Helsinki, 28 June 2022

Addressees
Registrant(s) of Octocrilene listed in the last Appendix of this decision

Registered substance subject to this decision (the Substance)
Substance name: Octocrilene
EC number: 228-250-8
CAS number: 6197-30-4

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:

A. Information required to clarify the potential risk related to Endocrine disruption

1. The Larval Amphibian Growth and Development Assay (LAGDA, test method: OECD TG 241, 2015) with the Substance, specified as follows:
   - Concentrations of the Substance must be monitored at least weekly or twice a week (if instability of the substance is proven), for at least one replicate in each treatment group, rotating between replicates of the same treatment group to ensure stable exposure conditions and avoid decrease of concentrations. Concentrations must be expressed as measured and nominal concentrations;
   - When relevant, data on assay performances, quality criteria and validations (limits of detection, quantifications, coefficient of variations, specificity) must be reported.

Deadlines

The information must be submitted by ***04 June 2024***.

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 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.

ECHA
EUROPEAN CHEMICALS AGENCY

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

1. Potential risk

1.1 Potential hazard of the Substance

Following its assessment of the available relevant information on the Substance, the evaluating MSCA and ECHA have identified the following potential hazard(s) which must be clarified.

In your comments to the draft decision, you highlighted the need to clarify the draft decision in distinguishing between human health and the environment.

The present request (LAGDA study) aims to clarify the concern for potential ED hazard for the environment and not to take any position for human health.

a) Potential endocrine disrupting properties for the environment

According to IPCS/WHO (2002) *"An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations".*

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

- it shows adverse effects(s) in (an intact) organism, or its progeny, or (sub)population;
- it shows endocrine activity, i.e. it has the potential to alter the function(s) of the endocrine system; and
- there is a biologically plausible link between the adverse effects and the endocrine activity, i.e. the Substance has an endocrine disrupting mode of action (ED MoA)

The available information in rat shows that the substance may have endocrine disruption properties related to the thyroid modality (T-modality).

1.1.a.1 Lines of evidence for adverse effects related to T-modality

1.1.a.1.1 T-mediated parameters

Thyroid follicular hypertrophy/hyperplasia and pale staining colloid were consistently observed in the available repeated dose oral toxicity studies in rats. These thyroid effects are reported below together with potential liver effects and/or general toxicity observed at the same dose levels:

- In a GLP-compliant 90-day dietary study, according to OECD TG 408 (1981) in Wistar rats (tested doses: 0, 750, 2250, 4500 and 15000 ppm equivalent to 53/63, 163/187, 315/365, 1027/1143 mg/kg bw/day in males/females), follicular hypertrophy associated with pale staining colloid were observed from 4500 ppm onwards in both sexes (7/10 and 4/10 in males and females at 4500 ppm and 10/10 in males and females at 15000 ppm). Increased absolute and relative liver weight were noted from 4500 ppm in females and at 15000 ppm in males. At the high-dose level, general toxicity was substantiated by a decreased of the final body weight - 10% and - 8% in males and females respectively (Unpublished study report, 1993).

- In a mechanistic subacute toxicity study in Wistar rats similar to OECD TG 407 and GLP-compliant at concentrations of 0, 1000 ppm, 3000 ppm and 10000 ppm over a period

of 14 days (subset B) and 28 days (subset A), corresponding to 63-72, 188-215 and 630-720 mg/kg bw/day, hypertrophy/ hyperplasia of follicular cells (5/10 animals) accompanied by altered colloid were observed in both sexes of both subsets at 10000 ppm. In subset B, high-dose group females showed also absolute and relative weight increases of the liver, while in subset A, an increase of the absolute relative liver weights of males and females were noted as well as a decrease of the final body weight in males (-10.8%) (Unpublished study report, 2019).

- In an EOGRTS (including a DART cohort extended to include a F2 generation and a DNT cohort) performed according to OECD TG 443 (2011) and compliant to GLP (except for the DNT part)[2] in Wistar rats (tested concentrations: 0, 750, 2100 and 7000 ppm equivalent to: 0, 46/56, 127/155, 425/498 in males/females during F0 premating period), a statistically significant increase of hyperplasia of follicular epithelial cells and loss of colloid were observed in 14/28 F0 males and a non-statistically significant increased incidence of activated thyroids in F1A males (7/20 vs 2/20 in controls) in the absence of any general or liver toxicity [a slight statistical increase of relative liver weight (7% and 10% in F0 and F1A males respectively)] was noted but not considered adverse in the absence of correlated histopathological or clinical findings. At 7000 ppm, hyperplasia of follicular epithelial cells and loss of colloid (18/28 and 17/28 in males and females respectively) associated with increased thyroid weights (25% to 30%) were noted in both sexes. At this dose level, increased absolute and relative liver weights were reported in both sexes of any generation as well as decreased final bodyweight (- 5% to - 10% according sex and generation) (Unpublished study report, 2019).

- In a dose-range-finding study, related to EOGRTS (tested concentrations: 0, 5000 and 15000 ppm equivalent to: 0, 279-399/351-392, 812-1271/919-1335 in males/females during premating period) increased hyperplasia of follicular epithelial cells and loss of colloid were observed in 18/24 and 21/24 animals at 5000 and 15000 ppm respectively correlated by a dose-related increase of thyroid weight. Increased relative liver weight was observed in both sexes from 5000 ppm while final body weight was impacted from 5000 ppm females (- 5%) and at 15000 ppm in males (-13%) (Unpublished study report, 2018).

In the absence of a 2-year rat study, it is not possible to conclude if longer exposure would lead to follicular adenoma/adenocarcinoma.

Thyroid effects and liver toxicity

In your comments to the draft decision, you argued that there is a strong evidence that the "*T-modality mode of action (MoA)* [is indirect] *via liver enzyme induction*" because in a subacute mechanistic repeated dose toxicity study in rats "*related increase of phase I and phase II liver enzyme activities (Cytochome P450 subtypes and T4 specific glucurosyltransferases), accompanied by an increase of serum TSH without significant changes in T3 and T4 levels*" and that at "*low dose levels, changes in body weights were mild and not found consistently anymore, however, histological changes in the thyroids were associated with substance related changes in liver associated parameters"*. It is acknowledged that the thyroid effects were mainly observed from dose levels where slight

[2] Due to the numerous major limitations and the insufficient documentation for assessment, the DNT part of the study is not considered as reliable (Klimish score 3), and it is arguably definable as in line with the GLP. For numerous endpoints, historical control data and positive control are missing, methods description is often incomplete and the statistical analyses are not appropriate. Raw data are not available for auditory startle response. Effects are dismissed without any explanation and the overall DNT assessment is considered inappropriate as reported.

effects on liver weight and/or on liver histopathological findings (centrilobular hypertrophy) and/or that liver enzyme induction are also observed consistently with the proposed mode of action (nuclear receptor activation leading to liver enzymes induction, further discussed below). However, the liver effects observed at those dose levels are not considered adverse. The LOAEL for a thyroid-based adverse effect was mainly based on the observed patterns of histopathological changes, in line with ECHA/EFSA for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 (ECHA/EFSA, 2018).
The lowest effective dose for the thyroid effects was 2100 ppm (127 mg/kg bw per day) observed in F0 males (statistically significant) and in F1A males (non-statistically significant) of the EOGRTS. At this dose level a 7% and 10% increase of relative liver weight was observed in F0 and F1A males, respectively, without any correlated liver histopathological or clinical changes. Such a 7 to 10% increase in relative liver weight is not considered adverse in the absence of correlated histopathological or clinical findings (WHO, 2015). Therefore, it cannot be concluded that thyroid effects are secondary to overt liver toxicity or general toxicity.

1.1.a.1.2 Parameters sensitive to, but not diagnostic of, T-modality

In your comments to the draft decision, you considered that the parameters sensitive to, but not diagnostic of, the T-modality cannot form a sound rationale to justify the requested LAGDA.
In accordance with the ECHA/EFSA ED Guidance (ECHA/EFSA, 2018) both T-mediated parameters and, to a lesser extent, parameters sensitive to, but not diagnostic of, T-modality are taken into consideration in the lines of evidence for the T-modality in a holistic approach. It is acknowledged that the later parameters are not specific to the T-modality. However, some parameters sensitive to, but not diagnostic of, T-modality (especially effects on the developing nervous system (ECHA/EFSA, 2018)) may be pieces of evidence of potential thyroid disruption which can support further request in particular dedicated to other taxonomic groups. Due to the conservation of hormonal regulation among vertebrates, the Substance is likely to interact with thyroid systems of any vertebrate species of the environment.

<u>Pre-implantation loss</u>

Lower mean number of implantation sites, and consequently, a lower number of pups delivered in high-dose group females (7000 ppm) were observed in the F0 generation (implantation sites: 10.7 versus 12.3 in controls; pups: 9.6 versus 11.4 in controls) and in the F1generation (implantation sites: 9.3 versus 10.7 in controls; pups: 9.3 versus 10.3 in controls). At this dose level a 9% and 6% decreased body weight was observed in F0 and F1 females at the end of the premating period (Unpublished study report, 2019). Lower mean number of implantation sites (9.7 versus 12.7 in controls) and a lower number of pups delivered (8.9 versus 12.1 in controls) were also reported in high-dose group females (15000 ppm) in the dose-range finding study (Unpublished study report, 2018).

In your comments to the draft decision, you cited literature which indicates maternal stress as contributors for decreased implantation rates ([redacted], 2015) as well as in-house historical control data on Wistar rats showing the time mated and subsequently transported rats had a reduction of 1.4 in implantation sites when compared to in-house mated animals without transportation. As a consequence, the mean number of delivered pups was reduced by 1.5, which is of a comparable magnitude to the changes observed after administration of the Substance at a dose of 7000 ppm. Since the Substance dependent changes in these reproductive parameters occurred at a maternally toxic dose level, you propose that a dependency between these effects (i.e. stress due to toxicity and

slightly reduced number of implantations) is possible and potentially independent from any T-modality.

As mentioned above, this effect is sensitive to, but not diagnostic of, T-modality. It is noteworthy, that no information is provided to substantiate that the potential stress induced by the general toxicity observed at 7000 ppm of the Substance is equivalent to stress induced by transport. Therefore it is only speculative. Furthermore, no effect on stress marker organs (i.e.: thymus, spleen, adrenals) were noted in high-dose females of both generations which could support that stress occurred.

You mentioned a follow-up study in Wistar rats with a comparable Substance application 10 weeks before mating until completion of the implantation phase (i.e gestational day 7) to obtain the mechanistic information based on fertility parameters, corpora lutea and implantation sites. To ECHA's knowledge, such a study has not been submitted. If such a study was available, it would not allow to take away the concern on potential endocrine disrupting properties for the environment related to the thyroid modality and the need for the LAGDA since the study proposed would be dedicated to evaluate the reprotoxicity of Octocrilene on mammals rather than dedicated to investigate adversity and/or endocrine activity related to the thyroid modality.

Pituitary histopathology

In the 90-day rat study, the number of hypertrophic cells in the pituitary gland in high-dose males (15000 ppm) was increased compared to controls (9/10 versus 2/10 in controls) (Unpublished study report, 1993). In your comments to the draft decision, you considered these changes in the pituitary as a potential consequence of the indirect T-modality MoA via liver enzyme induction. As you mentioned, histopathological effects on males may represent a line of evidence to support alteration of the hypothalamo-pituitary-thyroid-axis (HPT axis) induced by increased thyroid hormones (TH) clearance.
However, as you pointed out, these findings were not confirmed in any generation (F0 + F1) of the EOGRTS or in the associated range-finding study, which was dosed up to the same level (15 000 ppm). However, the dose levels tested in the EOGRTS (up to 7000 ppm) were lower and, while the high-dose level was the same in the range-finding study, the duration of exposure was lower (5 weeks vs 13 weeks) which may explain the discrepancies observed between these studies. Beside the increased TH clearance, alteration of the HPT axis and correlated histopathological changes in pituitary gland may also result from other causes of decreased serum TH levels (e.g. decreased TH synthesis due to inhibition of thyroperoxidase (TPO) activity).

Developmental neurotoxicity

As regards to the thyroid-related developmental neurotoxicity (DNT) concerns, while not required in OECD TG 443, learning and memory tests in the DNT cohort of the EOGRTS would have been of value to investigate cognitive functions in offspring. In your comments to the draft decision, you said that "*neither the developmental toxicity study in rats nor the OECD level 5 Extended One Generation Reproductive Toxicity study (EOGRTS) gave proof of any adverse developmental toxic effect, that can be based on a direct thyroid MoA".* Nonetheless, ECHA considers that the numerous limitations of the DNT part of the EOGRTS (not appropriate statistical analysis, absence of historical controls and positive control, poor reporting of the methods and lack of raw data for the auditory startle response (ASR)) hamper to draw any final conclusion on developmental neurotoxicity (DNT). Therefore, the results from the DNT cohort (part of the EOGRTS) are therefore considered as inconclusive., and no final conclusion can be drawn.

From a regulatory perspective and in a conservative approach the high dose (HD) is considered as a LOAEL. Indeed, a statistically significant decrease of the forelimb grip strength in HD males was noted as well as some effects in the ASR, the motor activity (MA) and morphometrics:

- The effects observeds in the ASR and MA (decreased max startle amplitude in HD males, decreased motor activity in HD females) did not reach statistical significance. However, they are considered biologically relevant (about 20% change) and adverse in the absence of appropriate statistical analyses and submission of historical control data (allowing the assessment of the biological relevance of the variation observed) and of positive control data (allowing assessment of the tests sensitivity).
- With regards to morphometrics, the effects in the measurements on hippocampus gyrus, corpus callosum and striatum measurements observed in HD males and females are considered adverse without additional statistical analyses (multivariate analysis of measurements in conjunction with other factors (age, sex, treatment), submission of historical control and of positive control data and more details on how and when low-dose and mid-dose tissues were processed to the block stage (Unpublished study report, 2019)).

In your comments to the draft decision, you challenged the relevance of additional learning and memory tests to investigate cognitive functions since according to the ECHA/EFSA Guidance (ECHA/EFSA, 2018), the data from the DNT and DIT cohorts of the EOGRTS are relevant to assess endocrine disruption, including thyroid related effects. We would like to emphasize that the DNT cohort of EOGRTS represents basic *in vivo* screening for DNT (limited animal number and the limited tests), but does not include all facets of a complete DNT study (OECD GD 151).

Concerning the developmental neurotoxicity parameters, it is acknowledged that additional learning and memory tests are not included in the currently in force EOGRTS guideline. It is well established that T4 decrease during critical windows of exposure may lead to neurological and cognitive impairments/auditory impairments. Uncertainties were raised (see above) about the results of the ASR test performed,. In the absence of other tests investigating cognitive functions (i.e. learning and memory tests) this uncertainty cannot be removed.

As regards to the ASR data, you have emphasized that you submitted the raw data to the evaluating member state upon their request. The raw data of the ASR were requested in order to perform an independent analysis with repeated-measures ANOVA, which is the most generally accepted approach according to NAFTA DNT Guidance (Moser et al., 2016) to confirm or infirm results provided by the unusual test implemented in the study report (Generalized Estimating Equations). The Dutch report you provided did not contain the raw data, nor historical control data or positive control data from the laboratory, which is not in line with GLP.
Observation of effects on parameters sensitive to, but not diagnostic of, T-modality and the lack of adequate investigation of potential effects on the developing nervous system support further request in particular dedicated to other taxonomic groups.

1.1.a.2 Lines of evidence for endocrine activity related to T-modality

1.1.a.2.1 In vitro *data*

No specific *in vitro* data have been submitted in the registration dossier.

From Toxcast/Tox21 data[3], the Substance was negative in TOX21_TSHR assays for both agonist and antagonist ways. The Substance was positive in TOX21_TR_LUC_GH3_Antagonist assay, but at high concentration AC_{50} = 26 µM, while the TOXCAST cytotoxicity thresholds were set at 5.463 µM and 30.742 µM for the lower bound and the mean respectively.

In your comments to the draft decision, you pointed out that the Substance was also negative as agonist and antagonist in the TSH receptor assay (TOX21_TSHR_HTRF).
The Substance was positive in TOX21_CAR_Agonist assay with an AC_{50} of 2.72 µM, but also positive in TOX21_CAR_Antagonist assay with an AC50 of 8.34 µM (borderline activity). The Substance was positive in TOX21_PXR_Agonist assay with an AC_{50} of 12.9 µM and was negative in TOX21_AhR_LUC_Agonist assay.

In addition, other possible thyroid-disrupting MoAs and alternative molecular initiating events (MIEs) cannot be ruled out. For example, no information is available on other relevant MIEs such as inhibition of Sodium-iodide symporter (NIS) or Thyroid peroxidase (TPO).

1.1.a.2.2 In vivo *data*

Hormonal changes

In the 14-d/28-d mechanistic study in rat (Unpublished study report, 2019), a two-fold increase of Thyroid-stimulating hormone (TSH) levels was observed in high-dose (10000 ppm) males (non-statistically significant) and females (statistically significant) at different time points, whereas a non-statistically significant decrease of T4 (10-25%) was observed at the same dose level.
In your comments to the draft decision, you considered that "*the current database showed, that hormonal changes are restricted to regulating TSH levels, whereas the effective TH concentrations are not dysregulated, since the mentioned T4 effects were not statistically significant, not supported by significant changes in T3 and not supported in P and F1 animals in the EOGRTS."* While it is acknowledged that no significant changes in T3 were observed in the 14-d/28-d mechanistic study in rat, ECHA considers the non-statistically significant decrease of T4 levels (10-25%) consistently observed at several time points in high-dose males and females biologically relevant.

These hormonal changes were not corroborated by the hormone analysis performed in the EOGRTS (Unpublished study report, 2019) in which hormonal levels were not impacted by treatment. It is noteworthy that in the EOGRTS, a high inter-individual variability was noted, the tested doses were lower (high dose of 7000 ppm versus 10 000 ppm in the mechanistic study) and the analyses were only performed at sacrifice contrary to the mechanistic study where thyroid hormones were measured at different time points.
While considered in a comparable range by you, the actual ingested doses are still lower in the high-dose group of the EOGRTS (about 200 mg/kg bw/d less) compared to those in the high dose of the mechanistic study.

Mechanisms that stabilize circulating T4 levels (i.e. TSH increase) may explain that the T4 decrease observed after 14 or 29 days, was not duplicated at sacrifice of EOGRTS adults. However, although the TSH increase was not observed in high-dose adults in the EOGRTS at sacrifice, thyroid hypertrophy/hyperplasia (indicative of previous TSH increase) was noted in both sexes of both generations. Also the coefficients of variation of TSH measurements in untreated controls of the EOGRTS were higher than 25% (i.e. higher

[3] https://comptox.epa.gov/dashboard/dsstoxdb/results?search=DTXSID9025299#bioactivity (2022-02)

than the value recommended in appendix B of ECHA/EFSA ED Guidance (ECHA/EFSA, 2018).

Regarding critical windows of potential hypothyroxinaemia (related to the development of the offspring nervous system), T4 hormone analysis in culled F1 and F2 pups was performed on PND4. While not statistically significant, lower T4 levels were observed in high-dose pups compared to controls in both generations (25% and 33% decrease in F1 and F2 PND4 pups respectively). However, the low number of high-dose culled pups was limited (pooled pup from 5 and 4 litters for F1 and F2 respectively) due to the pre-implantation loss observed at this dose level, compromising the reliability of the result.

No significant change was observed in TSH and T4 levels of PND21 pups of both generations. While the Substance has been detected in human milk in biomonitoring studies, the extent of pups exposure to the Substance during lactation is however unknown. Furthermore, since the concentrations in the diet have been lowered by half during lactation, the pups were exposed directly during the last week of the lactation period (when they began to eat for themselves) at lower doses (half doses) compared to parents. It should also be pointed out that the coefficients of variation of TSH (PND21 pups) and T4 (PND4 and PND21 pups) measurements in untreated controls of the EOGRTS were far above 25%.

The thyroid hormones (TH) levels during late pregnancy in dams (another crucial period for the development of the offspring nervous system) were not investigated as they are not required according to EOGRTS TG 443.

Thyroid-relevant enzyme induction

- (CAR/PXR-mediated) induction of endocrine-relevant enzymes

In the rat mechanistic study (Unpublished study report, 2019), induction of liver T4-UDPGT was substantiated by a 1.5-fold increased activity in mid-dose females (3000 ppm) and 2-3 fold increased activity in high-dose males and females (10000 ppm).

As mentioned in your comment to the draft decision, the authors observed a 2-fold increase of BROD (marker of CAR/PXR activation and induction CYP2B/3A subfamily enzymes) in both sexes and PROD (marker of CAR and marker of induction CYP2B subfamily enzymes) activities in males of the mid-dose group and a more extensive induction (>6-fold) of BROD/PROD activities in both sexes in the high-dose group. However, while an increase of measurements of T4-UDPGT activity represents a strong evidence of an increased TH catabolism (since TH are mainly catabolised through phase II metabolism (glucuronosyltransferases and sulfotransferases)), increased BROD/PROD activities (phase I metabolism enzymes not involved in TH catabolism) can only be used as surrogate events to support phase II induction since xenobiotic nuclear receptors activation up-regulates expression of both phase I and II metabolic enzymes.

- Deiodinases activity

In the rat mechanistic study (Unpublished study report, 2019), the liver type III deiodinase (D3) was induced (2-fold increased activity) in mid- and high-dose males while the liver type I deiodinase (D1) was inhibited (1.5-fold reduced activity) in high-dose males. It should be highlighted that D3 induction decreases the biological activity of TH via inner ring deiodination of T4 (thyroxine) and T3 (triiodothyronine) leading to rT3 (reverse T3, an inactive form of T3) and T2 (diiodothyronine), respectively.

In your comments to the draft decision, you mentioned that the observed changes in deiodinase activities could be a response contributing to regulate changes in TH plasma levels, which were affected by the Substance-mediated induction of metabolizing liver enzymes.

However, while the observed decrease of liver D1 activity may be a physiological response to the lowered serum levels of T4 (since D1 converts T4 to either T3 or rT3), the reasoning behind induced D3 activity as a response contributing to regulate increased TH clearance is speculative and is counterintuitive. Indeed, D3 decreases T4 level and T3 levels by deiodination of T4 and T3 leading to rT3 and T2 (diiodothyronine), respectively. It seems therefore surprising that increased D3 activity would compensate decreased TH plasma levels resulting from liver phase II induction.

The available information in rats show that the Substance has endocrine disruption activity (decreased T4 and increased TSH) indicative of a chemical pable of disturbing TH-signalling in rodents and potentially other taxonomic groups.

1.1.a.3 Mode of action analysis

From the mechanistic study in Wistar rats (Unpublished study report, 2019), you proposed the following mode of action (MoA): induction of liver enzymes (PROD, BROD, T4-specific UDP-glucuronosyltransferase) some of which is leading to an increased TH clearance in both sexes. This is usually associated with lower TH concentrations resulting in a decrease of TH negative feedback mechanism and thus higher TSH levels overstimulating the cell dynamic of the thyroid gland substantiated by a hypertrophy/hyperplasia of the thyroid follicular cells.

While the biological plausibility of the proposed MoA is strong (well-documented MoA), the empirical support of the proposed mode of action is considered moderate (formerly considered weak to moderate). In your comments to the draft decision, you did not agree that the given examples confirmed a weak to moderate empirical support of the proposed MoA by ECHA.

Reconsidering the overall dataset, the empirical support of the proposed mode of action is considered moderate and not strong as you proposed. Indeed, all the key events (KEs) except of the MIE were measured only in the mechanistic study in rats. While hypertrophy/hyperplasia of the thyroid follicular cells was consistently observed across studies, hormonal changes were only observed in the mechanistic study at 10000 ppm. No hormonal effects were noted in the EOGRTS up to 7000 ppm while thyroid hyperplasia was observed from 2100 ppm in males and at 7000 ppm in females. The analysis of the dose- and temporal-concordance between the key events of the postulated MoA was limited by the design and dose spacing in the available studies.

In your comments to the draft decision, you considered that ECHA focused *"on altered thyroid histopathology in mid dose F0 males (2100 ppm) of the EOGRTS in the absence of T4 and TSH changes and mentioned no altered T4/TSH levels in low and mid dose animals (1000, 3000 ppm) of the mechanistic study, disregarding that a significant increase of F0 males with altered thyroid histopathology at 2100 ppm was not confirmed in the F1 generation."* It is agreed that at 2100 ppm, the increased incidence of altered thyroid histopathology was statistically significant only in F0 males (20-week old at termination). However, while non-statistically significant, the effects observed in the F1A males (increased incidence of activated thyroids 7/20 vs 2/20 in controls) are considered

biologically relevant. Indeed, F1A males are sacrificed earlier compared to F0 (13-week old) which explains less marked effects.

In your comments to the draft decision, you added that "*in the mechanistic study, the dose groups 1000 and 3000 ppm did not show any relevant increase in animals with altered thyroid histopathology, which correlates well with the absence of changes in T4/TSH levels as the upstream key event*".

The direct comparison between F0 mid-dose males in the EOGRTS and the males from the dose groups 1000 and 3000 ppm in the mechanistic study is not relevant since the exposure duration is different. It sounds logical that for the same dose-level, a downstream key event as altered thyroid histopathology not observed after short exposure (mechanistic study) is noted after a longer exposure duration in the EOGRTS.

Regarding the upstream key events (decreased T4 and increased TSH), they were not observed in the mid-dose mechanistic study (3000 ppm) while the downstream key event thyroid hypertrophy/hyperplasia was observed from 2100 ppm in the EOGRTS F0 males, from 4500 ppm in males and females of the 90-day study and from 5000 ppm in males and females of the EOGRTS range finding study which also challenges the dose–response and temporal concordances.

In your comments to the draft decision, you stated that "*based on a strong empirical support, it was unambiguously demonstrated, that the effects observed in thyroids are due to enzyme induction in the liver considering that the NOAEL for thyroid effects was 2100 ppm"*. As discussed above, the thyroid findings in mid-dose males in the EOGRTS (2100 ppm) are considered to set the LOAEL for the thyroid effects (1.1.a.1.1).

Based on the MoA analysis performed by the eMSCA, while some uncertainties remain (mainly linked to the limited dose- and temporal-concordance of the key events), the available data seem to support the postulated MoA emphazing the need to evaluate if it occurs in other taxa and also if it leads to adverse effects in wildlife species.

Regarding the specificity of the proposed MoA, the presence of other possible thyroid-disrupting modes of action such as interference with TH synthesis (NIS or TPO inhibition) have not been investigated and alternative MIEs cannot be ruled out (many chemicals can activate multiple MIEs).

Anyway, according to the ECHA/EFSA ED Guidance for the identification of endocrine disruptors (ECHA/EFSA, 2018) where the adversity is based on T-mediated parameters (thyroid follicular hypertrophy/hyperplasia and pale staining colloid consistently observed in the available repeated dose oral toxicity studies), the biologically plausibility that the adverse effects are caused via a T-mediated MoA is high based on existing knowledge and the link is already pre-established in the absence of information proving the contrary (i.e. a fully developed non-ED MoA).

In your comments to the draft decision, you mentioned that studies consistently showed thyroid effects caused by an indirect T-modality mode of action (MoA) via the induction of liver enzymes, which leads to a rat-specific higher turnover of TH.

While it is acknowledged that rat may be more sensitive to thyroid hypertrophy and potential thyroid cancer resulting from T3 and T4 clearance, an increased TSH in rodents does indicate a chemical capable of perturbing TH signalling in other species (Noyes et al., 2019).

In your comments to the draft decision, you added that T3 and T4 levels remained in a physiological range in the mechanistic study. While non-statistically significant, the decrease of T4 levels (10-25%) observed at several time points in high-dose males and females is considered biologically relevant. No significant change in T4 levels was observed in adults of both generations in the EOGRTS which can be explained by the lower dose and the time of measurement (at sacrifice).

No significant change was observed in T4 levels of PND21 pups of both generations. While not statistically significant and based on limited number of pups, lower T4 levels were observed in high-dose PND4 pups compared to controls. It should also be pointed out that the coefficients of variation of T4 (PND4 and PND21 pups) measurements in untreated controls of the EOGRTS were far above than 25% advised which question the validity of the measurements and compromise the capability to identify treatment related modifications.

Based on the Adverse Outcome Pathways (AOP) network proposed by Noyes et al. (2019), beside the AOP "nuclear receptor activation leading to rat follicular thyroid tumors", major other outcomes of interest (e.g. neurological and cognitive impairments/ auditory impairments in mammals or metamorphosis impairments in amphibians) result from decrease in T4 and/or T3 levels (due to nuclear receptors activation or other MIEs). In your comments on the proposal for amendment you indicated that the paper by Noyes *et al.* (2019) does not change your view.

b) Conclusion on the potential endocrine disrupting properties

To summarise, in your comments to the draft decision, you considered that the Substance does not have a direct thyroid-related effect on the test organism and therefore that the need for any additional thyroid-related study in amphibians is not justified. You claimed that the observed thyroid effects are due to "*TH metabolism in the liver*" particularly activated in rodents while ECHA considers that other MIEs might be involved.

Indeed, from the available data of the Substance, two potential MIEs have been investigated and gave positive results:
- Nuclear receptor activation (TOX21_CAR_Agonist assay)
- Inhibition of deiodinase D1/Induction of deiodinase D3 (in vivo mechanistic study).

As regards to the adverse outcomes linked to neurodevelopment and due to a drop in T4 during critical periods, they have not been adequately investigated. Indeed due to severe limitations, the DNT part of the EOGRTS is considered inconclusive.

In addition, in the absence of substance-specific data that provide proof of the contrary, other taxonomic groups should be considered as sensitive to thyroid-disruption as the rat is. Indeed, it is well acknowledged that thyroid signalling, regulation and development are highly conserved in vertebrates and are comparable in mammals, hatching birds and amphibians (Buchholz, 2017), contrarily to what you commented. TH is essential for vertebrate development, hence it is important to consider the environmental impact of the Substance on the functioning of the TH nuclear receptor system during development. The total dependence of amphibian metamorphosis on TH allows this system to be a uniquely suitable model for evaluating the possible effects of the Substance on the TH signaling during development.
However, none of the available ecotoxicity studies provides information on apical and adverse effects specific to the T-modality in other taxonomic groups. The available and

current information are therefore not sufficient to draw a final conclusion on the potential hazard for the environment, i.e. to meet the conditions that define an endocrine disruptor for the environment as defined above. Further data need to be generated to investigate T-mediated parameters in other taxonomic groups (scenario 2a (i) of ED guidance (ECHA/EFSA, 2018)).

1.2 Potential exposure

According to the information you submitted in the chemical safety reports (CSR), 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 among other uses, the Substance is used by industrial workers, professionals and consumers. The Substance can be included into or onto a matrix and can be found in formulation of preparations, plastics, cosmetics, personal care products, perfumes and fragrances as stabilisers and UV filter. The available information indicate that the environmental emissions of the Substance can originate from industrial sites, from consumer uses of cosmetic products and can be related to long-life materials with low release rate and end use of plastics.

The Substance has a very low solubility in water (< 0.1 $mg.L^{-1}$), high lipophilicity (log Kow > 6), low volatilisation from water (Henry law constant= 3.1^{-04} $Pa.m^3.mol^{-1}$), high potential of sorption into sewage sludge, sediment and soil compartments (log Koc= 5.61), and is stable in environmental compartments (from biodegradation studies).
From the available scientific literature (Kameda et al., 2011, Poiger et al., 2004, Rodil et al., 2009), the Substance occurs in surface water and sediments. Concentrations of the Substance have been found in samples collected from across the entire planet, even in polar regions, revealing their global distribution (Cadena-Aizaga et al., 2020).

The Substance fulfills the criteria for persistence and toxicity but not for bioaccumulation. Continuous discharge in water could lead to raise in concentrations, leading to potential adverse effects.

Therefore, exposure of the environment occurs.

1.3 Identification of the potential risk to be clarified

Based on all information available in the registration dossier and information from the published literature, the Substance may be an endocrine disruption substance for wildlife species triggering disruption of the thyroid pathways.

The information you provided on manufacture and uses demonstrates a potential for exposure of the environment while publicly available information demonstrate presence of the Substance in 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 are not sufficient to conclude on the hazard and in particular endocrine disruption potential for wildlife species. Consequently further data are needed to clarify the potential risk related to endocrine disruption properties for the environment.

In your comments to the draft decision, you considered that, based on the available data, the thyroidal MoA of the Substance is not demonstrated and that accordingly there is no need for additional data. Moreover, you mentioned that the EOGRTS data available are suitable to inform on thyroid-related endocrine disruptive effects. You emphasized that the EOGRTS is the most predictive test for endocrine disruption adverse effect through EATS modalities and that performance of an OECD TG 241 is of no interest. As it is recognised that the EOGRTS is a very powerfull and strong assay, it does not provide specific data for environment which is of concern. Indeed, the Substance is manufactured or imported in the EU at a high aggregated tonnage, and can be found in formulation of preparations, plastics, cosmetics, personal care products, perfumes and fragrances as stabilisers and UV filter.

The Substance has a low volatilisation from water, is stable in environmental compartments, occurs in surface water and sediments from across the entire planet, even in polar regions, thereby revealing its global distribution. Continuous discharge in water could lead to raise in concentrations, leading to potential adverse effects.

Based on all the evidence of environmental exposure and of adverse effect on T modality, as detailed in section 1.1, the requested study to remove uncertainties of the potential adverse effect of the Substance on wildlife species via the T modality is justified.

1.4 Further risk management measures

An adverse effect, plausibly linked to endocrine activity, needs to be established for a substance to fulfil the definition of an endocrine disruptor. The guidance document regarding ED identification recommends to demonstrate "*the biological plausibility of the link between the adverse effects and the endocrine activity*" (ECHA/EFSA, 2018).

If the properties(s) 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 under Article 57(f) of REACH due to endocrine disrupting properties relevant for the environment,
- authorisation/ restrictions of the use of the substance for endocrine disruption properties.

This 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.

The LAGDA study (OECD TG 241), with the additional mechanistic parameters requested in this decision, will investigate apical endpoints to detect an adverse effect on the thyroid and provide additional evidence to support that any adverse effects observed are plausibly linked to endocrine activity via the thyroid pathway as required for identification of the Substance as an SVHC for its endocrine disrupting properties.

In your comments to the draft decision, you questioned the pertinence of requesting a LAGDA study (reasoning provided later in the decision) and the fact that it will improve risk management measure. As detailed in this decision, the requested information will allow to draw a definitive conclusion on the potential adverse effect of the Substance on thyroid and thyroid hormones on environmental species, as evidence is available that the Substance is able to exert some adverse effect on these parameters.

If the concern is confirmed, the next steps to ensure adequate management of the Substance will be the identification of the Substance as a substance of very high concern

under Article 57(f) of REACH, which will lead to stricter risk management measures to protect the environment and the species living in it. If the Substance is included in Annex XIV of REACH due to its ED properties for the environment, an assessment of the risk for the environment would be added to the scope for authorisation, according to Article 62(4)(d) of REACH.

Consequently the exemptions specified in Article 56(5) of REACH and relevant with regard to uses of the Substance (use in cosmetics in particular), would not apply. Therefore, an SVHC identification due ED properties for the environment 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.

2. How to clarify the potential risk

Several studies *in vitro* and *in vivo* in rats describe T-mediated effects as well as changes of thyroid hormone levels (see Section 1.1). The Substance was noted to induce hormonal changes in a mechanistic study in rat (Unpublished study report, 2019), a 2-fold increase of TSH levels was observed both in high-dose (10000 ppm) males (non-statistically significant) and females (statistically significant) at different time points. Non-statistically significant decrease of T4 (10-25%) was observed at the same dose levels and considered biologically relevant. In this study, induction of enzyme involved in TH catabolism was noted (increased T4-UDPGT activity in mid-dose females (3000 ppm), in high-dose males and females (10000 ppm) along with other CAR and PXR-regulated enzymes (BROD and PROD activities) from 3000 ppm onwards in both sexes. A putative adverse outcome pathway (AOP n°194) "Hepatic nuclear receptor activation leading to altered amphibian metamorphosis" is currently under development. Furthermore, liver type III deiodinase, D3, was induced (2-fold increased activity) in mid- and high-dose males while liver type I deiodinase, D1, was inhibited (1.5-fold reduced activity) in high-dose males. It should be highlighted that D3 induction decreases the biological activity of thyroid hormones via inner ring deiodination of T4 (thyroxine) and T3 (triiodothyronine) leading to rT3 (reverse T3, an inactive form of T3) and T2 (diiodothyronine) respectively. Deiodinases are involved in metamorphosis in amphibian and impairment of these MIEs will impact metamorphosis as reflected in adverse outcome pathways under development (AOP 189, 190 and 191). Considering that the HPT axis is highly conserved among vertebrates, all these evidence triggered ECHA to request an assay on amphibians to conclude on potential adverse endocrine effect of the Substance for the environment.

According to the ECHA/EFSA ED guidance (Section 3.3.1.4., ECHA/EFSA, 2018), no apical endpoints data have been identified to acknowledge that disruption of thyroid function leads to environmental adversity of the Substance at the population level. This concern therefore needs to be further clarified.

ECHA concludes that it is possible to gain such information with the conduct of the Larval Amphibian Growth and Development Assay (LAGDA, OECD TG 241) that includes apical as well as mechanistic endpoints linked to disruption of the T pathways and would allow to confirm or dismiss the ED properties of the Substance for the environment. Contrarily to what you commented, ECHA considers that the available data are sufficient to raise concern of the potential endocrine disruptive effect of the Substance and sufficient to request an OECD TG 241 assay to resolve the observed uncertainties.
Moreover, the necessity to obtain such data to conclude on the potential ED effect of the Substance is justified also by the high tonnage and the highly dispersive uses of the Substance that may impact large area and populations if apical adverse effects occurred at wildlife population level.

2.1 The Larval Amphibian Growth and Development Assay (LAGDA, test method: OECD TG 241; OECD 2015)

a) Aim of the study

As detailed in Section 1.1 above, information on the Substance is required to clarify if disruption of thyroid function occurs and leads to apical effects considered as adverse at the population level on wildlife species.

The OECD TG 241 (LAGDA) describes a toxicity test with an amphibian species that considers growth and development from fertilization through the early juvenile period. It is an assay that assesses early development, metamorphosis, survival, growth, and partial reproductive maturation. It also enables measurement of a suite of other endpoints that allows for diagnostic evaluation of suspected endocrine disrupting chemicals or other types of developmental and reproductive toxicants.

The requested study will allow evaluation of the effects of the Substance on endocrine system and especially on thyroid and thyroid gland function. It is the most sensitive developmental toxicity study while exposed through the environment to detect adverse effects linked to effects on thyroid as it includes many parameters related to endocrine activity/mode of action. Also it serves as a higher tier test with an amphibian, placed at Level 4 of the OECD Conceptual Framework on Endocrine Disrupters Testing and Assessment, where *in vivo* assays also provide data on adverse effects on endocrine relevant endpoints. It is the only OECD TG that can inform on both adversity and endocrine activity related to thyroid and thus, enable establishing the ED MoA.

The information requested from OECD TG 241 aims at clarifying the potential risk that the Substance poses and to clarify if the disruption of thyroid function leads to environmental adversity of the Substance at the population level.

Therefore, it is requested under the current substance evaluation.

b) Specification of the requested study

The study must be performed according to the OECD TG 241 (OECD, 2015). All quality criteria must be respected. Octocrilene is considered as a "difficult substance" that requires to be tested following the OECD GD n°23. According to the OECD TG 241, the number of replicates is doubled (8 replicates) for controls compared to each test concentration (4 replicates) in order to give adequate statistical power for the test. Contrary to your comments to the draft decision, this validated increase in number of controls is proportionate and will allow to draw a robust conclusion at termination of the assay.

Test material and concentration

The test material should be the Substance, as specified in Appendix C, and with the highest purity in order to avoid confounding effects of impurity(ies).

For the purposes of this test, results from existing studies must be considered in determining the highest test concentration so as to avoid concentrations that are overtly toxic. The concentrations used must be carefully chosen to ascertain the results and avoid physical effects of the Substance arising from the low solubility.

To better choose the adequate range of concentrations, the results from the Bioaccumulation study in Fish (Zebrafish, OECD TG 305), the Daphnia magna

Reproduction Test (OECD TG 211) and the Fish Short Term Reproduction Assay (OECD TG 229) and Androgenised Female Stickleback Screen (AFSS, variant of OECD TG 230) must be taken into account. If there are no relevant data to be used for dose level setting, it is recommended that results of a range-finding study are reported with the main study.

It is recommended to use a minimum of four chemical concentrations and appropriate controls (including solvent controls, if necessary). Generally, a concentration separation (spacing factor) not exceeding 3.2 is recommended.

The iodide content of water used in the study need to be checked in order to comply with the iodide levels commonly found in freshwater system to ensure the quality and robustness of the assay (generally comprised between 0.004 - 0.158 µM). The iodine content and supplementation of the test water must be checked and reported to comply with the recommendation of the paragraph 17 of the OECD TG 241 to ensure the success of the assay. Additionally, as indicated in paragraph 17 of the OECD TG 241, you may monitor iodine content in food as fresh water vertebrates cover their main iodine demand via the food.
In your comments to the draft decision, you commented on "*the absolute necessity to monitor and ensure stable concentration of the Substance all along the assay*". This comment is in line with ECHA's proposal and is appreciated.

Regular analytical monitoring of the Substance is required to know its exact concentration during the experiment and to evaluate its disappearance, should it occur. In your comments to the proposal for amendments you have specified that in case the stability of the test substance can be demonstrated, weekly monitoring should be sufficient. Therefore, based on your comments, the exposure concentrations of the Substance must be determined at least weekly (as indicated in the OECD TG 241), or twice a week (if instability of the substance is proven), for at least one replicate in each treatment group, rotating between replicates of the same treatment group. The flow rate to each tank should be constant in consideration of both the maintenance of biological conditions and chemical exposure and is recommanded to operate at least 5 tank turnovers per day. You can also follow the recommendation in the OECD Guidance Document (GD) 23, on Aqueous Phase Aquatic Toxicity Testing of Difficult Test Chemicals which provides indications to limit biodegradation of a test substance during assessment.

Route of exposure

The assay must be performed under flow-through condition in order to maintain stable exposure concentration in the system. This has to be verified during the assay by measuring concentration along the study.

Parameters to be measured

At NF stage 62, a larval sub-sample (up to 5 animals per replicate) is collected and various endpoints are examined (Table 1 below) and the remaining animals continue exposure until 10 weeks after the median time to NF stage 62 in the control. At test termination (juvenile sampling) additional measurements are made. When relevant, data on assay performances, quality criteria and validations (limits of detection, quantifications, coefficient of variations, specificity) must be reported.

One MSCA questioned in a proposal for amendement (PfA) the need to request as mandatory the additional parameters related to hormones measurements. In your comments to the PfA you agreed that there is no need to include further physiological measurements into the standard protocol.

Based on the PfA and your comments, those additional parameters originally requested were removed from the decision and are only recommended.

Parameters recommended to be measured

In order to fully take advantage of the test animals to investigate effects related to thyroid disruption, the following parameters are recommended to be measured, in addition to those requested in the guideline:

- Histopathology of the thyroid gland at the end of exposure
- Measurement of thyroid hormones (TSH (compatibility with the ELISA kit must be assessed prior the assay) and TSHβ gene expression, free T3, total T3, free T4, total T4) must be performed at NF62 (and time to reach this stage must be accurately reported) and at the end of exposure.
- Measurement of deiodinase D1, D2 and D3 activities and mRNA gene expressions in liver and brain must be performed at NF62 (and time to reach this stage must be accurately reported) and at the end of exposure.

Mechanistic data informative of the mode of action leading to the effects are therefore important in a possible future SVHC identification of the Substance as an endocrine disruptor for the environment in the aim to link the apical effects with endocrine activity in the suspected mode of action.

The list of parameters (requested or recommended) to be provided results from the analysis of the data currently available on the Substance, and which is specific to the suspected mode of action of the Substance for environmental species. Based on the available data, a thyroid-disrupting mode of action is suspected.

The measurement of thyroid hormones as an additional parameter is recommended in order to provide supporting information to investigate this thyroidal MoA in an environmental species (amphibians). In this specific case, these additional measurements are recommended to inform whether the potential adverse effects occurring on development and time to metamorphosis results from an impact of the substance on the thyroid gland histology/thyroid gland function and whether they do not occur through general systemic toxicity, as you commented (more related to hepatic enzyme induction).

In your comments to the draft decision "*in order to allow for a clear discrimination between a direct thyroid-mediated effect and a secondary effect of the substance*", you suggested to "*add both liver and kidney histopathology as additional endpoints of relevance*". Nevertheless these endpoints are already required in the OECD TG 241 and should therefore be performed without modifying the decision.

In your comments to the draft decision, you highlighted that the measurements of the additional parameters (TSHβ gene expression, deiodinase activities and RNA gene expressions) are not validated by an OECD technical guideline, and you suggested that "*The absence of currently validated and established methods suitable for Xenopus leavis was also confirmed by a most recent document on behalf of the European Food Safety Authority, EFSA ([redacted], 2020)*". ECHA agrees with your comment that these measurement are not part of an actual validated technical guideline. Nevertheless, the document published by EFSA highlighted in your comment does not exactly says that no methodology were validated for thyroid hormones, but says this for estrogens and androgens measurements "*Preferably, all three thyroid hormones, T3, T4 and TSH, should be measured in amphibians. Methods for measurement of T3, T4 and rT3 are relatively*

easily adaptable from established assays (e.g.for human or rat hormones). Anti-body-based methods for amphibian TSH require specific antibodies. Similarly, all relevant reproductive hormones should be measured within amphibian tests. However, not many methods have been described. Therefore, development and validation of suitable methods for measurements of estrogens and androgens in amphibians are urgently needed. Previous attempts to include hormone measurements in TG assays were not successful, possibly due to lack of proper validation of assays adapted from other species. Thus, development or adaption of methods in combination with detailed validation for the test species is strongly recommended". These measurement are increasingly performed in assessing the potential endocrine adverse effect of chemicals in literature and validated protocol and techniques are available (see Fini et al., 2017, Mughal et al., 2018, Spirhanzlova et al., 2019, Martínez-Guitarte et al., 2021). Moreover, the document published by EFSA highlighted that *"It is recommended to incorporate thyroid and reproductive hormone measurements into existing guideline tests at Level 4 of the OECD Conceptual Framework that assess effects attributable to an endocrine mode of action, such as the Larval Amphibian Growth and Development Assay (LAGDA) (OECD TG 241)"* which is indeed what is recommended in this decision.

In your comments to the draft decision, you indicated that "*the LAGDA study protocols aims for clarification on the adversity of ED-related physiological findings in an intact organism. Relevant effects will be covered within the definitive endpoints harmonized within the OECD test guideline. Therefore, the AMA is more suitable to identify a possible thyroid-mediated mode of action*". The OECD GD 150 details that the LAGDA could "*be used at any stage in the hazard assessment process, the most likely use scenario will be when there are some data available about the possible thyroid disrupting properties of a chemical*" indicating that this would not lead to a level 3 data gap.

As noted in section 1.4. above, an adverse effect, plausibly linked to endocrine activity needs to be established for a substance to fulfil the definition of an endocrine disruptor. Mechanistic data informative of the mode of action leading to the effects are therefore important in a possible future SVHC identification of the Substance as an endocrine disruptor for the environment in the aim to link the apical effects with endocrine activity in the suspected MoA. Deiodinases are involved in metamorphosis in amphibian and the impairment of these MIEs will impact metamorphosis as reflected in the adverse outcome pathways under development (AOP 189, 190 and 191). Regarding the alternative MIE "Inhibition deiodinase D1/Induction of deiodinase D3" and uncertainties raised by the mechanistic study of the dossier, measurement of deiodinase D1, D2 and D3 activities and RNA gene expressions in liver and brain is recommended.

In order to avoid bias, sampling for thyroid hormones should be performed at the same time (e.g. same hours in the morning or in the evening) for all animals. If it cannot be done, the distribution of time collection should be evenly distributed across groups (not all individuals of one group sampled concomitantly and all individuals of another group at a later time point).

- Measurement of vitellogenin (VTG)

Measurement of VTG concomitantly to phenotypic/genetic sex should be performed in order to ensure that the potential observed effects are arising from thyroid effects and not from other endocrine effects. It will also serve as control parameters allowing to conclude if effects are more linked to general toxicity or arising from ED effects. However, as available data primarily raise a concern for thyroid disruption, and also based on your comments on the draft decision that measuring this parameter is of no use as VTG is

correlated to sexual steroid hormones, it has been agreed that the measure of VTG is only recommended as an additional measurement, but it is not mandatory.

Table 1: Summary of mandatory or recommended endpoints to be measured

Endpoints	*Daily*	*Interim Sampling (Larval sampling NF62 stage)*	*Test Termination (Juvenile sampling)*
Mortality and abnormalities*	X		
Time to NF stage 62*		X	
Histo(patho)logy (thyroid gland)*		X	X**
Morphometrics (growth in weight and length)*		X	
Liver-somatic index (LSI)*			X
Genetic/phenotypic sex ratios*			X
Histopathology (gonads, reproductive ducts, kidney and liver)*			X
Vitellogenin (VTG)**		X	X
TSH**		X	X
TSHβ gene expression**		X	X
Free T3**		X	X
Total T3**		X	X
Free T4**		X	X
Total T4**		X	X
Deiodinase 1**		X	X
Deiodinase 2**		X	X
Deiodinase 3**		X	X

* Parameters to be measured - already mandatory in the OECD TG 241
** Parameters recommended only

In your comments to the draft decision, you questioned "*the relevance of the LAGDA to identify endocrine active substance on the HPT axis, especially analysis at NF62 on thyroid*". The OECD TG 241 is a validated test guideline for investigating a T mode of action (disruption in the HPT axis). This is apparent from the OECD TG 241: '*Endpoints evaluated during the course of the exposure include* (…) *endpoints designed to characterize specific endocrine toxicity modes of action targeting oestrogen-, androgen-, or thyroid-mediated physiological processes (i.e. thyroid histopathology, gonad and gonad duct histopathology, abnormal development, plasma vitellogenin (optional), and genotypic/phenotypic sex ratios).*' The validation of the OECD TG 241 for investigating the T mode of action is also recognised under the Revised OECD Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption. During the validation exercise for the LAGDA performed by the US EPA, they determined that examination of thyroid at NF62 was able to identify 100% of the individuals encountering adverse effect (glandular hypertrophy and extreme follicular cell hypertrophy and hyperplasia) after exposure to known thyroid adverse substance. Moreover the LAGDA assay holds a validated status at the OECD, meaning that this assay is considered sufficiently robust and reproducible to be used for regulatory purpose. Regarding thyroid hormone measurement, it is also highlighted by external publication of EFSA that "*Integrating hormone*

measurement for thyroid and reproductive toxicity in existing guidelines for endocrine disruptor testing allows to provide additional information without the use of additional animals. […] It is however recommended to integrate hormone measurements in OECD non-mammalian TGs, within Level 4 and 5 tests of the OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupters, where in vivo assays also provide data on adverse effects on endocrine relevant endpoints. […] For amphibians, there is currently only a level 4 TG available, the "Larval Amphibian Growth & Development Assay (LAGDA)" (OECD TG 241, OECD 2015b). Level 3 tests are not recommended for inclusion of hormone measurements since they have lower power to detect changes in hormone levels and are less informative with regards to adverse effects."

To address the missing information identified above, the OECD TG 241 will allow to identify information both on thyroid effects and developmental effects, which are required to conclude on the endocrine disruption properties, and to confirm whether the observed thyroid mode of action is of concern for the Substance.

Request for the full study report

You must submit the full study report which includes:

- a complete rationale of test design and
- doses of the Substance (reference and batch number) along the experiment
- all detailed images for metamorphosis change and identification
- interpretation of the results
- access to all information available in the full study report, such as implemented method, raw data collected, assay performance descriptors, interpretations and calculations, consideration of uncertainties, argumentation, etc.

In your comments to the draft decision, you argued that the reporting of quality criteria "*can certainly be provided for any standard data requirement for a given and approved OECD TG (e.g. OECD 231 and 241) but not necessarily for a non-established and non-validated method, as it is the case for the additional requested endpoints stated under c) – e). Therefore, we suggest that an AMA study should be performed following GLP-principals and OECD TG 231 test guideline requirements in order to clarify whether the substance has any adverse effect on the thyroid in amphibians and the underlying mode of action*". The fact that the requested data must be generated, partly, with non standard assay does not preclude the non-application and reporting of all criteria allowing to assess the quality of the exposure, sampling, preparation, measurement and analysis. The same high-quality procedure and reporting requested under GLP and standard OECD guidelines application has to be implemented to ensure the relevance and quality of the data.

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 for the Substance.

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

The request for The Larval Amphibian Growth and Development Assay (LAGDA, OECD TG 241) is:

- appropriate, because it will provide information which will clarify adverse effects potentially caused by endocrine activity of the Substance and especially effects on thyroid in wildlife species. This will enable the evaluating MSCA to conclude on potential ED properties of the Substance for the environment;

- the least onerous measure because there is no equally suitable alternative method available to obtain the information that would clarify the potential hazard, and this is the only OECD TG with such extensive parameters for endocrine activity and endocrine-mediated effects on thyroid gland and hormones levels at level 4 of the OECD Conceptual framework (CF) for environment according to Revised OECD GD 150. In particular, the OECD TG 231 Amphibian Metamorphosis Assay (AMA) also describes some specific thyroid function endpoints related to interaction with the HPT axis (thyroid histopathology and time to metamorphosis/ develomental phases) (OECD, 2009). However, the AMA test would not always allow a definitive conclusion on thyroid-adversity of the Substance compared to the LAGDA. Moreover, it cannot be excluded that for a substance with a specific MoA (that limits peak thyroid hormone levels in the developing tadpole, especially at later stages of metamorphosis) population relevant apical adverse effects can only be detected in the period of metamorphic climax that is only covered by LAGDA.

In your comments to the draft decision, you considered that if an additional study in amphibians is considered to be necessary by ECHA, this should be an Amphibian Metamorphosis Assay (OECD TG 231) as a T-mediated MoA has to be confirmed before adversity at population level needs to be investigated (which would be reflected within the LAGDA). After analysis of all available data, we concluded that data were insufficient to reach a definitive conclusion on the hazard potential of the Substance as an ED for the environment, especially in the view of further regulation of the substance through SVHC identification. Indeed, to reach a conclusion on a substance as meeting the criteria set out in Article 57(f) and compile an Annex XV dossier, an adverse effect at population level needs to be demonstrated. For the environment, the sole available test focusing on thyroid effect of a substance and described in the OECD GD 150 is the LAGDA as a level 4 test. In that view, the LAGDA assay is requested to generate these data and to definitively conclude on the ED hazard on the Substance for the environment.

In addition, the OECD GD 150 details that the LAGDA could "*be used at any stage in the hazard assessment process, the most likely use scenario will be when there are some data available about the possible thyroid disrupting properties of a chemical*" indicating that this would not lead to a data gap at level 3 of the OECD CF, contrarily to what you claimed. In order to avoid unnecessary animal testing by performing another assay at a later stage of the evaluation process, in case an AMA would be requested first, and to reach a definitive conclusion on the concern investigated, the LAGDA is the best option, the least onerous and the most informative. Indeed, the duration of the test is longer (112 days in the LAGDA vs 21 days in the AMA) providing a better assessment of toxicity, allowing other histopathological determination to check for toxicity before and after metamorphosis, a clearer and more accurate observation of delay in metamorphosis, and more concentration to be investigated in order to generate a more accurate estimation of dose-response effects.

In your comments to the draft decision, you considered that LAGDA significantly increases the number of test animals used with the requirement for additional test concentrations creating an animal welfare issue. In the LAGDA (OECD TG 241) one can read that : "*The test lasts about 16 weeks and requires a total of 480 animals, i.e. X. laevis embryos, (or 640 embryos if a solvent control is used) in order for the test to be powerful enough to evaluate the effects observed at the population level (e.g. growth, development and maturation of the reproductive system)*". A solvent control is necessary, therefore 640 embryos are needed for the performance of the test on the Substance. The AMA (OECD TG 231) states that : "*The best individual spawn(s) (2-3 recommended to evaluate the quality of the spawns) should be retained based upon embryo viability and the*

presence of an adequate number (minimum of 1500) of embryos. All the organisms used in a study should originate from a single spawning event (i.e., the spawns should not be co-mixed)". Finally, less tadpoles will be exposed for the AMA study (400 with a solvent control), even if the number of embryos recommended in order to guarantee survival of tadpoles at stage 51 will imply huge clutch sizes of 1500 embryos. When comparing the AMA to the LAGDA for proportionality of the requested test, the minimum amount of tadpoles needed in the AMA (320 larvae for three test concentrations plus the control) is lower compared to the LAGDA (480 larvae for four test concentrations plus control). However, as more (i.e. four or five) test concentrations are recommended for the AMA, the only significant difference between the tests is the number of replicates in the control: four for the AMA and eight for the LAGDA. However, in the LAGDA the higher number of replicates in the control addresses the variability better and, therefore, helps to ensure appropriate statistical power of the test. Moreover, if the AMA results are positive or inconclusive, a follow-up test may be required in the form of the LAGDA. Therefore, animal welfare is not a sound argument.

In a proposal for amendment, one Member State, while agreeing to the proposed LAGDA study, suggested to elaborate on another test available since 2019, the Xenopus Eleutheroembryonic thyroid assay (XETA; OECD TG 248). It is an aquatic screening test capable to detect thyroid disruptors if one of the following mechanisms of action is involved: metabolism by deiodinases, clearance/ hepatic metabolism, thyroid receptor agonist and thyroid receptor antagonist. Therefore, XETA does not cover other potential thyroid-disrupting modes of action such as interference with TH synthesis (NIS or TPO inhibition) that cannot be ruled out for the Substance. The XETA provides neither an unequivocal identification of the precise MoA activated by the Substance nor an associated apical endpoint relative to adverse outcomes as present in the AMA or in the more thorough LAGDA. Consequently the XETA test is not appropriate in this case to clarify the adverse effects potentially caused by endocrine activity of the Substance and especially effects on the thyroid in wildlife species.

In your comments to the proposal for amendment, you noted that you fundamentally disagree with the proposed modes of action detected by XETA. You have reiterated your reasons already provided in your comments on the draft decision why AMA should be requested instead of LAGDA.

d) Timeline for performing the assay

In your comments to the draft decision, you requested an extension of the timeline from 15 months, as indicated in the original draft decision, to 32 months. The requested extension was not substantiated with any evidence and, in consequence was not granted. Nevertheless, ECHA acknowledged that additional parameters than those listed in the OECD TG 241, developed and made available in the open literature, were recommended as detailed in section 2.1.b).
Therefore, ECHA has partially granted the request and set the deadline to 20 months., instead of the 15 months usually granted for performing this assay.

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 follow-up evaluation

Due to initial grounds of concern, the Member State Committee agreed to include the Substance (EC No 228-250-8, CAS RN 6197-30-4) in the Community rolling action plan (CoRAP) to be evaluated in 2012. France is the competent authority ('the evaluating MSCA') appointed to carry out the evaluation.

In accordance with Article 46(3) of REACH, the evaluating MSCA carried out its evaluation based on the information in the registration dossier(s) you submitted on the Substance on 29 March 2019 subsequent to a decision, and on other relevant and available information.

The evaluating MSCA completed its 'follow up' evaluation considering that further information are required to clarify potential risk on endocrine disruption.

Therefore, it submitted a further draft decision (Article 46(3) of REACH) to ECHA on 17 March 2020.

Decision-making

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

For the purpose of this decision-making, dossier updates made after the date the draft of this decision was notified to you (Article 50(1) of REACH) will not be taken into account.

(i) Registrant(s)' commenting phase

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

The evaluating MSCA took your comments into account (see Appendix A). The request(s) and the deadline were amended.

Amendment of the deadline

The deadline was extended to 20 months for the reasons explained in Appendix A, section 2.1.d).

(ii) Proposals for amendment by other MSCAs and ECHA and referral to the Member State Committee

The evaluating MSCA notified the draft decision to the competent authorities of the other Member States and ECHA for proposal(s) for amendment.
Subsequently, the evaluating MSCA received proposal(s) for amendment to the draft decision and modified the draft decision (see Appendix A).

ECHA referred the draft decision, together with your comments, to the Member State Committee. ECHA invited you to comment on the proposed amendment(s). You provided comments on the proposed amendment(s).

Your comments on the proposed amendment(s) were taken into account by the Member State Committee.

(iii) MSC agreement seeking stage

The Member State Committee reached a unanimous agreement in its MSC-78 written procedure and ECHA took the decision according to Article 52(2) and Article 51(6) of REACH.

After the deadline set in this decision has passed, the evaluating MSCA will review the information you 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[4].

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"[5].

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