Helsinki, 16 December 2024

Addressees
Registrants of the Registered substance subject to this decision ("the Substance"), listed in Appendix F of this decision[1].

Registered substance subject to this decision ("the Substance")
Substance name: 2,6-di-tert-butyl-p-cresol as listed on published CoRAP
EC number: 204-881-4

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 for the environment

1. The Larval Amphibian Growth and Development Assay (LAGDA, test method: OECD TG 241) (Request A.1) with the Substance, specified as follows:
   - When relevant, data on assay performances, quality criteria and validations (limits of detection, quantifications, coefficient of variations, specificity) must be reported.
   - Histopathology of the thyroid gland at test termination (juvenile sampling in addition to the larval one) must be performed.
   - Measurement of thyroid hormones in plasma (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 NF stage 62 and at test termination (juvenile sampling). Moreover, time to reach both stages must be accurately reported.

B. Information required to clarify the potential risk related to Developmental neurotoxicity and Endocrine disruption for human health

1. Developmental neurotoxicity study in rats via the oral route (dietary) (DNT, test method: OECD TG 426) (Request B.1) with the Substance, specified as follows:

   - Behavioral investigation and anxiety test must be performed at PND 25±2 and PND 60±2 on the same pups.

   - To assess if malnutrition (quantity and/or quality) could drive developmental neurotoxicity potentially observed and to assess if endocrine disruption could drive potential effects on milk:
     - *In dams:*
       - at LD10, milk nutritional quality, the Substance contamination levels, and metabolite levels must be measured;
       - mammary gland development must be characterized, at sacrifice of dams at LD21 (or a few days later i.e. when females are no longer lactating and before cyclicity resumes) by performing: Histology for the right 4th gland, and storage of the left 4th gland for molecular analysis of prolactin and

[1] Appendix F is removed from the version of the adopted decision published on ECHA's web page.

estrogen receptor 1, as well as milk synthesis markers (Caseins, lactalbumin) via RNA extractions and RT-qPCR analysis.
    - *In pups at PND11±1*: in addition to body weight and size (male and female) measurement, stomach must be sized and weighted.

- To assess if endocrine disruption could drive developmental neurotoxicity potentially observed:
    - *In dams at LD10 and LD21 (or a few days later depending on when dams have been culled)*:
        - hormonal measurements of free and total T4, circulating prolactin and estradiol level (plasma) as described in Appendix B, Section 2.1, b) must be performed;
        - histopathologic analysis and measurement of organ weight of the thyroid in dams in high dose and controls groups.
    - *In pups at PND11±1, PND25±2 and PND60±2*:
        - measurement of circulating IGF1, free and total T4 (if limited material available at PND11±1, only free).
    - *In pups at PND25±2 and PND60±2*:
        - measurement of estradiol and testosterone must be measured in plasma.

- To assess if hepatic metabolic enzymes activation could drive developmental neurotoxicity potentially observed:
    - *In dams after culling and in pups at PND25±2:* hepatic metabolic enzymes from these families must be assessed: cytochrome P450, deiodinase, UDP glucuronosyltransferase, Sulfotransferase, and the members transporters (OATP, MCT, TAT). The quantitative measurements must be performed by an appropriate method.

- For potential further analysis:
    - *In F1 pups at PND11±1, PND25±2 and PND60±2*: In addition to brain, liver and thyroid must be collected, together with the remaining plasma samples. The samples must be frozen and preserved at -80°C for further analysis.
    - *In dams after culling:* Blood must be collected for further measurement (see Appendix B below for further details).

Deadlines
You must submit information under request A and B above by **24 July 2028**.

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 studies in the corresponding endpoints 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 Appendices A and B 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[2] under the authority of Mike Rasenberg, Director of Hazard Assessment

[2] 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 and/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:
- There is a potential risk to human health and/or the environment, based on a combination of hazard and exposure information.
- Information is necessary to clarify the potential risk identified.
- There is a realistic possibility that the information requested would allow improved risk management measures to be taken.

In its comments to the draft decision, the lead registrant refers to an opinion from the Commission Scientific Committee on Consumer Safety ("SCCS") issued on 02 December 2021. The lead registrant indicates that, while the opinion states that there are remaining uncertainties in the ED evaluation, the use of the Substance in cosmetics was considered safe. The lead registrant also refers to an opinion from EFSA indicating that the use of the Substance as food additive is considered safe. The lead registrant considers that "*ANSES/ECHA are obliged by law to take into consideration the SCCS Opinion in the substance evaluation*" as:
- "*the cosmetic products use of substances is not exempted from REACH substance evaluation*".
- "*the REACH substance evaluation procedure and the approval of a substance under the Cosmetics Regulation require a risk assessment*".
- the lack of coordination with the SCCS "*can potentially amount to a violation of Article 95(3) REACH*".

The evaluating MSCA understands from the above comments that the lead registrant considers the SCCS and EFSA opinions as a sufficient basis to exclude any potential risk to human health and/or the environment. The evaluating MSCA emphasizes that these opinions point towards remaining uncertainties to conclude on the ED properties of the Substance. In particular, the SCCS opinion states that "[a]*lthough there are converging pieces of evidence suggesting that BHT might act on thyroid homeostasis through increased thyroid hormone hepatic catabolism, in the current knowledge, there is no direct proof that this mechanism holds true*" and emphasizes that "[e]*valuations are based on old studies, not always available, of poor reliability, with limited reports and not statistically powerful*". On this basis, the evaluating MSCA considers that the current decision does not contradict the earlier SCCS opinion since adequate information to clarify the potential ED properties of the Substance is lacking. Therefore, a potential risk to human health and/or the environment cannot currently be excluded. Furthermore, it is noted that SCCS opinions only address safety of cosmetic products to end-users (and not to workers) and do not evaluate environmental effects of substances used in cosmetics (Judgment of the General Court in cases T655/20 and T656/20). In that regard, the SCCS opinion states that "*Environmental concerns that substances used in cosmetic products may raise are considered through the application of Regulation (EC) No 1907/2006 ('REACH Regulation')*".

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

According to the amended CLP Regulation (Commission Delegated Regulation (EU) 2023/707 of 19 December 2022), *"An endocrine disruptor is a 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 a correlation between an endocrine activity and an adverse effect.

Following assessment of all available relevant information on the Substance, the evaluating MSCA has identified a potential hazard of endocrine disrupting (ED) properties for the environment, more specifically related to thyroid modality (T-modality) of the Substance, which must be clarified.

1.1.1 Adverse effects related to T-modality

Evidence based on in vitro data

De Abrew K.N. *et al*. (2022) compared the genomic signatures obtained with 5 different concentrations of the Substance to those obtained with various substances ("*a large external database containing signatures of other compounds (including many known endocrine disruptors)*") on 4 different cell lines. However, the genomic signature of the Substance was not compared with known Thyroperoxidase (TPO) or sodium/diiodide symporters (NIS) inhibitors which would have been useful to provide information regarding the identified concern.

In its comments to the draft decision, the lead registrant states that the relevance of the study by De Abrew *et al.* (2022) to justify the potential hazard is unclear as the study does not indicate an ED concern for the analogues.

The evaluating MSCA notes that among the three structurally similar substances showing high signature analogy with BHT displayed in the above publication, p-heptylphenol (CAS RN 1987-50-4) was identified as an endocrine disruptor for the environment and included in the candidate list of substances of very high concern for Authorisation on 12 January 2017. Therefore, the evaluating MSCA maintains that this piece of information is relevant. However, the evaluating MSCA acknowledges the absence of details on the method used in this study and on the way the conclusions are constructed. Therefore, the evaluating MSCA does not consider that the study, on its own, is sufficient to confirm the potential ED properties of the Substance.

Evidence based on in vivo data (T-mediated parameters)

As reported in the registration dossier, thyroid follicular cell hypertrophy/hyperplasia were consistently observed in the available repeated dose oral toxicity studies in rats using the Substance (Søndergaard D. and Olsen P. (1982), Price S.C. (1994) [later published under McFarlane M. et al. (1997)], Olsen P. et al. (1986)).

In addition, follicular adenoma/adenocarcinoma were reported in two 2-generation studies and long-term studies including carcinogenicity studies (Olsen P. et al. (1986); Price S.C. (1994)). Tumors reported in Olsen P. et al. (1986) included thyroid, pancreas, ovary, uterus, thymus, reticulo-endothelial system, and mammary gland, but incidence was not statistically significantly different from that in controls, respectively.

Søndergaard D. and Olsen P. (1982), which is an old and poorly reported study and a complicated design, explored the effects of the Substance on thyroid function depending on the type of diet given. The Substance was given to 10 Wistar rats in the diet at levels of 250 mg/kg bw/d for 30 days and absolute thyroid weight was increased in the 2 groups given different diets, both containing iodine. Other animals treated for 90 days with no specification of the diet displayed an increased ($p<0.001$) relative thyroid weight at 25 (n=10) and 250 mg/kg bw/d (n=30). The relative liver weight significantly increased at the high dose of 250 mg/kg bw/d only. **There is no co-morbidity between liver and thyroid effects as the thyroid weight was increased in both doses**, but the liver weight was unchanged at the lower dose.

As reported in the registration dossier, two long term-studies where rats were exposed daily to 0, 25, 100 and 250 mg/kg bw/d of the Substance in diet are available (Olsen P. et al. (1986), F0 (n=200) for 13 weeks and F1 (n=360) for exposure until 144 weeks), and McFarlane M. et al. (1997), (F0 (n=216) for 13 week exposure then F1 (n=360) until 98 weeks)). Olsen P. et al. (1986) described thyroid tumours at doses 100 and 250 mg/kg bw/d to be not statistically significantly different from that in control group of Wistar rats. It was reported thyroid hyperplasia as following: reduction of follicular size, absence or reduction of colloid, irregularities in the follicular outline, hyperaemia and increase in the number of follicular cells starting at 11 months at both 100 mg/kg bw/d (mild changes affecting 75-82% of the rats) and 250 mg/kg bw/d (marked changes affecting 100% of the rats) (McFarlane M. et al. (1997) in EFSA 2012). A comment provided by CEFIC-EBMA on this EFSA opinion indicated that at the 22 months interval, the animals in the 100 mg/kg bw/d group were observed to have thyroid hyperactivity in the absence of liver weight changes (but increased liver eosinophilia, glutathione-S-transferase and CYP2B).

1.1.2 Endocrine activity related to T-modality

Evidence based on in vitro data

From the US EPA CompTox Chemicals Dashboard (http://comptox.epa.gov/dashboard), the Substance is positive in ATG_PXR_TRANS assay with an AC_{50} of 20.56 µM, (expression in liver) that would suggest a mechanism of action (MoA) through CAR/PXR nuclear receptor also not demonstrated. In addition, the Substance is positive in CCTE_Simmons_AUR_TPO assay with an AC_{50} of 2.38 µM (well-characterized molecular-initiating events (MIEs) for adverse thyroid-mediated outcomes). Danish QSAR results are positive for TPO inhibition QSAR1 (Rat *in vitro*), TPO inhibition QSAR2 (Rat *in vitro*).

Evidence based on in vivo data

In Søndergaard D. and Olsen P. (1982), rats exposed to the highest dose of the Substance, 5000 ppm (corresponding to 250 mg/kg bw/d) presented a significant increase in the uptake of Iodine ^{125}I at all time periods studied (8, 26, 62 or 90 days). However, no change

in mean of 8 high dose group-rat blood levels of T3 and T4 measured after 90 days in the blood was reported.

In its comments to the draft decision, the lead registrant indicates that the above study was disregarded by the MAK commission (2012) for their assessment as the described effects at 25 mg/kg bw/d have not been detected in other longer-exposure studies (i.e., Olsen *et al.*, 1986; Hirose *et al.*, 1981).

The evaluating MSCA, who would like to emphasize that MAK, 2012 is a republication of (MAK, 2007), notes that the increased relative thyroid weights observed at 25 mg/kg bw/d in the study by Søndergaard D. and Olsen P. (1982) could not be observed in the other studies carried out with Wistar rats as this parameter was not measured at this low dose level. Therefore, carrying out further studies at this low dose would allow confirming whether 25 mg/kg bw/d can be seen as a NOAEL (as currently claimed by EFSA) or if it should be considered a LOAEL.

In an assessment review, rats exposed to the highest dose of the Substance (i.e., 250 mg/kg bw/d) presented a not statistically significant increase of serum T4 levels concomitantly with the thyroid hyperplasia observed at both 100 and 250 mg/kg bw/d (CEFIC-EBMA 1994).

[redacted] (2013a and 2013b) performed two tolerance studies on chickens and on weaned piglets respectively for determining Maximum Residue Level (MRL). These studies were assessed by EFSA for confirming the safety of the Substance (BHT) as a feed additive. The question of endocrine modulation was not assessed except some rare hormonal measurements in some timepoints on some animals:

- In [redacted] (2013b), 540 chickens were fed with a basal diet, containing 0, 150, 1000 or 1500 mg BHT/kg for 35 days which are equivalent to doses of 0, 11, 76 and 173 mg BHT/kg bw/d. As this was not the aim of the study, Thyroid-stimulating hormone (TSH), free thyroxine (FT4) and free triiodothyronine (FT3) were measured on nine animals per group only at day 35 only. No effect on these hormones was observed by the authors on chicken.
- In [redacted] (2013a), 144 pigs (80 castrated male (CM) and 64 female (F) piglets) were fed with the Substance in one of 4 dose groups. The animals were exposed to doses of 0, 150, 1000 or 1500 mg BHT/kg feed equivalent to 0, 6, 40 and 60 mg BHT/kg bw/d over 42 days. Only few animals were selected for T3/T4 testing as follows: TSH, FT4 and FT3 were measured at D0 and D43 (at D0, 20 observations (3 CM+2 F in control group, 3 CM +1F at 6 mg BHT/kg bw/d, 2 CM+3F at 40 mg BHT/kg bw/d and 2 CM+4F at 60 mg BHT/kg bw/d) and at D43, 48 observations (12 samples (6CM+6F) per dose and per control) though these observations were not performed on the same animals across the 2 timepoints). No adverse effects were observed related either to the thyroid hormones (TSH, FT3, FT4) or hepatic function (GOT, GPT, GGT).

A statistical analysis of data was performed by the evaluating MSCA on the 2 [redacted] studies to investigate the effect of the Substance on thyroid hormones in pig and chicken. Different parametric models were tested using ANOVA test (lm function), linear and nonlinear fitting (second degree polynomial). In addition, multivariate analysis (a non-parametric test (Wilcoxon-Mann-Whitney) was used on pig data to explore, for each group (control and treated at different levels), the effect of time on hormone level. The only statistically significant result was a decrease in T4 free thyroxine (FT4) found for pig at the highest dose tested (60 mg/kg bw/d) between day 0 and day 43 (Wilcoxon Mann Whitney, $p < 0.001$).

In its comments to the draft decision, the lead registrant states that "*without additional information* [on the statistical analysis performed by the evaluating MSCA], […] *it is not clear why this particular statistics is considered biologically most relevant*".

The evaluating MSCA requested the registrant to provide the performance assay for further analysis, without success. Retrospective statistical analysis was conducted on the data as described above. The statistical analysis was performed to circumvent the limited statistical power imposed by the experimental design of the studies (i.e., measured values are available at the group level only and not at the level of individuals) and not chosen as the biologically most relevant. Evaluating MSCA foresaw that the study design (and corollary bias) did prevent any definitive conclusions. However, the results of this statistical analysis indicate that the Substance induces a modulation of T4 in pigs: the concern on the thyroid modality may not be a rodent-specific effect.

Both studies ([redacted], 2013a and 2013b) have limited added value as they were not performed as toxicological assays. Based on the low doses tested, the sample size for measuring thyroid hormone modifications should have been increased, to be able to detect subtle effects. This would also have allowed the use of parametric hypothesis (at D0 in pigs by example), by considering sex as covariate. In pigs, individuals were identified at D0 but there was no follow up of the same animals at day 43 while in chicken, animals when only measured at day 35. It was not possible to follow individual temporal dynamic on thyroid hormones. No correlation with any gross macroscopy was achieved as individuals were not followed but only groups of animals. Therefore, these studies have limited statistical power due to their experimental design (as groups are followed instead of individuals). The fact that only one thyroid hormone effect is reported in these studies is sufficient to confirm the concern related to the Substance on this modality. No strong conclusion can be reached based on these studies and further analysis are needed.

The existing data show concern regarding the T-Modality. However, the number of reliable studies measuring thyroid hormones that can be used in the assessment is limited and do not allow to conclude on the Thyroid concern.

- T4 serum level was increased in a non-statistical manner at the only dose tested (CEFIC-EBMA 1994) in one study out of the only 2 rodent studies where it was measured.
- Free thyroxine (FT4) was modified in pig at the highest dose tested but not in chicken though the doses used were low and the study design not appropriate.
- T3 hormonal level was not modified in 2 rodents' studies. It was not modified in pig and chicken though the doses used were low and study design not appropriate.
- TSH hormonal level was not measured in any rodent study. It was not modified in pig and chicken though the doses used were low and study design not appropriate.

1.1.3 Mode of action of the Substance for the T modality

a. Direct thyroid mode of action via TPO inhibition

As mentioned above, the Substance is positive for TPO inhibition in the_ATG_PXR_TRANS assay and in CCTE_Simmons_AUR_TPO assay (well-characterized MIE for adverse thyroid-mediated outcomes) and in the Danish QSAR (which might be built on the assays mentioned above). Several *in vivo* studies report a possible endocrine disruption activity of the Substance on thyroid gland and thyroid axis. Thyroid hyperplasia or hyperactivity with increased T4 level measurements are reported in a study carried out on rats (CEFIC-EBMA 1994). Other old studies also performed in rats reported effects on the thyroid histology but without any further details on the levels of TSH, T3, T4 in plasma. Regulation of the thyroid hormone (TH) signalling axis is complex and there are multiple mechanisms

by which disruption of TH homeostasis may occur. The various molecular initiating events, integrated on the Adverse Outcome Pathways (AOP) network are described in Murk et al. (2013) and Noyes et al. (2019). There is no data available after the Substance exposure on expression of sodium/diiodide symporters (NIS) or activity of deiodinase enzymes converting T4 into T3 in peripheral tissues.

Available *in vitro* and *in vivo* mechanistic data, also few, seem to support the following MoA: interference with TH synthesis by TPO inhibition, and the adverse effect observed, thyroid hyperplasia. According to the ECHA/EFSA ED guidance (2018): "*Substances inducing histopathological changes (i.e., follicular cell hypertrophy and/or hyperplasia and/or neoplasia) in the thyroid, with […] changes in the circulating levels of THs, would pose a hazard for human thyroid hormone insufficiency in adults as well as pre- and post-natal neurological development of offspring".*

b. Alternative mode of action: Hepatic enzymatic induction that might lead to thyroid disruption

Søndergaard D. and Olsen P. (1982) proposed a *hepatic primary mechanism* to explain the effects on thyroid in rat: "*hepatic enzyme induction and increased catabolism of TH lead to a compensatory cascade involving an acceleration of iodine cycle in connection with the increased needs to maintain circulating levels*". Based on the observations in the report, there is no direct evidence that the toxic mechanisms on the thyroid are due to increased hepatic metabolism of TH. If such mechanism is true, T4 biological half-life would be expected to be shorter in the Substance-treated animals whereas opposite effects were observed with longer T4 half-life.

CEFIC-EBMA 1994 reported that an induction of CYP P450 2B was observed in rats exposed to high-dose of 250 mg/kg bw/d. In addition, EFSA in 2012 calculated an increase by 30-60% in total cytochrome P450 (CYP P450 2B) content in the animals receiving the high-dose of the Substance, and a dose-related increase in epoxide hydrolase (EH), glutathione-S-transferase (GST) and pentoxyresorufin-O-depentylase (PROD) activities, were reported starting at 21 days of age, statistically significant in the mid- and high-dose groups. There was also a marked induction of gamma glutamyl-transferases (GGT) activity of nearly all the high-dose treated rats, starting after 11 months of treatment.

Macroscopic liver effects:
A dose-dependent increase in hepatocellular carcinomas was observed in male rats from 100 mg/kg bw/d, as well as an increase in hepatocellular adenomas in males and females from 100 and 250 mg/kg bw/d (Olsen P. et al. (1986)). In McFarlane M. et al. (1997), immunohistochemical staining of liver sections revealed a marked persistent increase in hepatocellular content in rat treated with 500 mg/kg bw/d for 98 weeks.

Based on the results of the two 2-generation studies including carcinogenicity, EFSA in 2012 concluded that the Substance has tumour promoting effects in some animal models when used at high doses. The induction of hepatic enzymes activities and thyroid hyperactivity in the mid- and high-dose groups, together with the tumour data, lead to propose a NOAEL of 25 mg/kg bw/day. These *in vivo* data are correlated with *in vitro* data on cultured human hepatocytes treated with (2-200 µM) of the Substance. A concentration-dependent increase in mRNA levels for CYP2B6 and CYP3A4 was demonstrated up to around 12- and 7-fold, respectively, with no cytotoxic effects at these concentrations (Price R.J. et al., 2008).

In its comments to the draft decision, the lead registrant refers to the EFSA re-evaluation of the Substance (EFSA, 2012) which states that "*Given that the rodent is a sensitive*

model for measuring the carcinogenic influences of TSH and that humans appear to be less responsive, effects on rodents would represent a conservative indicator of potential risk for humans" and to Manibusan and Touart (2017) which states that "*The Agency (US EPA) has determined that the chemicals producing thyroid follicular cell tumours formed via disruption of thyroid-pituitary function in rodents are not likely to be carcinogenic to humans because of significant quantitative differences in anticipated levels of exposure. Rodents do not have the thyroxine binding globulin that humans have and therefore have a heightened sensitivity to perturbations of thyroid hormone levels. The rodent bioassay, therefore, is considered conservative for predicting carcinogenicity in the human population*".

The evaluating MSCA notes that a debate on the representativeness of rodents for human carcinogenicity is not relevant to the current request as it aims to evaluate whether the effects observed on rodent's thyroid are associated to thyroid disruption in other environmental species.

In its comments, the lead registrant also refers to a non-mammalian assay (Yu et al., 2018). In this 10-week growth study conducted to investigate the efficacy and tolerance of dietary exposure to the Substance, inflammation, apoptosis and hepatic disease related to oxidative stress was evaluated in largemouth bass (*Micropterus salmoides*). The registrant states that "*No effects on growth have been detected in fish fed up to 23.80 mg/kg bw/d of BHT which is several folds over the expected environmental concentrations*".

The evaluating MSCA emphasizes that the publication mentions that *Micropterus salmoides* are cultured under poor conditions leading to liver damage (probably due to an inappropriate, over-rich and oxidized diet). Under such conditions, the Substance provides effective protection on unhealthy animals having liver diseases that are not typically observed under natural conditions. The study also seems to indicate that the Substance induces phase liver I and II enzymes in fish which could lead to physiological alterations in "normal" fish. However, the study did not assess the induction of thyroid hormones.

While, as mentioned in the registrant comments, there are beneficial effects of antioxidants (e.g., protection against aging, anti-tumorigenicity and reduction of ischemia-reperfusion injury (Felter et al., 2021), high doses of some antioxidants have been associated with impaired reproduction, teratogenesis, growth retardation and alterations in hepatic metabolism (Hoffman, 2002). The evaluating MSCA does not dispute the consideration that the Substance may have beneficial antioxidant properties under specific conditions. However, in itself, such property does not allow excluding that the exposure to the Substance may be detrimental to specific life-stages (e.g. development stages).

Gaunt I.F. et al (1965) reported an increase in the relative liver weight observed in weanling Carworth SPF rats that received 150 mg/kg bw/d of the Substance in diet for periods of up to 16 weeks. A significant increase in relative liver weight was observed from 250 mg/kg bw/d (Søndergaard D. and Olsen P. (1982)).

It is not possible to conclude that the indirect mechanism involving increased catabolism of TH by the hepatic enzymatic induction is the (unique) MoA explaining the thyroid disruption observed. Indeed, results showing that liver enzyme induction does not lead to increased TH clearance in humans are needed to dismiss the decrease in TH observed in rats because of induction of hepatic metabolism for human risk assessment. Such observations do not exist for the Substance. The data are inconclusive regarding T4 pharmacokinetics (AUC, clearance, terminal half-life, T_{max}, C_{max}) and there is no quantitative data regarding circulating TSH that proves that TSH secretion is modified.

Therefore, it is only assumed that increased TH catabolism in liver occurs in humans. The link between the induction of hepatic enzymes and the disruption of the thyroid-pituitary function is not clearly evidenced.

In its comments to the draft decision, the lead registrant claims that the Substance has been proved to be an hepatic enzymatic inductor and refers to the study by Manibusan and Touart (2017). The registrant supports this assertion by indicating that the changes in TSH occurred at doses where increased Phase I/II activity as well as increased weight and microscopic changes in the liver were already established, and the absence of significant effects on circulating T4 provides evidence of the adaptive rather than adverse nature of the thyroid findings.

The evaluating MSCA notes that in the registration dossier, three studies showed effects on thyroid without liver effects (Søndergaard D. and Olsen P. (1982), Price S.C. (1994), Olsen P. et al. (1986)). In the study by Sondergaard D. and Olsen P. (1982), relative thyroid weights increased at 25 mg/bw/d when the relative liver weight significantly increased at the high dose of 250 mg/kg bw/d only. There is no other study to prove that both effects happen concomitantly.

Furthermore, the study by Manibusan and Touart (2017) corresponds to an evaluation of the adequacy of current test methods to inform regulatory risk assessments on whether a substance has the potential to disrupt endocrine-related pathways and to lead to human adverse effects. It does not provide any data specific to the Substance that could conclusively demonstrate that the Substance acts only via the postulated MoA.

The comments on the draft decision does not include any additional information to those already addressed in the draft decision allowing to reliably confirm that the effects observed on thyroid disruption are indirect and result from catabolism of thyroid hormones following hepatic enzymatic induction. As indicated in the ECHA/EFSA Guidance for the identification of endocrine disruptors (2018), "*potentially endocrine-related adverse effects observed at, or below the MTD or MTC, can be considered as secondary to other (non-endocrine) toxicities only if substantiated by the MoA analysis*". Currently available evidence does not demonstrate that an alternative non-endocrine mechanism of action is the most likely explanation and that the observed effects are not endocrine-mediated.

c. *Consideration of possible rodent-specific effects*

The toxicological paradigm of dismissing the rat as a predictor for any thyroid effects in humans is at least partly based on the premise that rats have much less (in some stages of their life) thyroxin binding globulin (TBG). TBG is the major and specific transport protein of TH in humans, other primates and dogs. TBG is a high affinity binding protein, which has a 1000-fold greater binding affinity compared to non-specific low affinity protein carriers which bind T4 (and T3 to a lesser degree). TBG is not present in rodents, birds, amphibians and fish (Larsson M. et al., 1985). The evaluating MSCA acknowledges sensitivity differences in rodents, particularly regarding the thyroid hormone binding proteins. In the rat, 75% of T4 is bound to Transthyretin (TTR) and 25% to albumin (Chanoine J.P. et al., 1992) in contrast in humans 75% is T4 is bound to TBG, 20% to TTR and 5% to albumin (Refetoff 2015 cited in Li A.A. et al., 2019). However, TTR is also a transporter vital to normal human embryogenesis. TTR aids trans-placental passage of maternal T4 to foetal circulation and inadequate TH delivery leads to developmental abnormality (Saha S. et al 2017). Therefore, the evaluating MSCA considers that human relevance of the registrant's proposed mode of action cannot be excluded, particularly during development. Moreover, the comments on the draft decision do not provide any evidence to demonstrate that this mode of action cannot occur in other taxa than rodents.

TTR has lower affinity for T4 and is therefore less efficient in binding T4 than TBG. Thus, in species in which TBG is not the major T4 carrier, the free fraction of the hormone is larger than in humans and total T4 half-life appears to be shorter (Leghait J. et al. (2009, 2010)). Therefore, the adverse effects observed in rodents following this MoA should be relevant for amphibians.

According to the ECHA Board of Appeal decision in case A-10-2022, 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 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 D.R. (2017)). TH is essential for vertebrate development; hence it is important to consider the environmental impact of the Substance on the actions 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 signalling during development. However, none of the available ecotoxicity studies provide information on apical and adverse effects specific to T-modality in other taxonomic groups.

In its comments to the draft decision, the lead registrant reiterates arguments provided in earlier consultation on the factors contributing to the greater sensitivity of the rat to long-term perturbation of the pituitary thyroid axis.

However, the comments on the draft decision do not provide any evidence to demonstrate that this MoA cannot occur in other taxa than rodents.

1.1.4 Overall conclusion

The evaluating MSCA considers that the available information is not sufficient to conclude on the potential ED hazard of the Substance for the environment. Therefore, further information to clarify the potential hazard for endocrine disruption via the thyroid pathway is necessary.

In its comments to the draft decision, the lead registrant challenges the above conclusion. In particular, the registrant refers to a 'Statement on potential endocrine disruptor activity of 2,6-di-tert-butyl-p-cresol' (2016) which concludes that "[t]*here is at least "reasonable evidence" ("biological plausibility") demonstrating that liver induction is the primary effect of BHT leading to the secondary effect thyroid activation in rodents. Consequently, the respective non-endocrine-related liver toxicity should not be considered as an adverse effect that is relevant for the identification of a substance as an endocrine disruptor*". To support this conclusion, the registrant provides an assessment of the existing body of evidence against the OECD conceptual framework (CF) described in the OECD GD 150.

With regard information available at CF level 1:

- No alerts for Estrogen/Androgen receptor binding, estrogenic and androgenic effects or thyroid receptor a & b (VEGA QSAR)
- Positive potential for TPO inhibition (rat in vitro) PXR and CAR binding based on human in vitro predictions (Danish QSAR)
- Medium probability of binding to LX-Rβ, A-R and M-R, and T-Rα (disruptome predictions)
- Low numbers of 'active' results for E and A bioassays (EDSP21 US EPA CompTox).

The evaluating MSCA notes that the QSAR predictions undertaken using the VEGA tool are not described in either the registration dossier or the comments to the draft decision. In

the absence of QMRFs and QPRFs describing these predictions, ECHA is not able to independently assess this information. The evaluating MSCA also considers that the disruptome predictions are of limited reliability as chemical structures are compared to only 18 structures, and weak performance of the model prediction for the mineralocorticoid (M-R) receptor is reported (http://endocrinedisruptome.ki.si/). Finally, it is noted that the registrants do not provide their interpretation of CF level 1 data. The evaluating MSCA considers that this information indicates that the Substance has an effect on thyroid activity. However, this information does not allow reaching any conclusion on the registrant's proposed mode of action and nor to demonstrate that this mode of action is rodent-specific.

With regard to information available at CF level 2, the lead registrant considers that "*mechanistic in vitro studies did not evidence with any reliability or consistency that* [the Substance] *had consistent activity on any endocrine modality*". In particular, the registrant states that:

- "*of the 11 reported assays within the EDSP21 US EPA CompTox dataset, only 5 reported potential activity on the Thyroid Peroxidase ("TPO")*". There is no other data measuring TPO activity *in vitro*
- "*mechanistic in vitro studies did not evidence with any reliability or consistency that BHT had consistent activity on any endocrine modality […] although the assay indicates an 'active' outcome*". The registrant identified "*additional parameters (concomitant evaluation of nonspecific luciferase activity and cytotoxicity) that should have been tested in parallel*"
- "*of those 5, the leading effect can be concluded to be cytotoxicity rather than TPO modulation in 4*"
- "*one study evaluating thyroid peroxidase inhibition [(The Amplex UltraRed-thyroperoxidase ("AUR-TPO") assay) showed] active result could be considered as relevant*" as those were observed below the cytotoxicity cut off. However:
  - "*The assay was* […] *poorly described within the ToxCast data set, with limited information on substance analytics* […], *no method detailed outside of linked publications outlining the approach, no reporting on deviations, and no critical evaluation or discussion of the results available*"
  - "[the assay] *can suffer with a high rate of false positive result [and] additional control assays to evaluate cytotoxicity and nonspecific signal loss are required alongside*. [..]*for the assay on BHT* […] *no further detail on either of these aspects is provided.*"
  - The registrant refers to Friedman et al. (2016) which state that "*this assay is well suited to HTS and prioritization but not necessarily for mechanistic evaluation of TPO inhibition*" and to Hassan I. et. al. (2020) "*the quantitative relationship between TPO inhibition in the AUR-TPO assay and the magnitude of TH change that would be expected as a result of this inhibition is yet to be elucidated*". The registrant therefore considers that the assay should be considered as providing pre-screening level information only.

The evaluating MSCA acknowledges the registrant's concerns on the available EDSP21 data for the modulation of TPO and does not dispute the fact that the few available data may have limitations in respect of this assay. Nevertheless, this data as part of all evidence including other levels of the CF, indicates a potential hazard for endocrine disruption via the T-modality that requires further clarification. OECD CF level 1 & 2 data available for the EAS modality also demonstrates a potential risk of affecting (anti-)estrogenicity and (anti-)androgenicity requiring further clarification which is addressed under Appendix B.

Regarding information available at CF level 3, the lead registrant states that "*the available studies were found to be of limited relevance and reliability*". To its knowledge, there is no available CF level 3 study on thyroid, neither on rats nor on any environmental species.

The evaluating MSCA agrees that limited information is available at OECD CF level 3 for the assessment for thyroid adversity. An OECD CF level 3 assay is available for the potential risk on developmental neurotoxicity, specifically relating to a mode of action affecting lactation. However, due to the limitations described in Appendix B, Section 1.1.2, this study is considered as an alert to a potential endocrine effect of the Substance affecting lactation which is further addressed under Appendix B.

With regard to information available at CF level 4/5, the lead registrant states that "*multiple comprehensive in vivo studies were available for review* […] *which demonstrated multiple lines of evidence that the liver is the primary target* […]".

However, for the reasons already explained under section 1.1.3. b), no conclusive evidence to support this claim has been provided.

Based on the above, the evaluating Member State considers that the information in the dossier and in the comments of the registrants does not provide conclusive evidence that the effects observed *in vivo* result from the postulated non-endocrine related pathway and that no additional MoA is involved. As indicated in the ECHA/EFSA Guidance for the identification of endocrine disruptors, an alternative non-endocrine mechanism of action should be demonstrated and in a comparative analysis found to be the most likely explanation to consider the effect as not endocrine mediated.

In its comments on the proposals for amendment, a member registrant reiterated that the Substance has no direct effect on thyroid homeostasis, and that the Substance affects the thyroid based on a secondary non-endocrine MoA. In support of this claim, the following information was provided in a dossier update:

- 2,6-di-tert-butyl-p-cresol: *In Vitro* Inhibition of Thyroid Peroxidase (TPO)-Catalyzed Iodination using Rat and Human Thyroid Microsomes;
- *In vitro* Interaction Studies of 2,6-di-tert-butyl-p-cresol with the human NIS (SLC5A5) and rat Nis (Slc5a5) Sodium Iodide Symporter.

The evaluating MSCA takes note of this new information and acknowledges that such information may constitute useful screening tools to identify potential disrupting chemicals that may induce hypothyroxinemia/hypothyroidism (see for e.g. ENV/JM/MONO(2014)23). However, such screening tool only evaluate direct pharmacodynamic interactions between the test compounds and specific mechanisms that may result in alteration of NIS and/or TPO expression. The evaluating MSCA emphasizes that several other mechanisms may be relevant and remain not investigated (for e.g., thyroid stimulating hormone receptor (TSHR) signalling or Na/K-dependent ATPase or dual / thyroid oxidase (DUOX) function). The evaluating MSCA is of the opinion that based on currently available knowledge and methods, it is not possible to demonstrate that the effect of BHT is exclusively resulting from a secondary non-endocrine MoA.

In addition to the issue described above, the evaluating MSCA has identified the following deficiencies affecting the reliability of the provided TPO inhibition study:

- two out of the four experiments were rejected by the study authors themselves;
- one of the accepted experiments was conducted with a concentration of H_2O_2 that was too high. The concentration of H_2O_2 in the final incubation sample is 250µM. The effect of this strong oxidant on the Substance, which is an antioxidant, is not assessed nor explained. Thus, the antioxidant effect of BHT could be hidden by an excess of H_2O_2;

- the accuracy of the quality control was flawed because several measurements of MIT were reported as outside the acceptability criteria (Table 9 of IUCLID study endpoint record)
- DIT was not measured, leading the bioassay to be less sensitive;
- it is claimed that BHT could not be tested at concentration above 3.16 mM due to its low solubility. However, information provided by BHT's suppliers indicate that BHT is soluble from 30 mg/mL (Cayman Chemical, 2023) to 60 mg/mL (Targetmol, 2023) in DMSO (136-272 mM) i.e. 43-86 times higher than the dose tested;
- the negative result obtained in this study is questionable as it is known that natural antioxidants inhibit TPO (Habza-kowalska, 2019). As BHT is used as an antioxidant, it is somewhat surprising that it would not display any effects on such test, supporting its weak sensitivity;
- the test is limited to a very specific part of the extensive mechanisms of action *in vivo* for which the physiological relevance of this system is questionable.

Regarding the NIS inhibition study, the evaluating MSCA has identified the following issues based on the IUCLID study endpoint record (study report not available):

- the maximal concentration of BHT used is 10 time less than perchlorate (the report claims some limits regarding nephelometry);
- the quantification method (colormetric or radioactive iodine with counts per minute indicated in the results)_is not clearly reported;
- only nominal concentrations are reported, thus real exposure concentrations are not known;
- the expression of transfected NIS was not clearly assessed (e.g. by Western blot) and any impact of BHT on NIS expression is not tested;
- no information is given on the potential metabolisation of BHT by the cells, even if exposed for short duration;
- the test evaluates only iodine uptake which is a very specific part of the potential extensive mechanisms of action *in vivo*.

In its comments on the proposals for amendment, a member registrant referred to an ongoing comparative *in vitro* hepatic enzyme activity assay to assess whether the proposed secondary non-endocrine MoA is relevant for humans. The evaluating MSCA takes note of the ongoing assay, however as this data is not currently available for assessment and due to the deficiencies observed in the submitted *in vitro* TPO and NIS assays, it cannot currently be concluded that there is no concern for the potential risk of endocrine disruption. Therefore, based on the above, the evaluating MSCA concludes that the additional information referred to in these comments and provided in a dossier update, do not change the assessment.

1.2 Potential exposure

According to the information submitted in the chemical safety reports (CSR), the aggregated tonnage of the Substance manufactured and/or imported in the EU is in the range of 10 000 – 100 000 tonnes per year.

Furthermore, it is reported that the Substance is used by consumers, in articles, by professional workers (widespread uses), in formulation or re-packing, at industrial sites and in manufacturing as food, feed additive, antioxidant and stabiliser, as lubricant and lubricant additive, as fuel and fuel additives, as laboratory agent, as anti-freezing agents, biocide substances, in anti-freeze, leather and washing and cleaning products, plant protection products and biocides and in cosmetics.

The available information indicates that the environmental emissions of the Substance can originate from all stages of the life cycle of the Substance, from industrial uses to wide dispersive uses (i.e., consumer uses).

The Substance has a low solubility in water (0.76 mg/L at 20°C), a lipophilic tendency (log K_{ow} = 5.1), medium volatility in water (Henry law constant: 1.13^{02} $Pa.m^3.mol^{-1}$), high potential of resorption into sewage sludge, sediment, and soil compartments (log K_{oc}: 4.362), relative stability in environmental compartments (from biodegradation studies).

From the available scientific literature (Wang W. et al., 2018, Liu R. et al., 2020, Wang W. et al., 2021, Fries E. et al., 2004), the Substance was detected in various environmental matrices including sea, sediment, and river water.

Furthermore, data from the Norman network (https://www.norman-network.com/nds/common/) indicates that the Substance was detected in surface water across seven countries (Georgia, Romania, France, Germany, Netherlands, Slovakia, Ukraine) between 2010 and 2020.

Therefore, significant exposure of the environment is expected to occur.

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 is suspected to be an endocrine disruptor in the environment according to the CLP and WHO/IPCS definition.

The information provided on manufacture and use demonstrates a potential for exposure of the environment, while publicly available information demonstrates presence of the Substance in environment.

Based on the potential hazard and the existing exposure information of the Substance poses a potential risk to the environment.

As explained above, the available information is not sufficient to conclude on the hazard, and particularly the ED properties in the environment. Consequently, further data is needed to clarify the potential risk related to endocrine disruption properties for the environment.

1.4 Further risk management measures

If the endocrine disruption properties of the substance are confirmed, the evaluating MSCA will analyse the options to manage the risk(s) and will assess the need for further regulatory risk management measures in the form of:

- Harmonised classification as an Endocrine Disruptor to the environment under the Commission Delegated Regulation (EU) 2023/707 of 19 December 2022 amending the CLP Regulation (EC) No 1272/2008 and/or identification as substance of very high concern (SVHC) under Article 57(f) of REACH due to endocrine disrupting properties relevant for the environment: The SVHC identification would trigger additional information duties of producers and importers to ECHA according to Article 7(2) of REACH and information duties in the supply chain and for consumers according to Article 33 of REACH.
- Authorisation or a restriction of the Substance to further limit the use(s) of the Substance: 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 will be performed

within each authorisation, according to Article 62(4)(d) of REACH. This would result in stricter risk management measures than those currently in place, such as improved measures at manufacturing sites, better waste management and revised instructions on safe use, if appropriate.

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.

2 How to clarify the potential risk

Two tests are requested in parallel in the decision. The OECD TG 241 is needed irrespective of the results of the OECD TG 426, because the effects that could be seen in the OECD TG 426 will not be sufficient to conclude on the potential endocrine disruption for the environment (population relevance).

In its comments to the draft decision, the lead registrant provides that "*the critical driver behind the effects observed in the previous rodent studies is the induction of hepatic enzymes), which is commonly observed in the rodent following exposure to xenobiotics*". The lead registrant emphasizes that "*it is important that* [the observed effects] *are understood from a mechanistic perspective to ensure that an appropriate assessment is made as to whether the observed effects are indeed meeting the criteria set for ED substances* (i.e., a direct effect on the thyroid) *or whether they are secondary effects* […]". The lead registrant further considers that "*for ED identification, the ED mode of action and the adverse effects must be proven first and then causality has to be demonstrated*". The lead registrant notes that "*the adverse effects* [of the Substance] *has not been established yet, as further data are requested*" as per this decision.

The lead registrant also claims that similar to Amphibian Metamorphosis Assay (AMA), the LAGDA is "*unable to separate thyroid hyperactivity from a precursor liver and increased liver enzyme activity* and notes that while the LAGDA presents additional parameters to the AMA "*which may serve alongside thyroid histopathology as a good indicator of expected liver hypertrophy,* [it] *is unlikely to provide definitive evidence of liver induced thyroid effects due to the rudimentary study design*". On this basis, the lead registrant considers that the requested study will no provide further information on the MoA than that already available and disagrees that the requested study (*i.e.*, the OECD TG 241) has a realistic possibility of clarifying the potential concern identified. The lead registrant concludes that the decision "*is directly addressing causality without considering the MoA as required by law and the* [ECHA/EFSA ED] *Guidance*".

The evaluating MSCA notes that the registrants do not dispute the fact that the Substance affects thyroid in existing rodent studies. Furthermore, as mentioned above, thyroid activity is demonstrated by CF level 1 and 2 data. As indicated in ECHA/EFSA guidance for the identification of endocrine disruptors, an alternative non-endocrine mechanism of action should be demonstrated and in a comparative analysis found to be the most likely explanation to consider the effects as not endocrine mediated. The evaluating MSCA also notes that Table 4.2.1 of Annex I to CLP setting out the criteria for ED ENV does not require "*causality*", but instead a "*biologically plausible link*" or a "*correlation*" between the ED activity and the observed adverse effects.

The evaluating MSCA emphasizes that, as already stated above, there is currently no conclusive evidence available demonstrating that the observed effects are secondary to increased thyroid hormone hepatic catabolism. Furthermore, the evaluating MSCA considers that any MIEs proposed in the AOP by Noyes et al. (2019) and inducing thyroid

activity are relevant to identify an endocrine disruptor. It is noted that several MIEs (including hepatic mechanisms) may lead to the same key event (i.e., variation in T4 and/or T3 levels) and to the same adverse effect. Therefore, any testing strategy relying solely on investigating whether the observed effects are secondary to increased thyroid hormone hepatic catabolism, would not conclusively demonstrate that no other endocrine-related MIE is involved nor allow conclusion on the inadequacy of the effects observed. In this context, the evaluating MSCA also emphasizes that the ED definition does not require one endocrine activity to be exclusive of the others (see page 40 of the ECHA-EFSA guidance paragraph "Multiple MoAs").

The aim of the current request is to evaluate if the observed thyroid adverse effects in rodents are relevant to amphibians. The evaluating MSCA maintains that the request for a Larval Amphibian Growth and Development Assay ('LAGDA'; OECD TG 241) is appropriate as it aims at clarifying whether the Substance may pose a risk to non-rodent species using a valid test method to examine the potential endocrine disrupting properties of a substance (Decision of 17.01.2023, SCAS Europe, para. 111-116). The requested *in vivo* measurements of thyroid hormones will further inform on the causality of adverse effects that may be observed in the requested study.

Finally, the lead registrant challenges the human relevance of the observed effects leading to the potential risk and states that "*the main difference between humans and rodents is the higher T4 half-life due to the thyroxine-binding globulin existing in humans, but not in rodents or amphibians*". The registrant indicates that the requested study will not allow clarifying human relevance.

The evaluating MSCA emphasizes that this request is not aiming to demonstrate relevance to human but rather than to conclude on the potential ED properties of the Substance for the environment.

2.1 Request A.1: The Larval Amphibian Growth and Development Assay (OECD TG 241)

a) Aim of the study

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

The requested Larval Amphibian Growth and Development Assay (LAGDA) aims at clarifying the potential risk that the Substance poses to the environment and to clarify if the disruption of thyroid function leads to environmental adversity of the Substance at the population level.

OECD TG 241 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 other so-called diagnostic endpoints on endocrine system and especially on thyroid and thyroid gland function allowing identification of suspected endocrine disrupting chemicals (EDCs) or other types of developmental and reproductive toxicants.

No apical endpoints data have been identified to acknowledge that disruption of thyroid function leads to adverse effect in non-mammalian environmental species while it is

identified in rodent. Environmental adversity of the Substance at the population level cannot be excluded. This concern therefore must be further clarified.
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.

b) Specification of the requested study

Study, quality, and statistics

The study must be performed according to the OECD TG 241 (OECD 2015). All quality criteria must be fulfilled. According to the OECD TG 241, the number of replicates is doubled (8 replicates) for controls compared to each of the four-test concentration (4 replicates) to give adequate statistical power for the test.

To optimise the test further regarding the potential endocrine disruptor properties of the Substance, the OECD TG 241 is requested with some additional parameters to be measured during testing. They are described in the sections below.

Test material and concentration

The requested study must be performed with the Substance as registered. For the purposes of this test, results from existing studies cited in this decision must be considered in determining the highest test concentration to avoid concentrations that are overtly toxic. To better choose the adequate range of concentration, and if there are no relevant data to be used for dose level setting, if a range-finding study is performed, the results must be provided. You must 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 needs to be checked 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 freshwater vertebrates cover their main iodine demand via the food.

Analytical monitoring

Regular analytical monitoring of the Substance is required to know its exact concentration during the experiment and to evaluate its disappearance should it occur. 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 recommended to operate at least 5 tank turnovers per day. You must 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 Substance is moderately soluble in water (0.76 mg/l at 20°C). Therefore, the exposure must take place as described in OECD TG 241 via testing in water. Use of a solvent must be avoided. The assay must be performed under flow-through condition to maintain stable exposure concentration in the system. This must 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) must be collected and various endpoints must be examined (as listed in the OECD TG 241). The remaining animals continue exposure until 10 weeks after the median time to NF stage 62 in the dilution water control. At test termination (juvenile sampling), the remaining animals (maximum 10 frogs per replicate) are euthanized, and the various endpoints (as listed in the OECD TG 241) are measured or evaluated and recorded. When relevant, data on assay performances, quality criteria and validations (limits of detection, quantifications, coefficient of variations, specificity) must be reported.

(i) Additional mandatory endpoints to be measured

To fully take advantage of the test animals to investigate effects related to thyroid disruption, the following endpoints must be measured in addition to parameters requested in the guideline:

- Histopathology of the thyroid gland at test termination (juvenile sampling).
- Measure of thyroid hormones in plasma (TSH (compatibility with the ELISA kit must be assessed prior the assay) and TSHβ gene expression, free T3, total T3, free T4, total T4) at NF stage 62 and at test termination (juvenile sampling). Moreover, time to reach both stages must be accurately reported.

Mechanistic data informative of the mode of action leading to the effects are therefore important in a possible future classification or 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 proposed additional mandatory endpoints are not validated by an OECD technical guideline. Nevertheless, a document published by *the European Food Safety Authority, EFSA (Martin et al., 2020)* highlighted that "*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). Antibody-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 measurements are increasingly performed in assessing the potential endocrine adverse effect of chemicals in literature and validated protocol and techniques are available (see Fini J.B. *et al*., 2017, Mughal B.B. et al., 2018, Spirhanzlova *et al*., 2019, Martínez-Guitarte J.L. *et al*., 2021).

The measurement of thyroid hormones as an additional parameter must be performed to provide supporting information to investigate this MoA in an environmental species (amphibians). 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)".

To avoid bias, sampling for thyroid hormones must 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 must be evenly distributed across groups (not all individuals of one group sampled concomitantly and all individuals of another group at a later time point).

Table 1: Summary of additional mandatory endpoints and their sampling time

Endpoints*	***Interim Sampling (Larval sampling NF62 stage)***	***Test Termination (Juvenile sampling)***
Histo(patho)logy (thyroid gland)		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

* All endpoints are analysed statistically

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.

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 Endocrine disruption of the Substance.

The tests requested do not aim to gather theoretical scientific information but to produce evidence to clarify a concern for ED properties, based on effects observed in available animal studies. They will also provide information on the plausibility of a threshold *versus* non-threshold effect.

In its comments to the draft decision, the lead registrant questions whether the OECD TG 241 has been validated for substances with antioxidant properties. The registrant explains that "*Frog skins keeps redox homeostasis by antioxidant peptides* […] *this is especially important at metamorphosis phase when ROS concentration is essential*." The registrant considers that while "*there are no studies on effects of antioxidant chemicals at metamorphosis stage* […] *a toxic effect might occur, which might lead a confounding interpretation in an in-vivo assay as AMA/LAGDA*".

The evaluating MSCA points out that metamorphosis and especially apoptotic changes of organs involved are tightly controlled by TH. However, the evaluating MSCA was not able to find any literature postulating that reduced reactive oxygen species (ROS) concentrations in the environment and/or on the skin can delay metamorphic changes. The lead registrant has provided no scientific evidence to support that a certain ROS concentration in the skin of tadpoles is essential to trigger the beginning of metamorphic

changes. Literature available shows that environmental factors like dryness may accelerate metamorphic changes to allow the tadpoles to escape this stressor (Robert J.D., 2021). Taking ROS as an environmental stressor, one may argue that this might trigger a faster metamorphosis towards a better antioxidant-equipped skin. However, testing with an antioxidant chemical should prevent such an accelerated metamorphosis and one would expect a normal development. The evaluating MSCA would like to stress that this discussion is very theoretical. Furthermore, the evaluating MSCA emphasizes 4-tert-octylphenol, identified as an antioxidant by some of its producers (DEFRA, 2008), has been used during the LAGDA validation (USEPA, 2013).

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

The requested study is:

- the most sensitive developmental toxicity study while exposed through the environment to detect thyroid-related adverse effects as it includes many parameters related to endocrine activity/mode of action. 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.
- able to provide apical as well as mechanistic endpoints linked to disruption of T pathways and would allow to confirm or dismiss ED properties of the Substance for the environment. The LAGDA 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.
- appropriate, because it will clarify if the Substance affects thyroid in non-rodent wildlife species. It will also provide information on adverse effects caused by the potential endocrine activity of the Substance. 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 appropriate parameters for endocrine activity and endocrine-mediated effects on thyroid gland and hormones levels at level 4 tests of the OECD Conceptual framework (CF) for environment according to Revised OECD GD 150.
  In particular, the OECD TG 231 AMA also describes some specific thyroid function endpoints related to interaction with the hypothalamus pituitary thyroid (HPT) axis (thyroid histopathology and time to metamorphosis/developmental phases) (OECD, 2009). However, the AMA does not provide equivalent information to a LAGDA as the period of metamorphic climax is only totally covered by LAGDA. Therefore, for substance showing a MoA impacting later stages of metamorphosis, the AMA may not allow reaching a definitive conclusion. Finally, LAGDA provides information on the liver somatic index, which may support the elucidation of the MoA.
- the Xenopus Eleutheroembryonic thyroid assay (XETA, test method: OECD TG 248) 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 are the MoA foreseen 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 definitive 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 its comments to the draft decision, the lead registrant disagrees with the information requested and proposed an alternative testing strategy in reference to the recommendations given in the Appendix A of the ECHA/EFSA Guidance to investigate the mode of action of the Substance and the human relevance of observed effects. In that regard, the registrant proposes the following stepwise approach:

Stage 1: *in vitro* studies

The first stage includes the following studies:
(i) the in vitro TPO assay in two species (human and rat) to investigate the potential of the Substance to directly interfere with TPO production (indicative of direct thyroid modality endocrine disruption)
(ii) the in vitro NIS assay in two species (human and rat) to investigate the transport of iodine via NIS to follicular cells in the thyroid (indicative of direct thyroid modality endocrine disruption)
(iii) a comparative hepatic enzyme activity test on pooled male and pooled female hepatocytes in rats and humans to determine the human relevance of the observed perturbation in liver enzymes reported in previous in vivo studies. The registrant states that "[i]f the MoA is via rodent-specific and non-human relevant CAR/PXR mediated induction of liver enzyme activity, then it can be expected that increased enzyme activity will be seen in rat but not human hepatocytes following [exposure to the Substance]".

The registrant states that "[o]*n the assumption that the TPO and NIS assays are negative, this would preclude these particular MIEs and render the in silico positive prediction for TPO as irrelevant* […] *This would leave a "medium probability" of binding to T-Rα as the only positive in silico Thyroid related alert at CF level 1* […]. *Of the other CF level 2 studies* […], *none of them showed consistent activity on any endocrine modality*." The registrant considers this would lead to the conclusion that there would not be any remaining concern for T modality from CF level 2 and 3 studies. The registrant further states that "[a] *positive result for increase phase I/II metabolism in the comparative hepatic enzyme assay, would further support the postulated mode of action*".

Stage 2: *in vivo* studies

In stage 2, the lead registrant proposes to conduct a 28-day sub-acute repeated dose toxicity study with a 14-day recovery in the Rat (according to OECD TG 407) with additional parameters of analysis, including:
(i) histopathology of the liver and thyroid, to evaluate morphological indicators of effect
(ii) cell proliferation via Ki67 immunohistochemistry, to evaluate potential hyperplasia following increased TSH production
(iii) hepatic enzyme activity, gene transcription and
(iv) measurement of T3 and T4 thyroid hormones.

In relation to stage 2 testing, the registrant states that "*in vivo tests may prove critical in providing an overall confident conclusion of no T-modality for human health (and the environment)*" and refers to a similar approach presented in the publication by Tinwell and Bars (2022).

In its comments to the draft decision, a member registrant also indicates that it supports the proposed approach. The evaluating MSCA notes that this strategy is aligned with the one recently published by ECETOC (Melching-Kollmuss et al, 2023) in which some of the legal entities who commented participated.

The evaluating MSCA takes note that the proposed alternative strategy is in line with Appendix A of the ECHA/EFSA guidance document 'additional considerations on how to assess the potential for thyroid disruption in human health', specifically on the investigation of increases in TH. However, proposed alternative strategy does not answer the same concern that the one identified by the evaluating MSCA: the strategy proposed by the registrant aims at defining species differences in hepatic enzyme activation while the one proposed in this decision aims at confirming if hazard and subsequent risk of the substance for human (OECD TG 426 requested in appendix B) and environmental species (OECD TG 241) exist. Contrarily to what is stated in the registrants' comments, the evaluating MSCA considers that these adverse effects can be due to (a combination of) different MoA including hepatic enzyme activation. Therefore, the strategy proposed by the registrant would not sufficiently clarify the potential hazard identified by the evaluating MSCA in this decision. For this reason, we believe the two tests required in that decision should be performed in parallel

The lead registrant also states that "[n]*o further testing is proposed directly on non-target organisms to investigate "population relevance*". The registrant considers that "[i]*t is sufficient that the substance meets the ED criteria in one taxonomic group to conclude that a substance meets the ED criteria for non-target organisms*" and that "[o]*nly where* […] *the criteria are not met for mammals as non-target organisms, would the assessment need to proceed to the other taxonomic groups, which may require the generation of additional data*".

The ECHA/EFSA guidance (see paragraph 3.3.1.4) reminds that "*the relevance of such effects* [development, reproduction in single species] *at the population level should be assumed when determining the adversity in the absence of appropriate scientific data demonstrating non-relevance*." However, the proposed rodent study focuses on neurotoxicity and lactation. Some ED parameters are added but are not the core of the study. In addition, rodent specificity of these effects is claimed for long by the registrants, who did not provide any rationale in support of using this kind of data for population relevance. Therefore, independent of the results obtained from the rodent study, the requested LAGDA study (which will inform on growth and development and on ED modalities) will be needed to conclude on ED for the environment.

In addition, the lead registrant states that "[w]*hilst the Alternative Testing Strategy also dictates that in the event of negative results for one taxonomic group, the assessment strategy should proceed to the other taxonomic group, that should only hold true if there is still a concern to validate*". The registrant argued that the standard information requirements currently require more studies which may be informative on ED properties for human health and mammals than for other taxonomic groups and implicitly suggested that we should focus on the requests on rodents before requesting data on amphibians.

The evaluating MSCA notes that ECHA/EFSA guidance instructs to assess whether an active plant protection product/Biocide product (PPP/BPR) substance can be considered non-ED for mammals first and, in a second stage, for the environment. The guidance also advises to consider whether the mammalian data can allow reaching a conclusion for non-target organisms. It is uncertain whether the data obtained from the rodent that focus on neurotoxicity and lactation (see appendix B) could be considered for population relevance. We are therefore not in the case described in the guidance. Moreover, contrary to the above-mentioned regulations, the Substance would not be subject to a non-approval in case it is found to be an ED for human-health and/or for non-target organisms. It is also noted that the downstream regulatory risk management actions triggered by the identification of a substance as ED for humans or as ED environment under REACH may

differ. Therefore, unless population relevance of observed effects in mammals can be demonstrated (which, as already stated above, is unlikely for the Substance) both potential hazards need to be evaluated in parallel. Taking into consideration the impregnation of BHT in the environment, it is important to act on BHT for environmental species together with humans.

The lead registrant refers to the OECD GD 150 and states that in case of a positive result in the LAGDA but a negative outcome to the *in vitro*/*in vivo* studies, there is "*strong evidence for endocrine activity/adverse effects, but not via an E, A, T, S mechanism*". The registrant considers that "*this is because the mechanism would be clarified as a secondary effect to primary liver toxicity* [and this] *data would confidently rule out a direct thyroid mechanism of action*." The registrant concludes that "[a]*ssuming the results* [of the proposed testing strategy] *are as expected, this would* […] *render the conduct of a LAGDA study as irrelevant to the environmental hazard assessment*".

In their comments, the registrants have claimed that the current evidence on thyroid effects are secondary to hepatic enzyme activation and are rodent specific. If it occurs in other species, it will not be rodent specific and would therefore be relevant for other environmental species. Regarding the relevance of the MIE, see our remarks based on Noyes above (Noyes et al., 2019).

Finally, the registrant also questions the adequacy of the LAGDA considering "*the large number of animals required*" and contradictory results obtained when conducting both an AMA and LAGDA on the same substance (Ortego et al., 2021).

The cited publication aims at comparing LAGDA with a protocol referred to as the "extended AMA". The authors explain that "*regulators and validating bodies such as the OECD should consider revising the existing amphibian test guidelines and endocrine disrupter assessment strategies to integrate the advantages of fixed‐stage designs for the identification of T‐mediated endocrine activity and adversity*." The evaluating MSCA disagrees that the cited publication indicates contradictory results when conducting both assays but rather considers methodological differences between the two test protocols. The evaluating MSCA notes that in the comments received in 2017 on the testing strategy to be implemented, the registrants commented that conducing an AMA is not appropriate as it is only an OECD CF Level 3 assay and it cannot give definitive information on the MoA. The evaluating MSCA agrees that the AMA is a screening test of level 3 which is intended to empirically identify substances which interact with the normal functions of the HPT axis. However, due to the relatively short exposure time in that test (i.e., 3 weeks) one cannot be sure whether effects seen on apical endpoints would result in adverse effects on development, growth or reproduction in the longer term. The AMA allows no unequivocal diagnosis of which type of thyroid disruption modality is occurring as opposed to the LAGDA which gives information on the MoA and related adverse effects. Therefore, the LAGDA serves as a higher tier test at OECD CF Level 4 (OECD 2012). Finally, the evaluating MSCA emphasizes that the requested hormonal plasma concentration measurements may inform on precise mode of action. Such data are valuable for ED identification (see 2.1.b.(i) for further details). A recent publication by US-EPA experts (Haselman et al., 2020), indicated that the plasma T4 levels are a good indicator of the expected *in vivo* outcome adverse for thyroperoxidase inhibition leading to altered amphibian metamorphosis.

Appendix B – Reasons to request information to clarify the potential risk related to Developmental neurotoxicity and endocrine disruption for human health

1. Potential risk

1.1 Potential hazard

Following assessment of all available relevant information on the Substance, the evaluating MSCA and ECHA have identified the following potential hazards which must be clarified.

1.1.1 Developmental neurotoxicity

The observed effects on the thyroid as described in Appendix A may contribute to the developmental neurotoxicity concern, too.

Signs of behavioural or neurodevelopmental effects were observed in different studies with the Substance. The Substance has effects in rodents during pre- and post-natal exposure, but the available studies are old and poorly reported.

The effects reported are growth retardation, delayed development, and changes in neurobehavioral parameters of offspring in two mouse studies (Stokes J.D. and Scudder C.L. (1974); Tanaka T., 1993), in two rat studies (Vorhees C.V. *et al.* (1981), Meyer and Hansen, (1980)) and in a rabbit study (FDA 1974). In the multi-generation study by Tanaka T. in 1993, CD-1 mice were administered doses of the Substance at 0, 0.015, 0.045, 0.135 and 0.405% BHT in food (which would correspond roughly to 0, 10-23 (later referred as 23 mg/kg), 25-68 (later referred as 68 mg/kg), 100-203 (later referred as 203 mg/kg), 250-608 mg/kg bw/d (later referred as 608 mg/kg) in diet). F0 was administered from week 5; F0 and F1 generations were mated at 9 weeks and F2 generation was examined to postnatal day (PND)4. No dose related findings were reported but effects were observed on reflexes and learning & memory were affected from 23 mg/kg bw/d in F2 generation (180-degree rotation) and in the F1 and F2 generations at 68 mg/kg bw/d (reduced activity) and in the F2 generation at the highest dose (negative geotaxis prolonged). Learning and memory impairment was described in rodent offspring. Neurodevelopmental (e.g., eye opening) and behavioural tests were performed in rat after 21 days of treatment with 333 mg/kg bw/d (Vorhees C.V. (1981)). A significant increase in surface righting time, delayed forelimb swimming development and a trend to a lower activity in open field tests were reported in rats exposed to 333 mg/kg bw/d**.** No effects except mortality/ reduction in live pup and decrease in birth weight from 14.9 mg/kg bw/d were reported in rabbit though the study had some limitations due to the variability of the strain used and unusual findings occurring in the control group (FDA 1974).

Two studies reported modified motor activity following the Substance exposure. Stokes J.D and Scudder C.L (1974) measured behaviour of mice in social environments and following isolation. Meyer and Hanson (1980) conducted a cross-fostering experiment in which young rats born from treated mothers (i.e., exposed *in utero*) were adopted by either untreated or treated mothers at birth. Especially, they reported that '*F1 animals* [exposed *in utero or not*] *nursed by dosed mothers appeared clinically to have a hyperactive behaviour when handled in the first week of the lactation period*'. However, the authors also noted that such behaviour could indirectly result from malnutrition caused by reduced lactation by dams or lack of suckling ability in pups.

In rats, Meyer and Hanson (1980) reported '*slightly higher incidence of average number*

of dead cells in the paramedian section of cerebellum, especially in the molecular layer of cortex and in the white matter, in the non-treated in utero F1-animals nursed by dosed mothers.' No changes in the available histopathological data for rat and mouse brain are reported (Vorhees C.V. (1981), Frawley J.P. et al. (1965) and Olsen P. et al. (1986)). However, the areas of the brain analysed were not specified and the staining method is considered not specific enough to observe subtle brain modifications.

Mice exposed to 714 mg/kg/d of the Substance for 21 days presented reduced time of sleeping, increased social and isolation-induced aggression, and a severe deficit in learning using an automated avoidance conditioning climbing screen (Stokes J.D and Scudder C.L 1974). Behavioural abnormality only was evaluated in an old and poorly conducted 2-years study in monkeys dosed twice at the NOAEL (50 mg/kg bw/d) (Allen J.R. (1976)): no effect was reported.

In the lead registrant's comments on the draft decision, it is acknowledged that the "*ED adverse effect of BHT has not been established yet, as further data are requested by ANSES to conclude whether the substance has adverse effects related to ED identification*".

The evaluating MSCA does not dispute the limitations of the available data but considers the available evidence as indicative of a potential of the Substance to induce developmental neurotoxicity that require further investigation.

Effects on development and weight

The available *in vivo* data on the Substance are old (in particular Frawley *et al*., 1965; Meyer O. and Hansen, 1980; Vorhees C.V. et al. (1981); Olsen P. et al. (1986); Mc Farlane M.C. *et al*. (1997)) but convergent on their effects on neurodevelopment. Multiple reasons could explain this effect such as thyroid disruption but also growth retardation.

Impaired growth of offspring of dams treated with the Substance was reported in one mouse study (Stokes J.D. and Scudder C.L. (1974)), and two rat studies (Vorhees C.V. et al. (1981), Meyer O. and Hansen E. (1980)). Maternal exposure (F0) to the Substance during gestation and lactation led to reduced weight of the offspring (F1 or F2) in rodents (in particular rats). According to some studies (Mc Farlane M.C. et al. (1997)), this effect (10-20% lower weight of pups exposed to the Substance) was significant after weaning and was maintained in adulthood. This effect seemed to be more pronounced in males than in females. This body weight deficit could correspond to a growth retardation observed in rats (Mc Farlane M.C. et al. (1997), Meyer O. and Hansen E. (1980)). It should be noted that these effects were significant at above 100 mg/kg/d.

It cannot be excluded that the accumulation of the Substance in human adipose tissue and/or the mammary gland adipose tissue (e.g., Hernández et al., 2009) and its presence in breast milk (Zhang R.J. et al. (2020)) may result in a direct effect on offspring.

In its comments, the lead registrant emphasizes that the effects observed were identified at relatively high dose. In accordance with EFSA and SCCS opinion *"based on the study of Olsen et al. (1986), the NOAEL for non-neoplastic effects was 25 mg/kg bw/day, based on the effects on litter size, sex ratio and pup body weight gain during the lactation period, in the reproduction segment of the study.*

The evaluating MSCA notes that this NOAEL is based on effects on litter size, sex ratio and pup body weight gain during the lactation period identified at 100 mg/kg bw/day and notes that these tested doses do not represent overdosing in the sense that they neither induced

over toxicity nor death (ECHA, 2022). Therefore, the evaluating MSCA disagrees with the registrants' comments that the effects observed were identified at relatively high dose.

Reasons for growth retardation: Potential effect on milk production and/or ejection

In rats, the Substance was detected in milk (Olsen P. et al. (1986), McFarlane M.C. (1997)). Mc Farlane M.C. et al. (1997) showed that the stomachs of pups at PND21 from dams given BHT were empty and contained very little milk, a sign of their under nourished state at all doses of 500, 750 and 1000 mg/kg bw/d (doses specified based on your comments) This may be due either to pups unable to suck maternal milk, to disrupted maternal behaviour or to disrupted milk production. No study evaluated the alteration of maternal behaviour. The altered milk production could be due to a direct effect on the mammary gland or indirect impact on the hypothalamic regulation by oxytocin of milk ejection and/or hypothalamic dopamine- and pituitary prolactin-mediated regulation of milk production. In the cross-fostering study by Meyer O. and Hansen E. (1980), the pups breastfed by a mother treated with the Substance showed a low weight (here of nearly 40% at dose of 500 mg/kg bw/d (specified based on your comments), independently of their gestational exposure. On the contrary, no weight deficit was observed when the pups were breastfed by untreated mothers. This argues for an effect related to breastfeeding rather than *gestational* exposure, however this cannot be concluded because it was based on a single study. Other studies in rabbits showed an effect of the Substance on litter size / litter viability. The study performed by FDA (1974) showed a harmful effect of the Substance at doses of 69.1 and 320 mg/kg/day on the number of live foetuses and the reduction of birth weight at 14.9 mg/kg/day.

In the F1-animals nursed by dosed mothers, a slightly higher incidence of average number of dead cells (pyknosis, karyorrhexis and hyperchromatosis of the nucleus membrane) was reported in the paramedian section of cerebellum. 3 litters were euthanised in extremis at 3 weeks old, showing tremor, wobbling movements and an under-nourished appearance. This might imply a lack of support for the causative direct action of the Substance in the brain, but rather an indirect effect due to starvation following reduced lactation or ability to intake milk (both of which involve mechanisms other than direct neurotoxicity).

Milk production and ejection are controlled by well-known hormones whose serum level analyses can inform on the Substance's MoA regarding potential lactational effects. In addition, the Substance and/or metabolites could influence the mammary gland structure, and consequently, a possible effect on milk production.

It is thus possible that the adverse effect on body growth of young rats observed after the Substance exposure either during gestation or more likely during lactation and possibly linked to neurodevelopmental effects observed during postnatal period, might result from several non-exclusive causes such as:

1. Effects on mammogenesis during lactogenesis.
2. Maternal metabolic disturbances modifying the quality of milk received by pups.
3. Impaired neuroendocrine hypothalamus-pituitary control of milk production or ejection.
4. Effects on maternal behaviour.
5. Altered pup's behaviour (reduced suckling force exerted on the nipple).

Recent data (Zhang Y. et al., 2020) also showed the presence of the Substance (approximately 1.52 ng/mL) in human breast milk suggesting a direct exposure of infants through maternal milk with possible effects on the development and post-natal growth of

young children *via* breastfeeding. Therefore, it appears important to understand the effects of the Substance on or via lactation.

1.1.2 Potential endocrine disrupting properties related to the adverse effects described

a) Estrogenic/androgenic activities potentially related to lactational effect

Evidence based on OECD CF Level 1 & 2 studies (in vitro)

The Endocrine Disruptor Screening Program of the US-EPA (EDSP21) based on CompTox Chemicals Dashboard for the Substance includes: 18 estrogen receptor bioassays (3 Active estrogen receptor over 18 total estrogen receptors; 15 inactive estrogen receptors over 18 estrogen receptor bioassays). Three bioassays gave positive results. 14 androgen receptor bioassays (5 Active androgen receptor bioassays /14 total androgen receptors; 9 inactive estrogen receptors over 14 total androgen receptors). Only one steroidogenesis bioassay and its viability control are available. It has been proposed by EDSP21 that no steroidogenic effect was foreseen for all endocrine disruption pathways.

The androgenic/anti-androgenic effects of the Substance (Pop A. et al. (2016)) and estrogenic/anti-estrogenic (Pop A. et al, (2018)), genomic and non-genomic effects, were investigated in human cell lines. Although the Substance did not seem to present any estrogenic effect even at high doses, it was shown that it might cause anti-androgenic effects (at around 50 µM).

Alofe O. *et al*. (2019) used the ER-positive, immortalized human uterine Ishikawa cell line and a range of concentrations (0, 1, 10, 100, and 1000 nM) for 6h below the reported limit of toxicity in humans. The publication reports cell proliferation in human uterine cells. Exposure to the Substance repressed the basal expression of all three estrogen-responsive genes: the progesterone receptor (PGR), growth regulation by estrogen in breast cancer 1 (GREB1), and natriuretic peptide precursor C (NPPC), which are reported to be regulated by estradiol in Ishikawa cells. If the expression of these genes is up regulated, this indicates E activity. The lowest concentration resulting in significant gene expression was 1 nM of the Substance. Maximal inhibition of PGR, GREB1, and NPPC expression changes was achieved at 10 nM. The Substance repressed ERβ expression 48 h after treatment.

In his comments to the draft decision, a member registrant provided references to two additional publications evaluating the estrogenic and androgenic activities of the Substance *in vitro*. The study of Wada et al. (2004) shows that the Substance had significant estrogenic activity at 50 µmol/L. In the report by Schrader and Cooke (2000), at 10 µM, the Substance completely antagonized the activation of DHT (50 pM), without having deleterious effects on cell viability. This supports that the Substance may act as an androgen antagonist. It was pointed out that "*these two in vitro studies* [show] *that BHT has an effect on steroidogenesis and anti-androgenesis. However* […] *the 3D-structure of BHT seems incompatible with such activation".* The registrant considers that *"taking into account all available data it can be concluded that BHT has no (anti-)estrogenic or (anti-)androgenic activity".*

The evaluating MSCA disagrees with the conclusion above but agrees that the *in vitro* data available show that the Substance has endocrine activities at quite high doses. BHT endocrine activity *in vivo* might be more complex than a direct ER or AR receptor (in)activation.

Evidence based on OECD CF Level 3 studies (in vivo)

The uterotrophic *in vivo* assay performed in Wistar prepubescent female rats (Pop O. et al. (2013)) showed that the Substance (75 mg/kg bw which is the dose with no toxicity) decreased the relative uterine weight of prepubescent female rats. The morphometric analysis results also showed a decreased endometrial epithelium cell height in the groups treated with the Substance.

In conclusion, absolute and relative uterus weights were significantly decreased by the Substance not affecting significantly endometrial epithelium thickness.

The available data alert on the potential ED effect of BHT on lactation. However, adverse effects and biological plausible link identifications are impaired by the limits of the old data available.

b) Neurodevelopmental effects potentially related to thyroid disruption or other endocrine modalities justifying extra parameters in the DNT

In rodents, the thyroid gland enlarges due to increases in TSH secretion, decreased serum T3 and T4 concentrations leading to induction of hypertrophy and/or hyperplasia of follicular epithelial cells. The available multigenerational studies conducted in rat with the Substance exposure indicate the occurrence of impaired growth and cognitive development. It was shown that developmental neurotoxicity can arise via thyroid disruption (Fang X. et al. (2017); Ghassabian A. et al. (2012) and it is now recognised that thyroid interferes with neurodevelopment (ECHA, 2015; WHO-UNEP, 2012). Therefore, even minimal modification of thyroid homeostasis could have an impact on future neuro-cognitive development (US EPA, 2011).

The registrants commented numerous times that the effects observed are secondary to hepatic metabolic induction. Please refer the first paragraph of the Appendix A 1.1.3.b for our answer.

Based on human evidence linking developmental hypothyroxinemia with altered brain development in children, increased catabolism of thyroid hormones (TH) in rats (decrease in total levels of thyroxin (T4)) is in the proposed AOP linked to adverse neurological development (US EPA, 2011). Since the foetus is dependent on maternal supply of TH, decreased maternal T4 in early pregnancy may lead to less T4 available for the foetus, and consequently to decreased T4 crucial for foetal brain with adverse neurodevelopmental effects. This effect on thyroid might explain some of the effects observed, including growth retardation and development described in the two generation studies.

The effect on neurodevelopment and behaviour could be due to a disruption of the thyroid axis but there is currently no conclusive evidence available.

These effects are observed together with growth retardation. Indeed, the available *in vivo* data on the Substance showed an effect of the Substance during gestation and lactation impacting the weight of the offspring (see above).

Evidence based on OECD CF Level 4-5 studies (in vivo)

BHT interferes with steroid hormones and compromises endometrial decidualization during early pregnancy (Sun et al., 2021) or diminishes the ovarian reserve (Hao et al, 2023).
Additionally, it is known that neurodevelopmental effects could be due to other endocrine disruption than thyroid. The role of sex steroid hormones in neurodevelopment, regulation of locomotor activity and learning and memory has been emphasized in the literature.

Indeed, well-established modulatory effects of sex steroids on learning and memory are described in both males and females (Mhaouty-Kodja, 2018). Impact of the sex and age through steroid hormones have been described on motor activity (Rosenfeld, 2017); grooming behavior and motor activity (Scimonelli, 1999). As BHT has been shown to interfere with estrogenic/androgenic activities (see point a above), the impact of these modalities on the neurodevelopmental adverse effect cannot be ignored.

1.1.3 Overall conclusion of the potential hazard

The Substance showed a neurotoxic potential in rodents during pre- and most probably during post-natal exposure, but the available studies are old and poorly reported. The effects observed are growth retardation, delayed development, and changes in neurobehavioral parameters of offspring.

The effects observed could have different origins, including thyroid axis modification. In addition, available *in vivo* data showed that the Substance exposure affected weight of the offspring. Whether this effect is concomitant or a cause of the developmental neurotoxicity of pups has not been explored, justifying that a developmental neurotoxicity study (DNT, OECD TG 426) modified with additional parameters is requested. It will allow to clarify if the Substance shows disruptive effects on thyroid functions in dams and pups, the measurement of hormones involved in the lactotrope axis to evaluate their role on lactation, the measurement of hormones involved in the somatotrope axis of the pups to evaluate their impact on pups' development and associated neurodevelopmental effects.

The available information is not sufficient to conclude on the hazard of the Substance and consequently, further information is necessary.

In its comments to the draft decision, the lead registrant explains that "*nine rodent generational studies are available*" but that only 6 studies "*report on neuro/behavioural developmental effects observed in pups following exposure either during gestation or lactation*". Out of these 6 studies, "*4 report adverse outcomes and 2 report no effect*" (Vorhees et al, 1981; Meyer and Hansen, 1980; Tanaka T., 1993; Price S.C., 1994.

On the study by Vorhees et al. (1981), the registrant states that:

- increased mortality following exposure to the Substance was observed during "*gestation or lactation*"
- reduced growth in pups was observed.

Overall, the registrant considers that "[i]*t is possible to raise major questions on the reliability* [of that study]"as the study was "*performed at very high doses compared to the NOAEL of 25 mg/kg-BW derived from a two-generation study in rats (Olsen et al., 1986)*"

On the study by Meyer and Hansen (1980), the registrant states that reduced growth in pups was observed and similar to the study above, the study was conducted at very high doses.

On the study by Tanaka T., (1993), the registrant does not report bodyweight or growth rates issues. However, the registrant states that the author indicates that "*growth retardation and developmental delay may be due to factors other than direct* […] *neurotoxicity, such as milk production or intake*". Again, the registrant indicates that the study was conducted at very high doses.

On the study by Price (1994), the registrant considers that "[i]*t is possible to raise major questions on the reliability* [of that study]" as "*Thyroid hyperactivity* [was] *observed at high doses together with liver effects secondary effect*".

The evaluating MSCA agrees with the registrants' comments stating that the available studies showing neurotoxic potential in rodents during pre- and most probably during post-natal exposure to the Substance, are of limited reliability. The lead registrant cites inconsistencies and 'extreme dose levels' for the effects. The available data cannot ensure if it is secondary to other toxicities or not. The purpose of this decision is to clarify the concern, its origins (including maternal toxicity, effect via milk or other) and clarify uncertainties. As mentioned above, findings on neurobehavior were reported from 23 mg/kg bw/d (Tanaka T., 1993) supporting possible effects below the current point of departure that is not considered by the evaluating MSCA as an 'extreme dose level'.

In his comments to the draft decision, the lead registrant also considers that there is "*no evidence in support of a direct effect on dams at the cerebral level*". The registrant justifies this claim by stating that:

- "[o]*nly one of the studies* [i.e., Frawley et al. (1965)] *observed an alteration in dams in that they had inadequate milk production, but notes there was no other alteration in the dam's behaviour, stating that there was no interference with lactation and nursing of the offspring*", and
- "[a]*nother of the studies reports 'empty stomach of pups at birth with very little milk' but notes that this could be 'due to inadequate milk production', implying no alteration in nursing behaviour* […]".

Similarly, the registrant claims that the "*slightly higher number of dead cells in the paramedian section of the cerebellum in pups* [is likely] *the result of malnutrition (an indirect effect, caused by e.g. reduced lactation in dams rather than by direct neurotoxicity of BHT*." [i.e., Meyer and Hanson et al. (1980)]. As mentioned in 1.1.1 above, malnutrition is recognised as a potential mode of action by the evaluating MSCA. The reason for such malnutrition is, however, unknown.

The evaluating MSCA does not agree with the registrants' comment related to:

- The study by Frawley et al. 1965). This study does not discuss nursing and maternal behavior but rather animal behaviour. Meyer and Hanson (1980) analyzed the behavior of pups from postnatal days 1 to 24 but not in dams. The evaluating MSCA emphasizes that the aim of the request is to see whether the Substance leads to neurodevelopmental effect on the pups (not on dams) and if so to understand its origin (direct, indirect, and/ or secondary to which type of effects).
- The exact study origin of the quotation was not found, however the evaluating MSCA notes that McFarlane et al. (1997) considered it '*likely that the failure of pups nursed by dams receiving very high doses of BHT to gain weight is due to an interference in milk production'*. This is taken into account in Section 1.1.1 reasons for growth retardation: Potential effect on milk production and/or ejection.

On '*neurobehavioural alterations (i.e. hyperactivity) observed in pups*', the lead registrant refers to the study by Meyer and Hanson (1980) and considers that the effects observed could result from "*malnutrition caused by reduced lactation by dams or lack of suckling ability in pups*". The registrant indicates that exposure to the Substance led to "*decreased sleeping and avoidance-based learning, as well as increased social and isolation-induced aggression*" and state that the slight increase in overall activity level was not statistically significant. The registrant therefore considers that "[i]*t could, therefore, be argued that the neurobehavioural alterations are indirect due to reduced sleep, rather than directly due to* [the Substance] *action*". The registrant also notes that "*other available studies report reductions in activity* [e.g. Vorhees, 1981]" and that, therefore, available evidence "*cannot be considered as consistent nor reliable in supporting the conclusion that administration of the* [Substance] *results in hyperactivity*".

The evaluating MSCA would like to emphasize that neurobehavioural alterations are often linked to hyperactivity, in particular related to-Deficit Hyperactivity (ADHD). However, it can also be related to hypoactivity (Godefroy et al., 2017). The evaluating MSCA agrees that the available data are not consistent but taken together, the effects reported indicate a potential hazard for the Substance and forms the basis for the request to clarify and characterize the neurodevelopmental alterations. The results of the requested study will elucidate the dose at which effects occur and any simultaneous effects for further consideration in risk assessment.

1.2 Potential exposure

Uses

According to the information submitted in all registration dossiers (25), the aggregated tonnage of the Substance manufactured or imported in the EU is in the range of 10000 – 100000 tons per year.

Furthermore, it is reported that among other uses, the Substance is used as:
- antioxidant (additive in animal feed products and in foodstuffs intended for humans),
- preservative / stabilizer (cosmetics and care and hygiene products, vegetable and animal oils and soaps).
- used in medicines ([redacted]), the Substance has been newly found in pharmaceutical products for babies, plant protection products, perfumes, waxes, cleaning products, biocides (disinfectants), paints, rubbers, and plasticizers, some of these uses not being covered by the REACH Regulation.
- lubricant.
- protection from oxidation of materials during prolonged storage

From the uses covered under REACH, there is a significant exposure of professional workers (widespread uses) at industrial sites and in manufacturing or re-packaging, of consumers (pregnant women and children as sensitive population) and of the environment because of its release from industrial uses and from article service life.

Exposure of consumers, workers and the environment to the Substance is proven as it is found in biological fluids.

Critical use of BHT in milk:

In their comments to the draft decision, the registrants consider that no concern for the Substance is indicated based on a weight-of-evidence assessment of available information, including a BHT residue evaluation study in lactating dairy cows' milk ([redacted] et al., 2014). In that study, measuring the Substance and its residues in cow milk, eight cows were administered 150 mg BHT/kg bw/d over 10 days. The Substance or its metabolites were not detected in the milk samples.

However, the evaluating MSCA has identified the following deficiencies with the study:

- the LOD of 5 µg/L is too high
- the exposure duration was short (only 10 days) while considering the current use of the substance in feed
- the study was conducted at different lactation periods of the cows
- Time zero samples were used as control, not allowing to assess the effect of time
- the health status of the cows is unknown and it is not reported whether the cows received treatments (e.g. hormone and/or antibiotics treatments). Therefore, the observed increase in number of cells/L of milk and the slightly increased in fat concentration cannot be interpreted.

The evaluating MSCA considers that the above deficiencies significantly limit the ability of this study to detect the Substance (or its metabolites) in cow milk. Moreover, Zhang Y. et al. (2020) reported the presence of the Substance in human breast milk (around 1.52 ng/mL) indicating also a contamination with the Substance and the registrants state in their comments that "*BHT is most probably present in the rat milk at such high doses, so is contributing to pup's toxicity as well*". The evaluating MSCA also raises that other studies such as Pattono et al. (2009) reported the presence of the Substance (and/or its aldehyde residue) in all conventional milk tested (cow, goat and sheep) and in 18/81 milk from an organic farm using the same GS/MS method than the [redacted] study with a LOD of 1 µg/L. For these reasons, the evaluating MSCA is of the opinion that the data provided during the commenting period are not sufficient to prove the absence of BHT in milk and to determine if a safe dose can be identified for this finding.

Biomonitoring data

Available biomonitoring data show that the human population is exposed to the Substance. A study by Schmidtkunz C. in 2020 reports the presence of the Substance in EU population and its metabolite in 98% of samples with a median urinary concentration of the Substance metabolite of 1.06 µg/L and 1.24 µg/g creatinine corresponding to calculated of intake. In another human biomonitoring project in Germany (Murawski A. et al. (2021)), from children and adolescents' quantifiable amounts of the acid metabolite was also found in almost all the children and adolescents sampled (median value was 2.18 µg/L creatinine) with a maximum concentration of 248 µg/L creatinine, i.e., quite higher than values reported in Schmidtkunz's study (2020). The median concentration was within the range of the values reported for adults and children from five different countries. No specific sources of exposure could be identified.

The registrant commented that epidemiological data exist showing '*no difference* [on thyroid hormones in blood] *were noted, between workgroups, neither exposed/non-exposed volunteers nor workers having worked short/long (up to 30 years) period in the factory*'. Mari et al (2018) and Verhage (1989), which examined the levels of thyroid hormones and potential thyroid effects in workers exposed to the Substance had weaknesses, such as single urinary measurement, high variability in exposure, limited number of volunteers, not homogeneous groups, no detailed description in TH and TSH assays that also prevent any solid conclusion on the Substance's capability to display endocrine activity in humans. There are too many biases and confounding factors in these studies to draw any solid conclusion.

Therefore, exposure to the Substance of workers/consumers/environment/sensitive population is ascertained by data. These data reflect aggregated exposure from all sources of the Substance which make it difficult to identify the source(s) of contaminations.

1.3 Identification of the potential risk to be clarified

Based on all information available in the registration dossiers and information from the published literature, the Substance may cause neurodevelopmental delay, during gestation but most probably (also) during breast feeding, through various mechanisms among which endocrine disruption. The effects and their causes need to be assessed in detail to be properly managed.

The information provided about manufacture and use demonstrates a potential for exposure of workers/consumers/sensitive population. Moreover, biomonitoring data show an important exposure of the human population to the Substance.

Based on this hazard and exposure information, the Substance poses a potential risk to human health.

As explained in Section B1.1 above, the available information is not sufficient to conclude on the potential hazard. Consequently, further data are needed to clarify the potential risk related to neurodevelopmental impairment, potentially through endocrine disruption mode of action of the Substance.

In its comment, the lead registrant mentions that "*the relevance of the experimental conditions to anticipated human exposure scenarios (i.e., dose, frequency and time) should be considered. In addition, chemically induced effects that are produced by short-term disruption in thyroid-pituitary function appear to be reversible, when the stimulus is removed." (EFSA, 2012)*".

The evaluating MSCA emphasizes that the statement in EFSA (2012) is not substance-related but rather general. In the case of the Substance, considering its wide use pattern, it cannot be excluded that continuous human exposure may occur. Furthermore, TH can act on very specific windows of exposure of embryos. This citation from EFSA is therefore considered inadequate in the current case.

In its comments to the draft decision, the lead registrant refers to the decision of the Board of Appeal in case A-005-2014 and state that:

- Substance evaluation is intended to assess risks that may occur in reality and not only theoretically
- ECHA must be able to demonstrate that the potential risk identified needs to be clarified.

In that regard, the lead registrant states that all supported uses in the BHT dossier "*demonstrated risk characterization ratios ("RCRs") below 1 and thus all uses were safe and neither workers nor consumers were exposed to any risk*". The lead registrant further states that this earlier draft decision only refers to "*potential health effects* [related to] *disruption of the thyroid hormone axis, but these potential effects were not supported by data which indicated that these* [effects] *could occur at doses below the derived DNELs*".

The lead registrant also states that in "*all* [available] *studies, the doses required to achieve any of the neurobehavioural observations reported were high (c.a. ≥ 500 mg/kg bw/day). The NOAEL for* [the Substance] *used to derive DNELs is 25 mg/kg bw/day; the same NOAEL level which has been used by EFSA to set the current ADI of 0.25 mg/kg bw/day*."

The evaluating MSCA emphasizes that effects on thyroid occurred from 25 mg/bw/d while increased liver eosinophilia, glutathione-S-transferase and CYP2B activities were observed at 100 mg/kg bw/d in the absence of liver weight changes. Furthermore, effects on neurobehaviour were observed at 23 mg/kg. Taking into account the limitations of currently available data as already described above, it cannot be excluded that relevant effects may be observed at lower dose. Therefore, the evaluating MSCA maintains that there is a potential risk that must be clarified.

The evaluating MSCA deems the present case as not comparable to carbon tetrachloride (ECHA Board of Appeal case A-005-2014) as the exposure to the substance BHT is more important than in the case of carbon tetrachloride and that there are no risk management measures for the extensive non-food and feedstuff uses of BHT in place yet. We are therefore in a case of 'potential risk'. The aim of requesting additional information is to clarify whether the 'potential risk' is an 'actual risk' (the decision of the Board of Appeal in case A-003-2018). It was previously reminded (the judgment of the General Court in case

T-125/17) that in order to demonstrate a potential risk, ECHA is not required to find the existence of a conclusive link between the substance and the identified adverse effects. ECHA needs only to establish that there was a potential risk of such a link existing.

1.4 Further risk management measures

If the potential properties of the substance are confirmed, the evaluating MSCA will analyse the options to manage the risk(s) and will assess the need for further regulatory risk management measures in the form of:

- Harmonisation of the classification, as defined in the CLP Regulation, for
  - Toxicity to reproduction based on developmental neurotoxicity (H360 or H361).
  - Endocrine disruption for human health under the Commission Delegated Regulation (EU) 2023/707 of 19 December 2022 amending the CLP Regulation (EC) No 1272/2008.
  - Effects on or via lactation (H362).
- Classification of the Substance as a category 1 reproductive toxicant would result in restriction of consumer uses of substances and mixtures containing 0.3% or more of the Substance via restriction Annex XVII entry 30. It would further result in stricter risk management measures and revised instructions on safe use under occupational health and safety legislation.
- Classification of the Substance as an ED based on the DNT study findings:
  - the results of this study would help determining whether this ED is a threshold /non-threshold to define its DNEL.
- If it is a non-threshold effect, a BMD10 may be more appropriate than a NOAEL
- Identification as a substance of very high concern (SVHC) and a subsequent authorisation or restrictions of the use.

All these potential 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.

2. How to clarify the potential risk

Two tests are requested in parallel in the decision. OECD TG 426 is needed irrespective of the results of the OECD TG 241, because it will provide specific information that can be used to clarify the potential risk for developmental neurotoxicity, and in addition, it may also clarify the potential risk of endocrine disruption for human health.

In its comments to the draft decision, the lead registrant disagrees that the requested study (i.e., the OECD TG 426) has a realistic possibility of clarifying the potential concern identified. In that regard, the comments already described under Appendix A, Section 2 equally apply to this request. In particular, the registrant claims that, to clarify the potential risk, the link between the induction of hepatic enzymes and its potential link to thyroid/pituitary function needs to be clarified as this would allow concluding on the relevance to clarify the potential risk. The registrant considers that the requested study would not inform on the toxicological mode of action. To address this aspect, the lead registrant describes a proposed MoA based on the published Adverse Outcome Pathways 08 and 107 which it considers plausible. The registrant states that further testing should aim at elucidating the MIEs and key events (KEs) for the observed effects *in vivo.*

The aim of the current decision is not to evaluate the MIEs or KEs or other endocrine activities but to produce adequate information on the adverse effects (i.e. neurodevelopment effect and lactational effect) following exposure to the Substance. The

MIEs or KEs for endocrine activities leading to such effects can be very broad, and to date it is impossible to propose *in vitro* tests to assess all of them.

2.1. Request B.1: Developmental Neurotoxicity Study (DNT) study, OECD TG 426, modified

a) Aim of the study

A developmental neurotoxicity (DNT; OECD TG 426) high tier test of level 4 of OECD Conceptual Framework on Endocrine Disrupters Testing and Assessment (OECD 2012), will provide state-of-the-art information on the neurotoxicological effects of the Substance repeated exposure during *in utero* and early postnatal development. With the additional measurements requested, this test will provide data to understand the mechanism(s) leading to the Substance's effects on pup neurodevelopment, such as mother behavior or effects on lactation or mammary gland in F0 dams that could explain growth retardation. Rats are the preferred species because the quantity of milk collected is higher compared to mice.

The modified DNT, as described in the schematic protocol (point b) below, will assess the effects of the Substance on modulations of various hormones involved in lactation in F0 dams and neurodevelopment in F1 pups. The extra parameters requested will provide further data to understand if the neurodevelopmental effects are the result of disruptions on the HPT axis in F1 pups, or of other endocrine modalities. Combined with the results of the LAGDA test dedicated to demonstrating an interference with HPT axis, it will clarify the role of the Substance on thyroid disruption in neurodevelopmental toxicity if the latter arises. This study will therefore allow to clarify if the Substance has an effect on neurodevelopment and if so, the extra-parameters highlight its potential modes of action.

Including the measurement of thyroid and liver parameters, and hepatic metabolic enzymes would allow to assess if the neurodevelopmental effects observed are (also) a consequence of thyroid disruption. Thyroid is functional at PND 15-21 in offspring. Behavioural testing according DNT OECD TG 426 will be performed in offsprings at PND 25±2 and PND 60±2. Food consumption and body weight of dams must be examined during lactation from LD1 to PND60. These timepoints should be adapted as indicated in the decision, where some flexibility is given (e.g. PND25±2 and PND60±2).

With this study, the mechanism of action of the Substance leading to the neurodevelopmental effects will be clarified and subsequently properly managed (see B.1.4).

Substances interfering with the sex hormone balance may also affect the developing brain. In the test requested, fetal exposure and the duration of dosing will allow to assess effects relevant to endocrine disruption. In addition, it will provide data on EATS-mediated effects related to reproduction, lactation, and neurodevelopment.

The tests requested do not aim to gather theoretical scientific information but to produce evidence to clarify a concern for developmental neurotoxicity and optimally use test animals to also address the concern for ED properties, based on effects observed in available animal studies. They will also provide information on the plausibility of a threshold *versus* non-threshold effect.

b) Specification of the requested study

The study must be performed according to the OECD TG 426 in rats by oral route (dietary). All quality criteria must be respected.

The study (DNT; OECD TG 426) is required to clarify the concern for ED and developmental neurotoxicity.

In pups

- At PND11±1, body weight and size (male and female) must be measured and the stomach must be sized and weighted. F1 pups sacrificed at PND11±1 are collected from supernumerary dams (n=10 dams from control group and n=10 dams from high dose group).
- At PND11±1, PND25±2 and PND60±2, measurement of circulating IGF1, free and total T4 (if limited material available in PND11±1, only free), must be performed in pups to specifically clarify the concern for effects on developmental neurotoxicity potentially related to thyroid disruption.
- Behavioral investigation of the same pups at sacrifice on post-natal days 25±2 and 60±2, including motor activity at PND25±2 and around PND60±2 (young adults), which is non-test guideline, to specifically clarify the concern for developmental neurotoxicity, and anxiety test at PND25±2 and PND60±2. Anxiety-state level tests in both adolescents and young adults (before learning and memory tests), which is non-test guideline, must be added since the anxiety state level can interfere with the motor and cognitive performance of animals. Information about their anxiety state level, using a dedicated paradigm, will allow a better interpretation of the obtained motor and cognitive data. The assessment of motor activity can be performed as indicated in the TG. What is important is to have data for this behaviour in juvenile and adult animals.

In addition to brain, collection of thyroid and liver, at PND11±1, 25±2 and 60±2 in F1 pups, together with remaining plasma samples must be frozen and preserved at -80°C for further analysis. The evaluating MSCA may decide on the need for further assessment of the metabolic substrates, such as glucose, NEFA (non-esterified fatty Acids), cholesterol and insulin. Further information may be requested in a later decision to clarify the concern. Collecting and storing this material will avoid additional animal testing.

Liver enzyme induction:

- Hepatic metabolic enzymes must be assessed in dams after culling and in pups at PND25±2, to specifically clarify the mechanism of action, e.g., the potential liver enzyme induction resulting in an increased catabolism of thyroxine. Enzymes from the families listed in Table 2 below, that are common to both rat and human, must be assessed; the recommended specific enzymes from each family to be tested are also listed in Table 2. A proposal for amendment (PfA) requested to add Dio3 in the table below because this enzyme is involved in the deactivation of thyroid hormones (Scimonelli *et al.* 1999). The evaluating MSCA agrees that it is important to assess this enzyme given its function in the thyroid hormone pathway and ability to link possible adverse effects with potential modes of action. Moreover, the evaluating MSCA consider that its inclusion would ensure the most efficient use of vertebrate animals. The quantitative measurements can be performed by an appropriate method e.g., at the mRNA levels of hepatic enzyme of phase I and phase II by qRT-PCR or at the protein level of hepatic enzymes by Western blot.
- Blood must be collected from dams after culling for further measurement (see below for further details).

Table 2: Required enzyme families to be measured and recommended specific enzymes to be tested

Enzyme family to be measured	Recommended specific enzymes to be tested in the Wistar strain
Cytochrome P 450	cytochrome P450, family 3, subfamily a, polypeptide 2 (CYP3A2), cytochrome P450, family 1, subfamily a, polypeptide 1 (CYP1A1), cytochrome P450, family 2, subfamily b, polypeptide 1 (CYP2B1), cytochrome P450, family 1, subfamily a, polypeptide 2 (CYP1A2)
Deiodinase	iodothyronine, type I (Dio1); type II (*Dio2*); type III (*Dio3*)
UDP glucuronosyltransferase	UDP glucuronosyltransferase family 1 member A1 (*UGT1A1)*, UDP glucuronosyltransferase family 2 member B1 (*UGT2B1),* UDP glucuronosyltransferase 1 family, polypeptide A6 (*UGT1A6)*
Sulfotransferase	sulfotransferase family 1B member 1 *(SULT1B1),* sulfotransferase family 1C member 1 *(SULT1C1)*
Members transporters (OATP, MCT8 and TAT1)	SLCO3a1 solute carrier organic anion transporter family member 3a1 (OATP3A1), SLC16a2 solute carrier family 16 member 2 (MCT8) (Chromosome X), SLC16a10 solute carrier family 16 member 2 ((TAT1)

The additional data will clarify the adverse effects on or via lactation and provide elements on the mode of action of the Substance.

The schematic protocol of treatment group and analysis on F0 dams and F1 pups are indicated in Appendix C.

In his comments on the draft decision, a member registrant states that the "*mode-of-action analysis of the proposed liver-thyroid axis within the framework of a DNT study is problematic and scientifically unsound*". To justify his statement, the registrant explains that enzyme induction shows maximum activation after c.a. 1-3 weeks with adaptation of the response at later time points. The draft decision requires that exposure to the Substance starts at 4 weeks pre-mating and continues throughout the mating period, gestation period and lactation period prior to analysis. The registrant concludes that this would "*impede the scientific interpretation of the data*".

In the opinion of the evaluating MSCA, if enzyme induction adapts, it is important to assess 'thyroid hormone' variations subsequently. Indeed, it would then be certain that these variations are not due to their catabolism. Therefore, it will not impede scientific interpretation of the data but rather facilitate it. Ultimately, the evaluating MSCA would accept satellite animals.

The registrant further commented that "[The] *conduct of mode of-action investigations in pregnant animals is challenging since pregnancy is a significant confounder*".

The evaluating MSCA recognises that pregnancy modifies thyroid hormones. This is the purpose of the request to define if the substance can interfere with hormone modulation. Treated animals will be compared to controls that are also pregnant animals. This request will allow to link potential adverse effects with potential MoA in real exposure situations (pregnancy). Indeed, it should be noted that pregnant animals and women are exposed via feed and food. The model of pregnant rats is therefore relevant.

Test material and concentration

The requested study must be performed with the Substance as registered. For the purposes of this test, results from existing studies in rodent must be considered for determining the highest test concentration, to avoid concentrations that are overtly toxic. Based on the study by Mc Farlane M.C. et al. (1997) reporting effects on the weight of pups and considering the recent ECHA review on EOGRTS, the evaluating MSCA would like to recall that doses should be high enough to produce toxicity: it is recommended that the highest dose tested is no lower than 250 mg /kg bw/ day to avoid testing at too low doses. If a lower top dose is used, a justification must be provided. Such justification must include the results of a range-finding study performed by the laboratory running the test. A minimum of three chemical concentrations must be used and appropriate controls (including solvent controls, if necessary) must be included.

The registrant states that the "*doses specified were derived from an older study (McFarlane et al., 1997)*" and that "*appropriate dose-range finding studies will need to be conducted to ensure the tolerability of the dose levels requested*".

The evaluating MSCA points that conducting a dose-range finding is left at the discretion of the facility as the potential need to adapt the doses. If this is the case, a detailed explanation must be provided.

Route of exposure

The Substance must be dosed via diet to mimic continuous exposure in food from 28 days prior to mating until postnatal day (PND) 25±2 at weaning. Three groups of doses must be included (1 group per dose, n=20 dams for each of the low to mid-dose groups and n=30 for the high-dose group) and one control group (n=30) with pups raised by their own mothers. Thereafter, pups must be exposed to a control diet from PND25±2 up to the day of sacrifice (at the end of behavioral analyses i.e. PND60±2) to evaluate developmental effects of the Substance after treatment cessation, on young adults that will have been exposed only during gestation and lactation.

In its comments on the draft decision, a member registrant states that "[t]*reatment prior to mating is not part of the guideline study design and defeats the purpose of focusing on developmental effects*".

The registrant also states that:

- with such dosing regime, no time-mated animals can be ordered, and mating would have to occur at the test facility
- the subsequent investigations on both dams and offspring will have to be performed in a staggered fashion throughout the study.

According to the registrant, this test design "*carries the inherent danger of lack of comparability*" as a result of an increased number of different tests to be performed on different days, the need to take in consideration mating of animals at test facility. The registrant also indicates that "*additional animals would* [be needed] […] *to ensure an adequate minimum number of litters per dose for the DNT main study*". Therefore, the registrant considers that dosing "*should be started on gestation day 6, as specified in the OECD TG 426*".

The evaluating MSCA emphasizes that the premating treatment is required because the effects observed in the studies cited in the report (Meyer and Hanson, 1980; Vorhees et al., 1981; Tanaka T., 1993; McFarlane et al., 1997) show that a pre-treatment is necessary. In addition, the analyses of lactating mothers and the potential accumulation

of BHT tissues and later in breast milk justifies this premating treatment period. Starting the treatment at gestational day 6 overlooks all the critical early period of brain development, as also highlighted in the TG 426. In particular, as no pre-implantation loss is expected with the substance, there is no rationale to start the exposure at GD6. Although organisationally constraining it is important to conduct the study with the windows of exposure mimicking the human one.

Then, the registrant highlights uncertainties with dosing via the dietary route as:

- "*a concentration will be provided in feed with the aim to achieve uptake concentrations*";
- "*dietary administration of the test substance should take into consideration the increased feed intake of dams during lactation*";
- "*adjustment of the feed concentration would be required to ensure constant dosing throughout the treatment period for both dams and pups*".

The evaluating MSCA acknowledges that dosing will be calculated on the basis of daily food consumption and weight of the animals and later adjusted to fit to the requested doses.

Based on the above, the evaluating MSCA maintains that the adaptations of the study are adequate as it will allow gathering data on the MoA linked to the adverse effects that may be observed. It would potentially avoid further animal testing and allow a hazard identification with data on potential confounding factors.

Strain

Based on your comments regarding genetic drift and necessary dose-range finding, we recommend performing the study on Wistar rats as both McFarlane et al. (1997) but also Meyer and Hanson (1980) used this strain. In Table 2 above, the names of the enzymes to be measured have been adapted to Wistar rats. Would another strain be used; it must be justified in detail and the hepatic enzymes to be measured must be adapted accordingly.

Monitoring of food consumption and body weight of dams

The monitoring of food consumption and body weight of dams during lactation must be done daily between lactation day (LD)1 and LD21. At LD21, the remaining F0 dams must be sacrificed.

Characterisation of milk nutritional quality on F0 sacrificed dams at LD10

Ten dams from the control group and 10 dams from the high-dose group must be used for collecting milk samples at lactation day 10.

Milk nutritional quality and the Substance contamination levels must be measured at LD10 to specifically clarify the concern for growth retardation and effects on lactation. At LD10, milk samples must be collected following injection of oxytocin via intraperitoneal route on live supernumerary dams (n=10 dams from control group and n=10 dams from the high-dose group) as described in method by Sevrin T. et al. (2020).

After 20 min dam/pups' separation and intraperitoneal injection of oxytocin to stimulate milk ejection, dams will be anaesthetized with 4% of isoflurane, and milk will be manually collected and stored at −20 °C until analysis.

The Substance and its metabolites must be analysed by mass spectrometry. The Substance can be analyzed by specific methods such as LC-MS or GC-MS described in Tsuji S. et al. (2004). A method by GC-MS is described in Rodil R. et al. (2012) to analyze the Substance and its metabolites: BHT-CHO (3,5-di-tert- butyl-4-hydroxybenzaldehyde), BHT-OH (2,6-di-tert-butyl-4-(hydroxymethyl)phenol), BHT-COOH (3,5-di-tert-butyl-4-hydroxybenzoic acid), BHT-Q (2,6-di-tert-butylcyclohexa-2,5-diene-1,4-dione). These degradation products must also be measured since they are also of concern (Oikawa S. et al. (1998).

Assessment of F0 dams' blood samples to be sacrificed at LD10 and LD21 (or a few days later i.e. when females are no longer lactating and before cyclicity resumes)

Blood samples must be collected before animal sacrifice.

Analysis of plasma prolactin levels (e.g., see Sevrin T. et al. (2020) for methodology if needed) and estradiol levels (n = 10 per group) must be performed to allow following their kinetics between LD10 and LD21 to clarify the concern for effects on lactation potentially related to estrogenic/androgenic interference. Free and total T4 will also be measured.

Thyroid assessment of F0 dams at LD10 and LD21 (or a few days later i.e. when females are no longer lactating and before cyclicity resumes)

Following a PfA received during the MSCA consultation, it was advised to perform histopathologic analysis and measurement of organ weights of the thyroid in dams at all points of sacrifice: at LD10 (high dose and controls and at LD21. Although not included in the study at earlier stages, the evaluating MSCA agrees that its inclusion would allow a more comprehensive investigation of the thyroid modality and ensure the most efficient use of vertebrate animals. This PfA was acknowledged by the registrant. In general, they do not agree to the addition of any parameter beyond the specification of the OECD TG 426.

Histologic and molecular analysis of the mammary glands of F0 dams sacrificed circa LD21:

After weaning (circa LD21, i.e. when females are no longer lactating and before cyclicity resumes), the remaining F0 dams (n = 80 for the 4 groups) must be sacrificed using an appropriate method.

The 4th mammary glands must be collected at the weaning sacrifice.

- The right 4th mammary gland must be sampled and fixed for histology as described in the review by Matouskova K. et al. (2022) and the technique described by Mayer J.A. et al. (2008).
- The left 4th mammary gland must be immediately frozen in liquid nitrogen before storage at −80 °C for further RNA analyses. RNA extractions and RT-qPCR analysis (RNA levels for prolactin receptor and for estrogen receptor 1 as well as synthesis markers (Caseins, lactalbumin) must be performed (you can refer to Campo Verde Arboccó F. et al. (2016) for further details if necessary). The evaluating MSCA acknowledges that such measurements will not be conducted under GLP and this may not be seen as a reason to reject the information.

Assessments to be performed on all necropsied pups

All pups body weight and size must be reported (males and females).

F1 pups sacrificed at PND11±1:

F1 pups sacrificed at PND11±1 are collected from supernumerary dams (n=10 dams from control group and n=10 dams from the high-dose group). Males and females F1 pups must be treated separately. PND11±1 pups' stomach weight ratio (full / empty) must be reported (males and females).

In its comments to the draft decision, a member registrant notes that "*pups sacrificed on PND 10 are generally perfusion-fixed, therefore either the investigations would have to be done on perfusion-fixed organs (including organ weights, which is very uncommon), or additional animals would have to be used for these investigations. In perfusion-fixed animals, the stomach weights would only be determinable as is, thus with as much content as was present at the time of fixation. Emptying the organ following fixation is not possible*."

The evaluating MSCA notes that it is not requested to perfuse pups for fixation. Pups should not be fixed for stomach content analysis, nor for further sample collections (that should be stored at -80 for gene expression analysis).

Blood samples of pups must be collected for hormone concentration measurement of circulating IGF1, free and total T4 (if limited material available at PND11±1, only free) (Sevrin, 2020). Blood samples are pooled if necessary (n=1 sample at least /sex/litter, n=3 if possible) and measurement must be duplicated (i.e. 2 measurements of each sample).

In its comments to the draft decision, a member registrant notes that these measurements "*require a large sample volume for analysis*" and that "*even taking total blood volumes will not suffice to measure all parameters on the plasma of the pups individually. Thus, pool samples would have to be used* […]". The registrant asks "*that the request be revisited once the technical feasibility has been evaluated both theoretically as well as in a preliminary study*".

The evaluating MSCA acknowledges the technical challenges in conducting measurements on individual pup. Therefore, the text above was modified to allow conducting measurements on pooled samples.

F1 pups sacrificed at PND25±2 or PND60±2:
F1 (n=1 per sex per litter) will be sacrificed at PND25±2. The remaining F1 must be sacrificed at adulthood at the end of behavioral analyses (i.e. PND60±2 when behavioral tests have been performed). On PND60±2 pups, blood samples must be collected individually.

Individual or pooled blood samples (as specified above) of each time point must be distributed in order to:

- Measure hormone concentration of circulating IGF1 (as a suitable marker of somatotropic axis), for free and total T4 (if limited material available in PND11±1, only free).
- Measure estradiol and testosterone in plasma (at least n=15 per sex per treatment).

The registrant also notes that the study "*by Sevrin et al. describing the method only analyzed these parameters in adult dams*".

The evaluating MSCA notes that this study is not referenced in relation to the analysis of blood samples in pups. However, based on the publication provided by the registrant (Marty et al., 2022), offspring serum thyroxine together with offspring serum triiodothyronine and thyroid stimulating hormone appear relevant to predict the likelihood

for neurodevelopmental effects, when maternal serum thyroid hormone levels do not show a causal relationship with statistically significant neurodevelopmental effects. Based on this publication, it appears that registrants are aware of methodologies to measure the hormones mentioned in this reference in pups.

A PfA to modify the study design was received during MSCA consultation, requesting further conventional and sensitive parameters to address a concern for EAS-mediated activity, such as anogenital distance and nipple retention after birth, and mammary gland histology, or measures of certain hormono-sensitive organs such as the weight of uteri in females and seminal glands in males. The basis for the concern was provided in the PfA and is included in Section 1.1.2 b) neurodevelopmental effects related to thyroid disruption or other endocrine modalities. Therefore, it is recommended to measure hormono-sensitive organs such as the weight of uteri in females and seminal glands in males, mammary gland histology as well as anogenital distance and nipple retention as non-invasive markers of endocrine activities in pups. It is noted however that in accordance with paragraph 33 of OECD TG 426, sexual maturation evaluation is required for at least one male and one female per litter. This PfA was acknowledged by the registrant. In general, they do not agree to the addition of any parameter outside the OECD TG 426.

Behavior investigations of F1 pups

Pups' body weight/ health, clinical observations, sexual maturation for those used for behavioral investigations must be monitored from PND1 to PND60 as requested in the OECD TG 426 standard protocol.

At PND25±2 and at PND60±2, pups must be tested for anxiety in addition to the parameters specified in OECD TG 426. An analysis of anxiety state level, using elevated plus or Zero mazes, must be assessed since an increased anxiety state level can impact learning and memory processes as previously reported (Darcet F. et al. (2014); Maloney E. et al. (2014)).

In its comments, a member registrant states that:

- "[m]*otor activity measurements can be performed on the same pups at different time points, however these cannot at the same time be the pups sacrificed intermittently*."
- "*Learning and memory tests as well as anxiety tests should not be conducted on the same animals*" and "*Both anxiety as well as learning and memory tests are generally maze tests and thus conducting multiple times as requested in the draft decision might falsify the results*". Therefore "*the anxiety level testing should be performed either on separate animals (requiring however additional animals to be included) or should be deleted from the request*".

The evaluating MSCA notes that assessment of learning and memory, anxiety-related behavior and locomotor activity should be conducted on the same animals. Indeed, modifications in the anxiety state level and locomotor activity can interfere with the memory performance of animals. This should be taken into account in the interpretation of data. The anxiety state level that should be measured corresponds to the basal anxiety-state level, without the use of any aversive stimulus.

An analysis of anxiety state level, using e.g. elevated plus or Zero mazes, must be assessed since an increased anxiety state level can impact learning and memory processes. In their comments on the PfAs, the registrant stated *"The proposal now requests anxiety testing using the elevated plus or zero mazes to assess learning and memory processes."* Contrarily to what is stated in the registrants comments on PfA, anxiety testing using elevated plus or Zero mazes were already present in the draft

decision submitted to them for comments. The inclusion of this parameter is in addition to the requirements for learning and memory testing, which is included as a requirement in the test guideline.

Request to keep tissues and serum
In a tier 2 and if some results need to be confirmed or clarified, some histological and molecular investigations could be requested on frozen organs preserved from dams and pups from the current test. Blood samples must be kept for possible additional hormonal measurements and metabolic profiles in pups for 5 years if further information is necessary. If necessary, these investigations would confirm elements for a possible human health mode of action clarification if the conclusions provided by the actual decision are unclear.

Request for the full study report
You must submit the full study report which includes:
- a complete rational 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, data and statistical analyses for behavioral tests, 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 Substance.

In its comments on the draft decision, a member registrant indicated that "*several of the additions requested will defeat the purpose of obtaining a guideline-conformant study, especially with regards to the time points specified and the mating design*". A member registrant also states that "[…] *due to the complex study design, no experience and subsequently also no historical control data for many parameters and time points will be available in laboratories. Taken together with the small group sizes specified as well as the new need to establish several of the measurements, this will hamper proper statistical analysis and potentially impact the validity of the study overall*". During the PfA commenting period, these concerns were further substantiated. It was pointed out that "*Without a baseline for comparison, it becomes difficult to determine whether observed changes are within normal variability, leading to potential false positives (identifying an effect when none exists) or false negatives (failing to identify an effect).*" and that "*[r]are outcomes, such as specific organ toxicity or developmental malformations, might be mistakenly attributed to the test substance if no historical incidence data exist to provide context.*" They advocated that "*[r]egulatory authorities may question the reliability of study findings if they are not supported by robust historical control data. This can delay decision-making or require additional testing to confirm results*" and a member registrant indicated lack of legal certainty and transparency and request that the "*rules of interpretation*" are detailed in the decision.

The evaluating MSCA acknowledges that validated and standard parameters usually have well-defined background and historical control values. Those standard parameters will be assessed within the GLP framework and allow to ensure the validity of the conducted study. The additional parameters will be compared with the existing database from the literature. Based on the validity of the conducted study, the evaluating MSCA considers that it will be possible to conclude that the results obtained on the additional parameters are the consequence of the treatment and not artefacts. Furthermore, the evaluating MSCA considers that these additional parameters are required to clarify the concerns identified for the Substance. The evaluating MSCA considers historical control data as important and

valuable in identifying whether an assay is conducted correctly. However, the absence of appropriate historical control values does not invalidate a study. The evaluating MSCA will relate the results of each parameter with each another, in addition to findings from other available studies (e.g. literature) and using the concurrent control as baseline. Results will be combined to decide if they are biologically relevant, meaningful and logical based on the knowledge gathered and detailed above. GLP parameters will be included in any interpretation.

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

The registrant's alternative strategy (Appendix A, section 2.1 (c)) aims to resolve the thyroid effects as secondary to liver effects. This question is very specific to one of the possible MoA of the potential concern and cannot clarify the concern. For the DNT request, the registrant has not proposed an alternative strategy to address the behavioural/delayed neurodevelopmental concern, which may/may not have an endocrine MoA.

The request is:

- Appropriate because it will provide information which will clarify the effects on neurodevelopment. This is the only test which is specifically capable to address simultaneously concerns on developmental neurotoxicity and integrated ED-related aspects. This will enable the evaluating MSCA to conclude on developmental neurotoxicity.
- Considering that the Substance shows potential disruptive effects on hypothalamic pituitary (thyroid and somatotropic) axes, which could be linked to neurodevelopmental toxic effects, the modified DNT TG 426 will also clarify effects on lactation and on mammary gland. Further exploration of the effect on progeny will demonstrate prenatal effects of the Substance on the capacity of pups to develop. Furthermore, the DNT study will provide clear evidence on the hypothesis of perturbations on hypothalamus pituitary thyroid axis that will be considered together with the results of LAGDA test (OECD TG 241) dedicated to demonstrating an interference with HPT axis. This level 4 in *vivo* test will provide definite evidence on thyroid function disruption and its relevance to human.
- The least onerous measure, because there is no equally suitable alternative method available to obtain the information that would clarify the potential hazard. The possible alternative of an *in vivo* study on dogs or monkeys is more expensive and should only be the very last recourse. Another option would have been to add a cross-fostering design to the requested study to distinguish the effects of the Substance in utero from those via lactation. However, it would require more animals. Consequently, there is no other study available at this stage that will generate the necessary information and does not need to test on vertebrate animals.

In his comments to the draft decision, the lead registrant disagrees with the information requested and proposed an alternative testing strategy already described in Appendix A, Section 2.1(c). The reply provided in that section equally apply to this information requirement.

The registrant also states that "[a] *CF Level 5 study investigating adversity (i.e., the OECD 426 neurodevelopmental toxicity study) is therefore only appropriate if the Alternative Testing Strategy suggests a primary, human-relevant mechanism."*

The evaluating MSCA notes that the activation of liver hepatic enzyme, if demonstrated to be rodent specific, does not allow concluding with certainty that this mechanism is responsible for the adverse effects observed with the Substance. Neither can it be claimed

to be the only MoA. As the registrants repeatedly pointed to uncertainties concerning the problematic of the relevance of rodent model (specific liver induction mechanism and sensitivity of rodent claimed by the registrants), it was in the past proposed to perform an *in vivo* study on animal model physiologically comparable to human, such as in pig. Indeed, the registrant had informed the evaluating MSCA that a pig study was planned to fulfil the obligation of the Feed Additives Regulation (Regulation (EC) No 1831/2003 as assessed by EFSA's FEEDAP panel[3]. The evaluating MSCA requested extra analysis and parameters to be added. This request was rejected by the registrant due to technical limitations. In order to give due consideration to all relevant information, evaluating MSCA decided to wait for the data of this study to be provided. As demonstrated in the decision, the pig data do not allow to draw any robust conclusions because the study used was not designed appropriately (extra parameters and analysis not added). The evaluating MSCA restarted an evaluation and a request in rodents as it seemed impossible to request Pig studies.

Finally, the registrant claims that the request for an OECD TG 426 infringes the legal certainty principle due to the high uncertainty and complexity of the requested adaptation to the standard design of the OECD TG 426. The registrant considers that this is reflected by the lack of expertise in the EU as supported by letters from two laboratories attached to the comments to the draft decision.

The evaluating MSCA clarifies that the requested study is to clarify a concern with information relevant enough to set up the required risk management measures, if necessary. Previous decisions have confirmed that under substance evaluation, it may be appropriate to make alterations to recognised test methods, for example, the examination of parameters which do not need to be examined to meet standard information requirements. This helps to ensure that information generated pursuant to a substance evaluation decision meets real information needs (Board of Appeal Decision of 23.09.2015, Case A-005-2014, Akzo Nobel and Others, para. 88; Decision of 12.07.2016, Case A-009-2014, Albemarle Europe, para. 156; Decision of 25 September 2018, Case A-008-2017, SI Group-UK, para. 91). This decision does not oblige registrants to provide information which they can neither assuredly obtain nor generate themselves (Decision of 06.08.2018, SI Group and Others, Case A-006-2016, para. 102). The principles of proportionality and legal certainty is therefore applied. In addition, for previous decisions it was acknowledged that there are no legal grounds to annul an obligation simply because it is predicated upon its technical feasibility (Decision of 12.07.2016, Case A-009-2014, Albemarle Europe, para. 191-193).

[3] The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)

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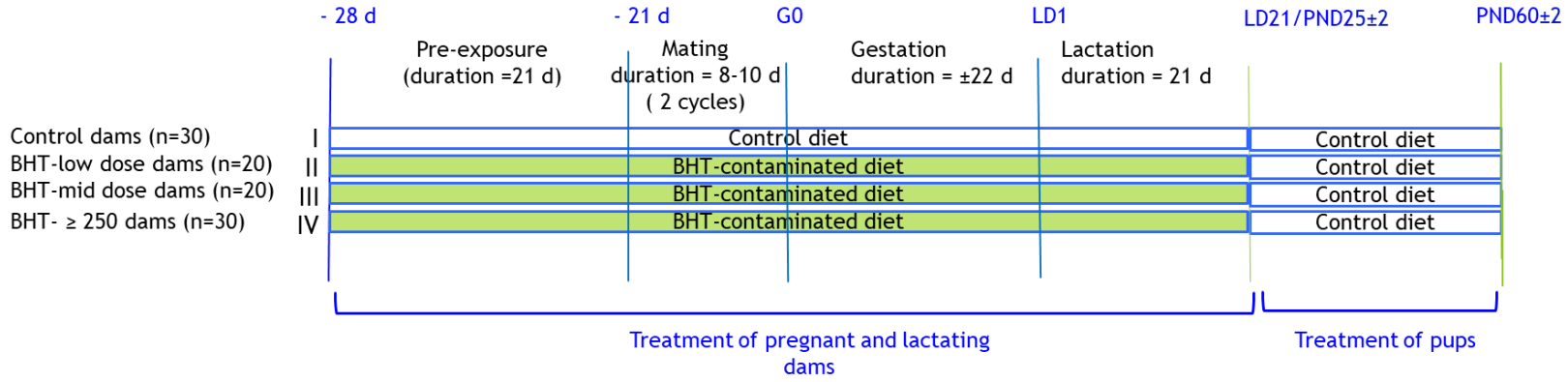
Appendix C: Scheme of the DNT OECD TG 426 modified/ treatments and analysis on FO dams and F1 pups emphasizing the parameters requested in addition to the standard protocol

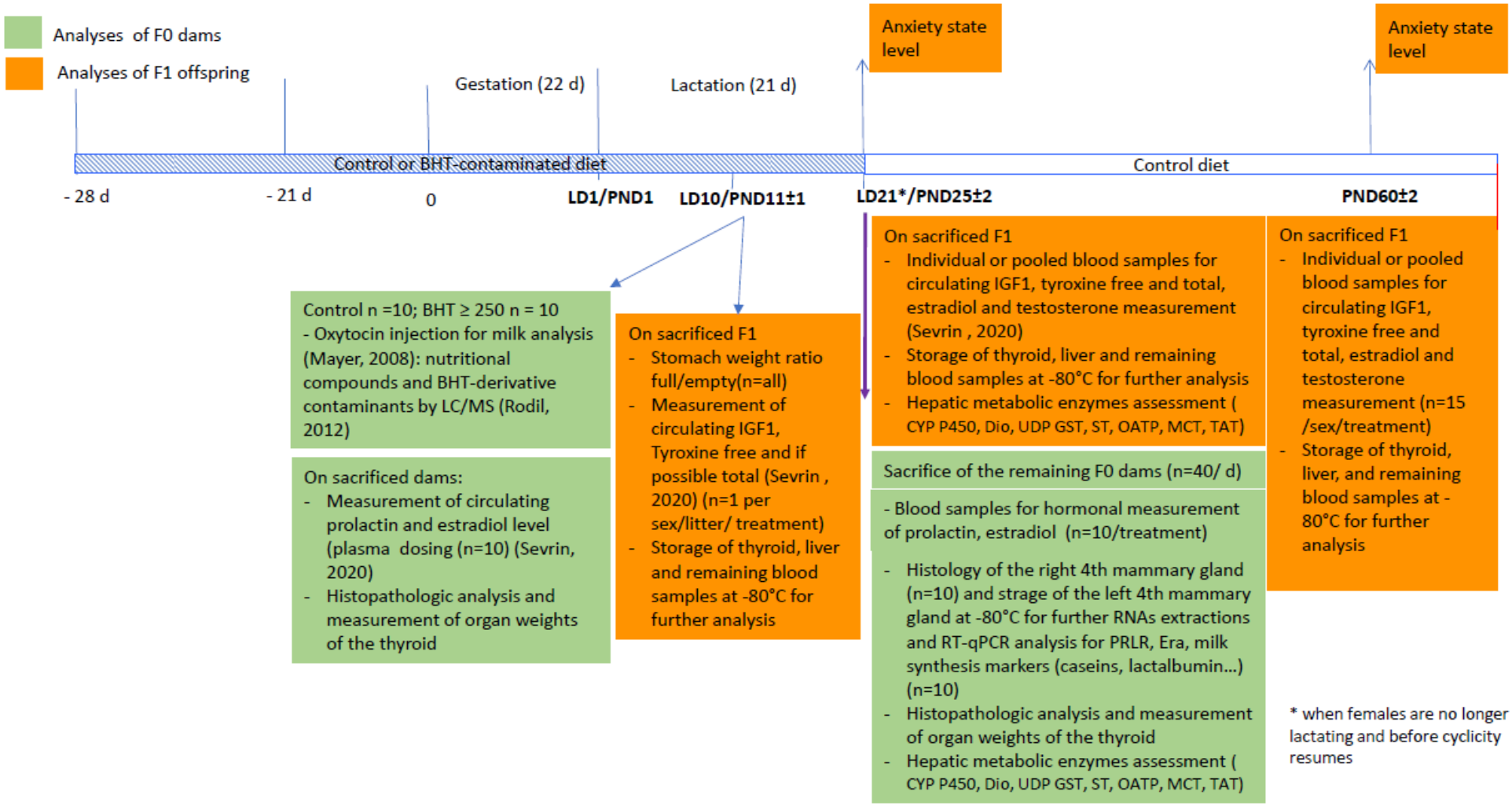
DNT OCDE 426 MODIFIED / Treatments

Treatment groups and periods of treatment
Control BHT- low dose BHT- mid dose BHT ≥ 250 mg/kg bw/d



ECHA
EUROPEAN CHEMICALS AGENCY

DNT OCDE 426 MODIFIED/ ANALYSIS on F0 dams and F1 pups



Appendix D: 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.

Evaluation period

Due to initial grounds of concern for Endocrine disruption, the Member State Committee agreed to include the Substance in the Community rolling action plan (CoRAP) to be evaluated in 2016. France 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 and on other relevant and available information.

Following a first evaluation, during which the composition of the Substance and an endocrine disruption property were assessed, an initial draft decision was submitted to the registrants for comments in April 2017. Following the comments submitted in June 2017 and in follow-up to the endocrine disruption expert group (ED-EG) meeting in December 2017, the evaluating MSCA took the decision to withdraw the draft decision and await data generation ongoing under other legislation for related concerns (*i.e.*, studies relating to the identification of degradation products, a tolerance study on chicken, and on pigs). The registrant indicated the relevance of these studies to the substance evaluation and the evaluating MSCA discussed protocol amendments to these planned studies to address the registrants' concerns regarding thyroid adversity as secondary to liver enzyme induction in spring 2018. However, the request for study modifications made by the evaluating MSCA could not be accommodated by the registrant. Indeed, it was argued that the modifications requested were too important, the contracted laboratory did not have the capabilities to perform such a study, and a suite of further reputable laboratories contacted about conducting such a study indicated no historical experience with such a study design in mini pigs. Nevertheless, the evaluating MSCA considered that the data from these studies could be relevant to the concerns identified in the substance evaluation. Before deciding whether further animal studies were required to clarify the identified concerns, the evaluating MSCA decided to wait for the information to become available and, in the meantime had several interactions with the lead registrant. The registrant provided the data in an update to the registration dossier in June then completed in October 2019 and the full study report data for assessment by the evaluating MSCA in January 2022.

After reviewing the newly generated information and as explained in this decision, the evaluating MSCA concluded that it does not allow clarifying the identified concerns. After re-evaluating the available body of evidence, the evaluating MSCA initiated a new testing strategy to clarify the concerns on both endocrine disruption and on developmental neurotoxicity. This new strategy was presented in November 2021 during the ED-EG 16th meeting. During the consultation of the ED-EG, the registrant presented new data on cow milk ([redacted], 2014). The registrant provided access to the study on 13th November 2023. This was found to be relevant to the substance evaluation and needed to be analysed.

After this process, the evaluating MSCA submitted a new draft decision (Article 46(1) of REACH) to ECHA on 9 February 2024.

Decision-making and Registrants comments

ECHA notified the registrants of the draft decision on 03 July 2023 and invited them to provide comments. ECHA received their comments and forwarded them to the evaluating MSCA and the evaluating MSCA took these comments into account.

In its comments to the draft decision, a member registrant requested an extension of the deadline from 26 months to 40 months. The registrant provided the following justification for the extension:

- dose-range finding studies are required prior to the main study;
- some additional parameters required for evaluation will require preliminary work in the selected laboratory;
- the requested studies may require involving more one laboratory and therefore may require setting-up a multi-site study;
- the difficulty in finding (an) appropriate laboratory(ies) and the anticipated long-lead time.

ECHA acknowledges the complexity associated to the requested study and currently longer lead times in contract research organisations. Therefore, ECHA has extended the deadline from 26 to 40 months.

In its comments to the draft decision, the lead registrant claims that the decision would infringe the EU law principles of good administration and legal certainty. In particular, on the duration of the substance evaluation procedure, the registrant considers that ECHA has violated the right to good administration enshrined in Article 41(1) of the EU Charter of Fundamental Rights as the duration of the substance evaluation procedure (i.e. 8 years) is not in line with Article 46(4) of REACH. The registrant considers that the substance evaluation procedure should have been completed either in 2017 or 2019 because:

- while there is no statutory deadline under REACH for a MSCA to review comments on a draft decision, the evaluating MSCA should do so within a reasonable period of time to comply with Article 46(4) of REACH;
- the restart of the substance evaluation was not justified by the fact that the "new" and "substantive" information came to light during the decision-making process. In that regard, the registrant refers to the Board of Appeal decision in case A-017-2014.

ECHA acknowledges the exceptionally long time taken in the current substance evaluation. However, ECHA does not agree that the substance evaluation should have been completed in either 2017 or 2019. Instead, there were valid reasons for the long processing. For example, as the registrant repeatedly pointed to uncertainties concerning the liver induction mechanism and sensitivity of rodent, it was proposed to perform an *in vivo* study on animal model physiologically comparable to human, such as in pig. Indeed, the registrant had informed the evaluating MSCA that a pig study was planned to fulfil the obligation of the Feed Additives Regulation (Regulation (EC) No 1831/2003. ANSES requested extra analysis and parameters to be added. This request was rejected by the registrant due to technical limitations. In order to be able to assess with all the data in hand, the evaluating MSCA decided to wait for the data of this study to be provided. As demonstrated in the decision, the pig data do not allow to draw any firm conclusions because the study used was not designed appropriately (extra parameters and analysis not added). ANSES restarted a classical evaluation and a classical request in rodent as it is impossible under REACH to request a pig studies.

More specifically, as mentioned above, new and (potentially) substantive information became available to the evaluating MSCA during the evaluation stage. The evaluating MSCA decided to wait for this information to guarantee an efficient use of animal testing. It should also be noted that the evaluating MSCA expected to receive performance test on chicken and winglet and additional raw data that was not provided. Under Article 47 of the

REACH, the evaluation of a substance must "*be based on all relevant information submitted*" and the evaluating MSCA could not ignore this obligation. This explains the overall duration of the evaluation phase. ECHA also emphasizes the number of interactions held between the evaluating MSCA and the registrants. The registrants were also informed on the progress of the evaluation through the consultation at the ED-EG in open sessions (ED-EG 10th and 21st meeting).

Finally, regarding legal certainty and the legitimate expectations claimed by the registrant, settled case-law clarify that, the right to rely on the principle of the protection of legitimate expectations presupposes that precise, unconditional and consistent assurances originating from authorised, reliable sources have been given to the person concerned by the competent authorities of the EU[4]. The Court has also held that the mere length of time in reaching a decision does not create a legitimate expectation in the outcome[5], and where the proceedings is in progress, there is no legitimate expectation that will have a particular outcome[6].

In the present case, both principles above have been respected by the regular exchanges held between the evaluating MSCA and the registrants of the Substance. As explained above, these exchanges aimed at identifying the information needed to clarify the identified concerns while taking into consideration the registrants feedback and requests.

No assurances from the evaluating MSCA or ECHA that based on the registrants' comments or any further communication with them the substance evaluation process would be closed with no decision requesting further information. Delays in the evaluation were also foreseeable and cannot come as a surprise to the registrants, also given that they submitted substantial new information and were informally in contact with the evaluating MSCA. This is also due to the fact that ECHA publishes the status of each substance evaluation case[7]. For this substance, the status is marked as "ongoing".

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.
Subsequently, the evaluating MSCA received proposal(s) for amendment to the draft decision and modified the draft decision.

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

ECHA referred the draft decision, together with the registrant(s) comments, to the Member State Committee.

ECHA invited the registrant(s) to comment on the proposed amendment(s).

The registrant(s) comments on the proposed amendment(s) were taken into account by the Member State Committee. In addition the registrant(s) provided comments on the draft

[4] see, inter alia, judgments of 22 June 2006 in *Belgium and Forum 187* v *Commission*, C-182/03 and C-217/03, EU:C:2006:416, paragraph 147, and 7 April 2011 in *Greece* v *Commission*, C-321/09 P, EU:C:2011:218, paragraph 45

[5] case C-121/86

[6] 246/87, para 17, case C-83/99

[7] Substance evaluation - CoRAP - ECHA (europa.eu)

decision, which were not taken into account by the Member State Committee as they were considered to be outside of the scope of Article 52(2) and Article 51(5).

MSC agreement seeking stage

The Member State Committee reached a unanimous agreement during its MSC-88 meeting and ECHA took the decision according to Article 52(2) and 51(6) of REACH.

Follow-up evaluation

After the deadline set in this decision has passed, the evaluating MSCA will review the information the registrants 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 E: Technical Guidance to follow when conducting new tests for REACH purposes

Test methods, GLP requirements and reporting

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[8].

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 and other parameters relevant for the property to be tested.

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[9].

[8] https://echa.europa.eu/practical-guides

[9] https://echa.europa.eu/manuals