composition(s) of the Substance,
   - the impact of each constituent/ impurity on the test results for the endpoint to be assessed. For example, if a constituent/ impurity of the Substance is known to have an impact on (eco)toxicity, the selected Test Material must contain that constituent/ impurity.

2. *Information on the Test Material needed in the updated dossier*

   a) You must report the composition of the Test Material selected for each study, under the 'Test material information' section, for each respective endpoint study record in IUCLID.
   b) The reported composition must include all constituents of each Test Material and their concentration values.

This information is needed to assess whether the Test Material is relevant for the Substance and whether it is suitable for use by all members of the joint submission.

Technical instructions on how to report the above is available in the manual "How to prepare registration and PPORD dossiers"[6].

[5] https://echa.europa.eu/practical-guides
[6] https://echa.europa.eu/manuals