Helsinki, 27 August 2019

Substance name: 2,4-di-tert-butylphenol (2,4-DTBP; herafter, 'the Substance')
EC number: 202-532-0
CAS number: 96-76-4
Date of latest submission(s) considered: 21 March 2018
Decision/annotation number: Please refer to the REACH-IT message which delivered this communication (in format SEV-D-XXXXXXXXXX-XX-XX/F)
Addressee(s): Registrant(s)[1] of 2,4-di-tert-butylphenol

DECISION ON SUBSTANCE EVALUATION

In accordance with Article 46(1) of the REACH Regulation (Regulation (EC) No 1907/2006), you must submit the following information on the Substance:

1. *In vivo* Mammalian Alkaline Comet Assay (oral route, gavage, with rats) on tissues as specified in appendix 1; test method: OECD TG 489.

2. Fish sexual development test (FSDT); test method: OECD TG 234; with Japanese medaka (*Oryzias latipes*) or zebrafish (*Danio rerio*), including gonadal histopathology. The study must be performed using five test concentrations, and if the test species is Japanese medaka, genetic sex must also be determined.

You must provide an update of the registration dossier(s) containing the requested information, including robust study summaries and, where relevant, an update of the chemical safety report by 27 February 2021.

In addition to the robust study summaries, you must submit the full study reports for the information required under point 1 and 2 of this section by the same deadline, by attaching it to the relevant endpoint study record in IUCLID.

The deadline takes into account the time that you may need to agree on which of the registrant(s) will perform the required tests (three months is allocated for this).

The reasons of this decision and any further test specifications of the requirements are set out in Appendix 1. The procedural history is described in Appendix 2. Further information, observations and technical guidance as appropriate are provided in Appendix 3. Appendix 4 contains a list of registration numbers for the addressees of this decision. This appendix is confidential and not included in the public version of this decision.

[1] The terms registrant(s), dossier(s) or registration(s) are used throughout the decision, irrespective of the number of registrants addressed by the decision.

Who performs the testing?

Based on Article 53 of the REACH Regulation, you are requested to inform ECHA who will carry out the studies on behalf of all registrant(s) within 90 days. Instructions on how to do this are provided in Appendix 3.

Appeal

This decision can be appealed to the Board of Appeal of ECHA within three months of its notification. An appeal, together with the grounds thereof, has to be submitted to ECHA in writing. An appeal has a suspensive effect and is subject to a fee. Further details are described under: http://echa.europa.eu/regulations/appeals

Authorised[2] by Christel Schilliger-Musset, 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.

Appendix 1: Reasons

Based on the evaluation of all relevant information submitted on the substance and other relevant available information, ECHA concludes that further information is required to enable the evaluating Member State competent authority (eMSCA) to complete the evaluation of whether the substance constitutes a risk to human health and/or the environment.

The eMSCA will subsequently review the information submitted by you and evaluate if further information should be requested in another decision to clarify the concern, according to Article 46(3) of REACH.

Consideration of your general comments on the original draft decision

You commented that the draft decision fails to identify a potential risk justifying the need to conduct additional studies.

According to information in the registration dossier the Substance is used as fuel additive and as intermediate. Significant exposure to workers, consumers and the environment cannot be excluded.

The eMSCA acknowledges that it is difficult to assess which fraction of the exposure to the Substance comes from the registered uses. However, a qualitative assessment shows that the environment and humans (through the environment) are exposed to the Substance. The following 52 studies provide information on exposure to the Substance (see 'References' for the reference list on exposure sources of the substance):

- 27 studies identify **natural sources** of the Substance
  - Mainly as antioxidant, anti-fungal or bactericide component
  - Found in plants, but also in bacteria or in invertebrates
- 14 studies detected the Substance in **plastic** or in **food/water in contact with plastic** or in **rubber**
  - Found in several types of plastic, i.e. polyethylene, polycarbonate, PET and polypropylene
  - Found in rubber gloves and occupational rubber
- 6 studies found the Substance in **water**
  - In rivers in Romania, Hungary and France
  - In drinking water in China and USA (probably due to plastic pipe migration)
- 1 study found the Substance in **humans**
  - Biomonitoring in pregnant women in USA
- 2 studies found the Substance in **animals**, following a secondary contamination
  - In mice
  - In fish
- 2 studies identified **other sources** of 2,4-DTBP
  - In household dust
  - In wound bandages

The eMSCA takes note of your comment about the conditions under which further information can be required under article 50(1) of REACH.

Based on previous Board of Appeal decisions, you argue that the three following conditions have to be fulfilled to request further information:

(1) Show that there is a potential environmental and/or health risk (real and not only theoretical)
(2) Prove that the potential risk needs to be clarified
(3) The information requested has a realistic possibility of leading to improved risk management

The eMSCA considers that these three conditions are fulfilled:

(1) The potential environmental hazard consists of the endocrine disrupting (ED) activity of the Substance (estrogenicity, anti-androgenicity, thyroid), which has been shown *in silico* (theoretical) but also *in vitro* (real). *In vivo*, some changes observed such as delayed preputial separation or increase of testis weight in different studies suggest a potential endocrine activity. Moreover there are some indications that the Substance is toxic for reproduction (for more details, see endpoint 2).
The potential health hazard consists of the positive result found in the *in vitro* Mammalian chromosome Aberration Test (OECD TG 473) with S9 metabolic activation (for more details, see endpoint 1).
The potential hazard is thus demonstrated. A qualitative assessment (see above) shows that the environment and humans are exposed to the Substance. This combination of exposure and hazard information shows that there is a potential risk for the environment and/or human health related to the intrinsic properties of the Substance.

(2) The potential risk identified under (1) needs to be clarified. Moreover, the FSDT test will clarify the potential environmental ED hazard and allow refinement of the risk assessment for the environment, while the comet assay will clarify the potential mutagenicity health hazard.

(3) Currently, you have self-classifed the Substance as Aquatic chronic toxicity 1, Eye damaging 1 and Skin irritant 2. The substance is not PBT nor vPvB.
If the ED concern for the environment is confirmed, identification of the substance as a substance of very high concern (SVHC) and subsequent inclusion into Annex XIV would lead to improved risk management measures for the environment.

If the mutagenicity concern is confirmed, the classification of the substance as mutagen and potential SVHC identification with inclusion into Annex XIV would lead to improved risk management measures for the human health.

Therefore, the identification of a potential risk is based on a combination of exposure and hazard information.

ECHA
EUROPEAN CHEMICALS AGENCY

1. *In vivo* Mammalian Alkaline Comet Assay (oral route, gavage, with rats)

The concern(s) identified

Available data indicate concern for genotoxicity with regard to the metabolite(s) of the substance. Positive results were found in the *in vitro* Mammalian Chromosome Aberration Test (OECD TG 473) (registration dossier, study report, 1998) with S9 metabolic activation: a significant number of cells with aberration were found, after 20h exposure duration, at the highest doses of 5 and 6 µg/ml in the first and second test, respectively. No significant chromosome aberration was seen in the test without metabolic activation. Cytotoxicity was seen at 5 µg/ml with metabolic activation, and at 6 µg/ml without metabolic activation.

An *in vivo* Mammalian Erythrocyte Micronucleus (MN) Test (OECD TG 474) (registration dossier, study report, 2008) has been performed with the Substance. The results of this test (absence of micronucleus induction) can however not be used to dismiss the genotoxicity concern as it was not demonstrated that the Substance reached the bone marrow. Indeed, no significant decrease in the ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) (PCE/NCE ratio) was observed in the treated animals compared to control animals during the evaluation of the micronucleus test. The PCE/NCE ratio for females treated until 800 mg/kg bw/day did not change and a T-test performed on the PCE/NCE ratio for males at 1000 mg/kg bw/day (0.43 +/- 0.08) and the male control (0.53 +/- 0.06) showed that the difference was not statistically significant (P=0.1).

Moreover, no plasma or blood analysis have been performed to check for the presence of the test substance or its metabolites. In addition, no toxicokinetic data are available to demonstrate bone marrow exposure or rapid elimination of the substance and its metabolite(s).

The genotoxicity concern is for mammalian cells, as two Bacterial Reverse Mutation Tests (OECD TG 471) (registration dossier, study reports, 1991 and 2015) were conducted and showed negative results with and without metabolic activation.

Why new information is needed

Further information is needed, taking into account the existing data which show a concern for genotoxic potential of the Substance and the widespread use of the substance.

What is the possible regulatory outcome

The results of the study will clarify the genotoxic potential of the substance. This can possibly lead to a classification for germ cell mutagenicity and related risk management measures.

Considerations on the test method and testing strategy

The *in vivo* alkaline comet assay is the method of choice to investigate further the uncertainties upon genotoxicity for the following reasons:

- The concern is based on positive results in an *in vitro* Mammalian Chromosome

Aberration Test (OECD TG 473) with metabolic activation (registration dossier (study report, 1998)). The chromosome aberration test (OECD TG 473) detects structural chromosomal aberration (e.g. breaks, deletions, rearrangements).
- The comet assay can detect single and double-stranded breaks, which can lead to chromosome aberrations.
- The comet assay presents an increased sensitivity for detecting low levels of damage that might otherwise go undetected by the standard assays (Vasquez MZ, 2010 and Tice RR *et al.*, 2000).
- DNA damages can be tissue specific and the comet assay will allow investigation of several organs at the same time (Hartmann A *et al.*, 2004).
- Short lived metabolite(s) may not be detected with *in vivo* micronucleus assay, because they do not reach the bone marrow (Cliet I *et al*, 1993).
- The comet assay can measure oxidative DNA damage *in vivo* (Ding W *et al.*, 2014).
- Significant gender difference in toxicity was observed in rats in the *in vivo* MN test (registration dossier, study report, 2008); possibility of sex specific mutagenicity can be detected by comet assay (Ding W *et al.*, 2014).

The following tissues must be investigated:

- glandular stomach and duodenum[3]

Reasons:
As set out in the OECD TG 489, the glandular stomach and duodenum are recommended as tissues to examine site of contact effects after oral exposure. Moreover, according to the test guideline, duodenum may be considered more relevant for humans. In view of the following possible variables; different tissue structure and function of the stomach and duodenum; different pH conditions; probable different absorption rates of the substance and possible breakdown product(s) between these two tissues; type of substance and its possible breakdown product(s), the eMSCA considers that it is necessary to sample both tissues to increase the reliability of the analysis of genotoxicity at the site of contact.

and

- Liver

Reasons:
As set out in the OECD TG 489, the liver is recommended as the primary site of xenobiotic metabolism, and an often highly exposed tissue to both parent substance and metabolites. Furthermore, liver toxicity has been shown in the 28-day repeated dose study in rats (OECD TG 407) (registration dossier, study report, 2000).

You are reminded that according to Annex IX, Section 8.4., column 2 of the REACH Regulation, if positive results from an *in vivo* somatic cell study are available, "the potential for germ cell mutagenicity should be considered on the basis of all available data, including toxicokinetic evidence".

For this reason, based on a proposal for amendment from one MSCA, it is recommended to prepare slides from single cell/nuclei suspensions from gonadal tissues and store them

[3] the duodenum is the most appropriate part of the intestine to be tested, as it is the first part of the intestine and directly connected to the stomach. The duodenum tissue sampled may contain a small part of the jejunum.

under suitable conditions for an appropriate amount of time. In case a positive result is obtained from any of the somatic tissues in the comet assay it is recommended to analyse the gonadal slides.

With respect to possible outcomes, a positive result in whole gonads is not necessarily reflective of germ cell damage since gonads contain a mixture of somatic and germ cells. However, such positive result would indicate that the substance and/or its metabolite(s) have reached the gonads and caused genotoxic effects.

A negative or inconclusive result in whole gonads cannot be used to conclude on the germ cell genotoxicity as the sensitivity of the comet assay in gonadal cells has not been validated to detect germ cell genotoxicity.

You must submit the full study report of the required information in your dossier update. Indeed a complete rationale and access to all available information (implemented method, raw data collected, interpretations and calculations, consideration of uncertainties, argumentation, etc.) are needed to fully assess the provided information and to efficiently clarify the concerns.

Consideration of alternative approaches

The request for the *in vivo* Mammalian Alkaline Comet Assay is suitable and necessary to obtain information that will allow clarifying whether there is a potential risk for human health. More explicitly, there is no equally suitable alternative way available of obtaining this information. It is noted that there is no experimental study available at this stage that will generate the necessary information and does not need to test on vertebrate animals.

According to ECHA Guidance on information requirements and chemical safety assessment (version 6.0, July 2017), after a positive result in OECD TG 473, three tests (OECD TG 474, TG 475 and TG 489) can be used for follow-up. As bone marrow exposure was not demonstrated in the available OECD TG 474 (registration dossier, study report, 2008), the comet assay is considered as the most appropriate method to clarify the concern for genotoxicity.

Consideration of your comments on the original draft decision

You did not agree with the statements in the draft decision implying the *in vivo* mammalian erythrocyte micronucleus test is not reliable for assessing the mutagenicity potential for 2,4-DTBP. You claimed that the high dose male rats did show a decreased PCE/NCE ratio (PCE/NCE ratio of 0.43 compared to 0.56 in vehicle control group) clearly indicating that the bone marrow was reached. The negative result from this study was consistent with the lack of a positive result in the *in vitro* mutagenicity assays.

The eMSCA notes that the high dose male rats in the *in vivo* mammalian erythrocyte micronucleus test did not show a statistically significant decreased PCE/NCE ratio contrary to the comment submitted by the registrant.

The PCE/NCE ratio decreased from 0.53+/-0.06 (and not 0.56 as indicated by the registrant) to 0.43 +/-0.08 for males. No plasma or blood analysis have been performed to check for the presence of the substance or its metabolites and no toxicokinetic data are available to demonstrate bone marrow exposure or rapid elimination of the

substance and/or its metabolites.

Moreover, the PCE/NCE ratio for females treated until 800 mg/kg bw/day did not change.

Therefore, it cannot be concluded that the bone marrow was reached in the study.

Furthermore, you mentioned that bioassays with whole water extracts from PET bottles containing 0.1 to 0.8 µg/L of 2,4-DTBP did not express cytotoxicity or genotoxicity (Bach *et al.*, 2013).

The eMSCA however notes that in Bach *et al.*, 2013 the *in vitro* micronucleus study was performed with HepG2 cells. A metabolic activation has not been done for testing HepG2 cells as they have a metabolism. However, they have a very low metabolism compared to human primary liver cells. Therefore, the eMSCA considers they are not suitable to test the metabolites of a compound (According to Gerets *et al.*, 2012).
Moreover, in this study several substances were identified in the bottled waters at very low test concentrations (<0.2% of initial concentration with the hightest concentration of 1.8 µg/L in the bottled water after 10d exposure at 60°C). Therefore, the eMSCA questions the sensitivity of the method used and thus the reliability of the study.

Moreover, you explained that lack of mutagenicity potential of the Substance is supported by the available results from other alkylphenols. Therefore in your opinion, the overall weight of evidence suggests no genotoxicity or mutagenicity potential for the category of alkylphenols.

The eMSCA underlines however that the request for the comet assay is based on positive results found in the *in vitro* Mammalian Chromosome Aberration Test (OECD TG 473) (registration dossier, study report, 1998) with S9 metabolic activation. This test was performed with 2,4-DTBP itself.

Therefore, no conclusion should be drawn on the mutagenicity potential of 2,4-DTBP based on results from other alkylphenols.

Consideration of proposals for amendment (PfA) and your comments

One Member State considered it might be more accurate to request a transgenic rodent (TGR) study (OECD TG 488) rather than a comet assay since the sensitivity of the comet assay has not yet been evaluated for germ cells.

ECHA does not agree to request a TGR due to the observed difference in toxicity between male and female rats. Therefore, the genotoxicity should be tested in both genders. The TGR is well suited for the study of gene mutation induction in male germ cells but not for the evaluation of female germ cells as stated under paragraph 31 of OECD TG 488.

Significant gender difference in toxicity was observed in rats in the in vivo micronucleus test (registration dossier, study report, 2008). Sex specific mutagenicity can be detected by the comet assay (Ding W. *et al*, 2014).

You also disagreed with this PfA and proposed conducting a new in vivo mammalian erythrocyte micronucleus test instead of the comet assay.

The In Vivo Mammalian Alkaline Comet Assay (OECD TG 489) is considered as more appropriate because it would deliver an added value, such as information about genotoxcity at site of contact and at primary site of xenobiotic metabolism by examining glandular stomach, duodenum and the liver respectively. Furthermore, the comet assay may identify sex differences (OECD TG 489, Annex 2).

Conclusion

Therefore, based on the substance evaluation and in accordance with Article 46(1) of the REACH Regulation, ECHA concludes that you are required to carry out the following study using the substance subject to this decision: *In Vivo* Mammalian Alkaline Comet Assay (oral route, gavage, with rats) on tissues as specified above; Test method OECD TG 489.

2. Fish sexual development test

The concern(s) identified

Several non-guideline *in vitro* assays and QSAR model predictions suggest that the Substance may have endocrine activity (related to estrogenicity, anti-androgenicity, and thyroid hormone levels).

Available QSAR data
QSAR data, corresponding to the **OECD CF level 1**, with 2,4-di-tert-butylphenyl point to an oestrogenic binding potential. Moreover, Thyroid Receptor binding is expected. Limited antiandrogenic probability is shown.

Data on E modality
- Strong ER binder (OECD toolbox) due to the fact that MW is > 200 and MW =<500 and to the cyclic molecular structure with a single non-impaired hydroxyl group,
- The battery approach of the Danish (Q)SAR Database for ERα binding Balanced Training Set (Human *in vitro*) is positive and within the applicability domain

Data on A modality
- AR antagonist was positive in the Danish (Q)SAR Database but was not within the applicability domain,
- Molecular docking (endocrine disruptome) predicts moderate probability of AR antagonist

Data on TR modality
- The battery approach of the Danish (Q)SAR Database for the binding affinity for Thyroid Receptor α Binding and Thyroid Receptor β Binding was positive and within the applicability domain,
- Endocrine disruptome predicted a moderate TRα and β binder potential.

Thus, QSAR data identify some concerns for endocrine disrupting properties of the substance.

Available *in vitro* data
Several *in vitro* assays, corresponding to the **OECD CF level 2**, showed weak oestrogenic, interference with the steroid binding protein (SBD) and potential anti-androgen activity of the Substance:

In vitro data on interference with Steroid Binding protein
A reliable *in vitro* assay performed with **fish** extracts/protein demonstrated interference with the steroid binding protein (SBP):

- Interference with the Steroid Binding protein (SBP) was demonstrated in a ligand-binding study with the plasma steroid-binding protein (SBP) of Rainbow Trout at concentrations between 25 nM–250 mM (Tollefsen, 2007). SBP is known to bind 17β-estradiol and testosterone with high affinity and moderate capacity, and is thus supposed to regulate the transport, cellular uptake, excretion and bioavailability of steroids.
  Log Inhibitory Concentration (IC50) of the Substance was -3.23 mol/L (IC50 5.9 x 10^{-4}), with a RBA of 2.7 x 10^{-4}% compared to 17β-estradiol. Therefore, the Substance may interfere with SBP and may modify the steroid hormone homeostasis.

In vitro data on Estrogen modality
- In a reliable (Rel.2) non-guideline study Akahori *et al.*, 2008 used a recombinant human hERα ligand binding domain to detect ER binding. The substance was tested at a concentration between 10^{-11} and 10^{-4} M. The relative binding affinity (RBA) of the Substance was calculated to be 0.00155%, LogRBA was -2.81, compared to 2.00 for 17β estradiol, indicating weak estrogen activity.

- In addition, in the TOXCast/Tox 21 database, 15 of the 18 high-throughput ER assays used for the Estrogen Receptor Model, were run with the Substance. Results showed one positive hit for ER antagonist (Tox21_ERa_BLA_Antagonist_ratio).

  It is noted that in the ER ToxCast model prediction (in EDSP 21 dashboard), both AUC (Activity for receptor area Under the Curve), agonist and antagonist, are equal to 0 (positive activity if AUC≥0.1) indicating no agonist or antagonist activity. However, the model does not correctly identify very weak compounds, whose activity is outside the concentration range tested (Judson *et al.*, 2015).

A reliable *in vitro* assay performed with **fish** extracts/protein demonstrated estrogen binding:

- Tollefsen and Nilsen, 2008 demonstrated estrogen binding in a receptor competitive binding assay using Rainbow trout (rt) livers extracts: the substance, tested at concentrations between 250nM to 7.5mM, was able to bind rtERs, showing IC50 of 2.2 x 10^{-4} mol/L (logIC50 of -3.66 ± 0.07 mol/L) and a RBA of 1.6 x 10^{-3} %(IC50 of 3.5x10^{-9} and RBA 100% for the control 17β-estradiol).

The following three studies are merely mentioned as supportive information due to their limited reliability:

- Creusot *et al.*, 2013 developed an Effect-directed analysis (EDA) to identify

endocrine disruptive chemicals in a multi-contaminated river sediment. Active compounds were first isolated using a multi-steps fractionation procedure, followed by final fractionation step using an hERα affinity column (MELN cell line) allowing the selection of estrogenic active substances. The Substance was identified by using GC-MS. The substance was found in the fractions with the highest estrogen activity. However the fractionation method has its limitations because of co-occurrence of several biological activities in the same fraction which makes specific identification of the active chemical difficult. Nevertheless the results are in line with the findings of Akahori *et al.*, 2008.

- Jonker *et al.*, 2016 also used an EDA to investigate estrogenic activity of compounds from plastics from electronic's casings. Fractionation was run in parallel with a reporter gene assay using human VM7Luc4E2 cells to detect estrogenicity and with ToF mass spectrometry to identify the bioactive substances. This assay is however not able to quantify activity. The Substance was found to activate estrogenic response. As already explained above, the fractionation method has its limitations and the study is therefore considered of low reliability. Nevertheless the results are in line with the findings of Akahori *et al.*, 2008.

- No oestrogen antagonist activity was detected in a non-guideline stable transfected ER reporter gene assay with MVLN cells (concentrations between 10^{-7} to 10^{-4}M) (Satoh *et al.*, 2008a). The ER competitive binding assay, also part of this study, indicated that the Substance weakly bound ER (IC50 of 2.7×10^{-4} M) at a concentration near cytotoxicity. Therefore, the authors were unable to clarify the ER antagonist activity of the Substance.
  Also no oestrogen agonist activity was detected in the reporter gene assay. However, the reliability of this study is highly questionable:
  - reported controls were not tested in the same experiment but were taken from a previous study (Satoh *et al.*, 2005).
  - All tested substances were negative in the agonistic assay. Therefore, it cannot be excluded that there might have been a performance issue or that false negatives are recorded.

<u>*In vitro* data on Androgen modality</u>

- 9 out of the 11 high throughput AR assays used for the Androgen Receptor Model (Kleinstreuer *et al.*, 2017) were run with 2,4-di-ter-butylphenol (TOXCast/Tox 21 database). Two positive hits (OT_AR_ARSRC1_0960 and NVS_NR_hAR and) were recorded.

  An agonist AUC =0.0185 and Antagonist AUC =0.0276 was estimated in the AR ToxCast model prediction (in EDSP 21 dashboard), from which it can be inferred that there is a weak potential of androgen receptor activity but the outcome is however considered inconclusive (positive agonist or antagonist activity if AUC >0.1, inconclusive if AUC between 0.01 and 0.1, Kleinstreuer *et al.*, 2017).

The following two studies are merely mentioned as supportive information due to their limited reliability (limitations mentioned above):

- Strong anti-androgen activity of the substance was shown in a non-guideline stable transfected reporter gene assay using 2 different CHO-K1 cell lines (AR-

EcoScreen and C-luc) (Satoh *et al.*, 2008a). The study was performed at concentrations of 10^{-6} to 10^{-4}M. In the AR-EcoScreen an IC50 was determined of $4.1x10^{-5}$M, while the C-luc cells were less affected at this concentration. No adrogenic agonistic activity was seen. However, AR binding was observed in a competitive binding assay with an IC50= $6.0x10^{-5}$ M.

- Creusot *et al.*, 2013 used an EDA to identify biologically active endocrine chemicals in a multi-contaminated river sediment. A MDA-kb2 cell line was used to assess the androgenic and anti-androgenic activity of chemicals. No androgen antagonistic activity was noted but it is suggested that their detection in individual fractions was impeded due to the many different chemicals that were distributed over many different fractions. Authors concluded that in order to identify anti-androgenic chemicals further investigation is needed e.g. by using normal phase-based HPLC.

In vitro data on Aromatase

- Regarding aromatase activity, from information available in the TOXCast/Tox 21 database, the Substance is considered active in 1 aromatase study (TOX21_aromatase_inhibition).

- On the other hand, Satoh *et al.*, 2008b compared two methods for aromatase activity: an enzyme linked immunosorbent assay (EIA) and a radioisotope (RI) assay, and they determined that EIA is ten times more sensitive than RI. The result of RI was negative for the Substance, but they did not perform the EIA with the substance.

In vitro data on Thyroid activity

- 2,4-di-ter-butylphenol was active in 2 high throughput studies for thyroid receptor in the ToxCast/Tox 21 database.

In vivo available data

Non-mammalian (wildlife) OECD CF assays of level 3, 4 and 5 are not available.

However, mammalian *in vivo* findings corresponding to OECD CF level 4, although not conclusive, point towards potential reproductive toxicity that may be endocrine related:

- Slight delay in preputial separation at the highest dose (3000 mg/kg bw/d), but decreased body weight could have influenced the onset (Hirata-Koizumi *et al.*, 2005), similar to OECD TG 407)
- Significant reduction in live birth index (85.0% at 250 mg/kg vs 96.6% in the control) (English translated summary of Japanese study introduced during consultation period, Study report of 2011, OECD TG 421)
- Significant and dose-dependent increase in relative testis weight at 150 and 300 mg/kg/d (Registration dossier, Study report, 1980, OECD TG 408/415)
- Non-significant increase in adrenals weight in males at the highest dose (300 mg/kg bw/d), not observed in females (Registration dossier, Study report, 2000, Japanese guideline)

A Prenatal Developmental toxicity study (OECD TG 414) which involves repeated dosing of the developing fetus is ongoing. The OECD TG 414 allows to detect changes in the

male and female genitalia and could therefore provide further information on the substance.
High concordance between fish and rats was seen with respect to identifying chemicals that impacted specific endocrine pathways of concern (Ankley and Gray, 2013).

In summary, based on the results of the above QSAR data and *in vitro* assays, the Substance may have weak oestrogenic activity, may modify steroid hormone homeostasis in fish by affecting the ligand binding of the SBP, may bind to thyroid receptor and its interference with androgen receptor and potency for aromatase inhibition is unclear. Together with the (inconclusive) reproductive findings in rodents, an ED concern for the environment cannot be ruled out.

Why new information is needed

Taking into account the above findings concerning the potential endocrine activity and reproductive toxicity in rodents, the widespread use of the Substance, endocrine disrupting effects may be possible *in vivo* and thus new long term information is needed to elucidate the potential endocrine properties for aquatic organisms (fish).

The literature shows that there are multiple sources of environmental exposure to the Substance (for more details see Appendix 1: reasons).

Furthermore, the Substance is considered not inherently biodegradable (0 % degradation within 28 days (OECD TG 302C)) (Registration dossier, study report, 1991). A ready biodegradability test is not available for the Substance.

In an aerobic mineralization study in surface water (registration dossier, study report, 2016) according to OECD TG 309, it was shown that <5% of the Substance mineralizes.

The Substance has a BCF value of 436 L/kg (OECD TG 305) (Registration dossier, study report, 1992).

Furthermore, the eMSCA consulted the ED expert group (November 2017 – open session in presence of representative of Registrant(s)). Based on the received advice, the eMSCA concluded that further testing is necessary to clarify ED concern for the environment.

In addition, it is noted that at present no aquatic long term study with fish is available.

What is the possible regulatory outcome

The requested Fish Sexual Development Test with the Substance will elucidate environmental ED adverse effects, which could lead to an identification of the substance as SVHC (ED for the environment) according to Art.57(f) and possible inclusion in Annex XIV of the REACH Regulation.

Furthermore, acute toxicity studies and QSAR data show that fish might be the most sensitive species (although of similar magnitude). For examination of endocrine effects only, three test concentrations are sufficient in the FSDT. However, the use of five concentrations will allow the determination of a NOEC/EC10 for fish that may lead to a more accurate risk assessment for the environment (if NOEC fish<NOEC algae). This would allow to evaluate more appropriate risk management measures that would further reduce the risk (e.g. reduction of exposure).

Due to its wide dispersive use as a fuel additive and high tonnage band, exposure to the aquatic compartment seems likely.

Considerations on the test method and testing strategy

A Fish Sexual Development test (OECD TG 234) is an *in vivo* assay (OECD Conceptual Framework Level 4) providing apical information on phenotypic sex ratio which is fixed during fry or juvenile stages of the species used in this test. The study must be performed with five test concentrations in order to provide a reliable NOEC/ECx for Risk Assessment purposes (as explained above). In addition, this is based on the assessment of the risk for the environment for the industry category '2 chemical industry, basic chemicals' and use category '28 Fuel additives' for manufacture, formulation, industrial use and private use (based on 1000 T/year) which was performed by the eMSCA using EUSES. RCRs in this assessment were found to be above 1 for all uses.

Furthermore gonad histopathology must be examined to enhance the sensitivity and the statistical power.

If the test species is Japanese medaka, genetic sex must also be determined.

You must submit the full study report of the required information in your dossier update. Indeed, a complete rationale and access to all available information (implemented method, raw data collected, interpretations and calculations, consideration of uncertainties, argumentation, etc.) are needed to fully assess the provided information and to efficiently clarify the concerns.

Consideration of alternative approaches

In order to identify a substance as an endocrine disruptor it should be demonstrated that it alters the function(s) of the endocrine system (mode of action), causes an adverse effect in an intact organism or (sub)population and that there is a biologically plausible link between the MoA (mode of action) and the adverse effect.

Use of Fish Short Term Reproduction Assay (OECD TG 229) has been considered.

However, the purpose of the requested study is to elucidate further the endocrine mode of actions as well as to determine potential endocrine adverse effects. The Substance is a weak estrogen and thus the Fish Sexual Development Test (OECD TG 234) is considered more suitable than OECD TG 229, due to the need for information on in vivo adverse effects and its higher power to detect those effects, its longer exposure period and that OECD TG 229 may not present exposure during the sensitive window. The Fish Sexual Development Test provides endpoints relevant for the population (e.g. sex ratio) and the statistical power is much higher compared to the OECD TG 229 test.

For animal welfare reasons and the higher risk for an inconclusive result in the OECD TG 229 for a weak estrogen it is considered more appropriate and proportionate to perform a Fish Sexual Development Test.

Consideration of your comments on the original draft decision

You agreed to perform the test.

Consideration of proposals for amendment (PfA) and your comments

One MSCA in its PfA proposed to delete the request for the FSDT as they considered it more appropriate to request a level 3 study (according to the OECD ED framework). ECHA disagrees as reliable available *in vitro* assays (WoE) show that the substance interferes with the Steroid Binding protein (SBP) and has the capacity to bind to the oestrogen receptor in fish, although weak. The OECD TG 229 screening study is an *in vivo* assay providing data merely about the endocrine mechanism(s)/Pathway. The ED concern for 2,4-di-ter-butylphenol is based on a MoA alert and thus further information is needed on possible adverse effects.

Due to the weak endocrine activity, exposure during sensitive life-stages (early-life immature sexual development phase) is of crucial importance for detecting endocrine effects in this case. OECD TG 229 uses fish which are in the mature reproductive phase of the fish life cycle and may not represent exposure during the most sensitive window and due to the small group sizes used in this study there is low power to detect effects. Moreover, the exposure time in the screening study is relatively short (21d) in comparison to FSDT (60dph). A (false) negative result in an OECD TG 229 will therefore not annul the potential ED concern but will require further testing with the more sensitive life stage and a longer exposure period resulting in the use of even more vertebrate animals.

Furthermore OECD GD 150 does not represent a testing strategy as it is restricted to a single step when further testing is recommended or proposed for consideration. It only recommends the most appropriate assay that could be performed if authorities need more evidence to support a regulatory decision.

Based on the above, ECHA is of the opinion that the FSDT is the most appropriate assay to investigate the ED concern of 2,4-di-terbutyl phenol.

You disagreed with this PfA as you believe conducting the FSDT will answer the concern on endocrine activity without potentially having to conduct multiple vertebrate studies.

Conclusion

Therefore, based on the substance evaluation and in accordance with Article 46(1) of the REACH Regulation, ECHA concludes that you are required to carry out the following study using the substance subject to this decision: Fish sexual development test; test method: OECD TG 234; with Japanese medaka (*Oryzias latipes*) or zebrafish (*Danio rerio*), including gonadal histopathology. The study must be performed using five test concentrations. If the test species is Japanese medaka, genetic sex must also be determined, as specified above.

References

Akahori Y *et al.*, 2008, Relationship between the results of *in vitro* receptor binding assay to human estrogen receptor a and *in vivo* uterotrophic assay: Comparative study with 65 selected chemicals, Toxicol. *In Vitro*., 22(1), pp.225-231.

Ankley G.T. and Gray L.E., 2013, Cross-species conservation of endocrine pathways : a critical analysis of tier 1 fish and rat screening assays with 12 model chemicals, Environ. Toxicol. Chem., 32(5), pp.1084-7.

Bach *et al.*, 2013, Effect of temperature on the release of intentionally and non-intentionally added substances from polyethylene terephthalate (PET) bottles into water: chemical analysis and potential toxicity, Food Chem., Aug 15;139(1-4):672-80.

Cliet, I. *et al.*, 1993, Lack of predicitivity of bone marrow micronucleus test versus testis micronucleus test: comparison with four carcinogens", Mutation Research, 292, 105-111.

Creusot N *et al.*, 2013, Effect-directed analysis of endocrine-disrupting compounds in multi-contaminated sediment: identification of novel ligands of estrogen and pregnane X receptors, Anal Bioanal Chem., 405(8), pp.2553-2566.

Ding W. *et al.*, 2014, Sex-specific dose-response analysis of genotoxicity in cyproterone acetate treated F344 rats", Mutation Research, Vol. 774, pp. 1-7.

Gerets H. *et al.*, 2012, Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. Cell Biol Toxicol, 28:69–87

Hartmann A *et al., 2004,* Use of the alkaline *in vivo* Comet assay for mechanistic genotoxicity Investigations, Mutagenesis , 19(1), pp.51-59.

Hirata-Koizumi *et al., 2005,* Elevated susceptibility of newborn as compared with young rats to 2-tert-butylphenol and 2,4-di-tert-butylphenol toxicity, Congenit Anom (Kyoto), 45(4), pp.146-153.

Jonker W *et al.*, 2016, Highly Selective Screening of Estrogenic Compounds in Consumer-Electronics Plastics by Liquid Chromatography in Parallel Combined with Nanofractionation-Bioactivity Detection and Mass Spectrometry, Environ Sci Technol., 50(22), pp.12385-12393.

Judson R *et al.*, 2015, Integrated Model of Chemical Perturbations of a Biological Pathway Using 18 *In Vitro* High Throughput Screening Assays for the Estrogen Receptor, ToxSci Advance Access., August 13, pp.1-42.

Kleinstreuer *et al.*, 2017, Development and Validation of a Computational Model for Androgen Receptor Activity, Chem. Res. Toxicol., 30, pp.946–964.

Satoh *et al.*, 2005, Androgenic and antiandrogenic effects of alkylphenols and parabens assessed using the reporter gene assay with stably transfected CHO-K1 celles (AR-EcoScreen System), Journal of Health Science, 51(5), pp.557-568.

Satoh *et al., 2008a,* Endocrine disruptive effects of chemicals eluted from nitrile-butadiene rubber gloves using reporter gene assay systems, Biol Pharm Bull., 31(3), pp.375-379.

Satoh *et al.*, 2008b, *In vitro* screening assay for detecting aromatase activity using rat ovarian microsomes and estrone ELISA, Biol Pharm Bull., 31(3), pp.357-362.

Tice RR *et al.*, 2000, Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing, Environ Mol Mutagen., 35(3), pp.206-221.

Tollefsen KE and Nilsen AJ, 2008, Binding of alkylphenols and alkylated non-phenolics to rainbow trout (Oncorhynchus mykiss) hepatic estrogen receptors, Ecotoxicol Environ Saf., 69(2), pp.163-172.

Tollefsen KE, 2007, Binding of alkylphenols and alkylated non-phenolics to the rainbow trout (Oncorhynchus mykiss) plasma sex steroid-binding protein, Ecotoxicol. Environ. Saf., 68(1), pp.40-48.

Vasquez MZ., 2010, Combining the *in vivo* comet and micronucleus assays: a practical approach to genotoxicity testing and data interpretation, Mutagenesis, 25(2), pp.187-199.

Literature references on exposure sources of 2,4-di-tert-butylphenol:

Detected in	Source	Reference
Blood of pregnant women	Human Biomonitoring	A Suspect Screening Method for Characterizing Multiple Chemical Exposures among a Demographically Diverse Population of Pregnant Women in San Francisco. Wang A, Gerona RR, Schwartz JM, Lin T, Sirota M, Morello-Frosch R, Woodruff TJ. Environ Health Perspect. 2018 Jul 24;126(7):077009.
Myriapodes (Scolopendra subspinipes)	Natural	Antioxidant effects of quinoline alkaloids and 2,4-di-tert-butylphenol isolated from Scolopendra subspinipes. Yoon MA, Jeong TS, Park DS, Xu MZ, Oh HW, Song KB, Lee WS, Park HY. Biol Pharm Bull. 2006 Apr;29(4):735-9.
Chinese eaglewood	Natural	[GC-MS analysis of volatile constituents from five different kinds of Chinese eaglewood]. Mei WL, Zeng YB, Liu J, Dai HF. Zhong Yao Cai. 2007 May;30(5):551-5.
Cactus leaves (Perskia bleo)	Natural	Cytotoxic components of Pereskia bleo (Kunth) DC. (Cactaceae) leaves. Malek SN, Shin SK, Wahab NA, Yaacob H. Molecules. 2009 May 6;14(5):1713-24.
Cogongrass (imperata cylindrical)	Natural	Chemical interaction in the invasiveness of cogongrass (Imperata cylindrica (L.) Beauv.). Xuan TD, Toyama T, Fukuta M, Khanh TD, Tawata S. J Agric Food Chem. 2009 Oct 28;57(20):9448-53.
Rhizobacteria	Natural	Root treatment with rhizobacteria antagonistic to Phytophthora blight affects anthracnose occurrence, ripening, and yield of pepper fruit in the plastic house and field. Sang MK, Kim JD, Kim BS, Kim KD. Phytopathology. 2011 Jun;101(6):666-78.
Sweet potato	Natural	2,4-Di-tert-butylphenol from sweet potato protects against oxidative stress in PC12 cells and in mice. Choi SJ, Kim JK, Kim HK, Harris K, Kim CJ, Park GG, Park CS, Shin DH. J Med Food. 2013 Nov;16(11):977-83.
Pseudomonas monteilii (bacteria)	Natural	Purification, characterization, and *in vitro* activity of 2,4-Di-tert-butylphenol from Pseudomonas monteilii PsF84: conformational and molecular docking studies. Dharni S, Sanchita, Maurya A, Samad A, Srivastava SK, Sharma A, Patra DD. J Agric Food Chem. 2014 Jul 2;62(26):6138-46.
Aspergillus terreus (fungus)	Natural	The overproduction of 2,4-DTBP accompanying to the lack of available form of phosphorus during the biodegradative utilization of aminophosphonates by Aspergillus terreus. Lenartowicz P, Kafarski P, Lipok J. Biodegradation. 2015 Feb;26(1):65-76.
Camphor tree (Cinnamomum camphora)	Natural	Acaricidal activity of compounds from Cinnamomum camphora (L.) Presl against the carmine spider mite, Tetranychus cinnabarinus. Chen Y, Dai G.

		Pest Manag Sci. 2015 Nov;71(11):1561-71.
Bacteraia (Serratia marcescens)	Natural	Assessment of 2,4-Di-tert-butylphenol induced modifications in extracellular polymeric substances of Serratia marcescens. Padmavathi AR, Periyasamy M, Pandian SK. Bioresour Technol. 2015;188:185-9.
Magnolia denudate	Natural	Larvicidal activity of Magnolia denudata seed hydrodistillate constituents and related compounds and liquid formulations towards two susceptible and two wild mosquito species. Wang ZQ, Perumalsamy H, Wang M, Shu S, Ahn YJ. Pest Manag Sci. 2016 May;72(5):897-906.
Bacteria (Lactococcus)	Natural	2,4-Di-tert-butyl phenol as the antifungal, antioxidant bioactive purified from a newly isolated Lactococcus sp. Varsha KK, Devendra L, Shilpa G, Priya S, Pandey A, Nampoothiri KM. Int J Food Microbiol. 2015 Oct 15;211:44-50.
Flower (Emilia sonchifolia)	Natural	Emilia sonchifolia extract activity against white spot syndrome virus and yellow head virus in shrimp cell cultures. Maikaeo L, Chotigeat W, Mahabusarakam W. Dis Aquat Organ. 2015 Jul 23;115(2):157-64.
Biofilm	Natural	Effect of 2, 4-di-tert-butylphenol on growth and biofilm formation by an opportunistic fungus Candida albicans. Padmavathi AR, Bakkiyaraj D, Thajuddin N, Pandian SK. Biofouling. 2015;31(7):565-74.
Flower (Spergularia marina)	Natural	A phenyl lipid alkaloid and flavone C-diglucosides from Spergularia marina. Cho JY, Kim MS, Lee YG, Jeong HY, Lee HJ, Ham KS, Moon JH. Food Sci Biotechnol. 2016 Feb 29;25(1):63-69.
Bacteria (Streptomyces mutabilis)	Natural	Activity of 2,4-Di-tert-butylphenol produced by a strain of Streptomyces mutabilis isolated from a Saharan soil against Candida albicans and other pathogenic fungi. Belghit S, Driche EH, Bijani C, Zitouni A, Sabaou N, Badji B, Mathieu F. J Mycol Med. 2016 Jun;26(2):160-169.
Bacteria (Bacillus subtilis)	Natural	*In vitro* and *in vivo* antibiofilm potential of 2,4-Di-tert-butylphenol from seaweed surface associated bacterium Bacillus subtilis against group A streptococcus. Viszwapriya D, Prithika U, Deebika S, Balamurugan K, Pandian SK. Microbiol Res. 2016 Oct;191:19-31.
Fungus (Fritillaria unibracteata)	Natural	Fungal endophyte-derived Fritillaria unibracteata var. wabuensis: diversity, antioxidant capacities *in vitro* and relations to phenolic, flavonoid or saponin compounds. Pan F, Su TJ, Cai SM, Wu W. Sci Rep. 2017 Feb 6;7:42008.
Betel leaves (piper betle)	Natural	Impact of Storage Conditions on the Stability of Predominant Phenolic Constituents and Antioxidant Activity of Dried Piper betle Extracts. Ali A, Chong CH, Mah SH, Abdullah LC, Choong TSY, Chua BL. Molecules. 2018 Feb 23;23(2). pii: E484.
Bacteria (Bacillus	Natural	Research on Volatile Organic Compounds From Bacillus subtilis CF-3: Biocontrol Effects on Fruit Fungal Pathogens and

subtilis)		Dynamic Changes During Fermentation. Gao H, Li P, Xu X, Zeng Q, Guan W. Front Microbiol. 2018 Mar 14;9:456.
Cinnamon bark (cinnamomum cassia)	Natural	Supercritical carbon dioxide extract of Cinnamomum cassia bark: toxicity and repellency against two stored-product beetle species. Wang Y, Dai PP, Guo SS, Cao JQ, Pang X, Geng ZF, Sang YL, Du SS. Environ Sci Pollut Res Int. 2018 Aug;25(22):22236-22243.
Indoor dust (urban and rural) in China	Other - Dust	Occurrence of synthetic phenolic antioxidants and transformation products in urban and rural indoor dust. Liu R, Lin Y, Ruan T, Jiang G. Environ Pollut. 2017 Feb;221:227-233.
Wound closure tape	Other - Bandage	Identification of phenolic dermal sensitizers in a wound closure tape. Myers LP, Law BF, Fedorowicz A, Siegel PD, Butterworth LF, Anderson SE, Sussman G, Shapiro M, Meade BJ, Beezhold D. J Immunotoxicol. 2007 Oct;4(4):303-10.
Drinking water - HDPE pipelines	Plastic	Volatile organic compounds in natural biofilm in polyethylene pipes supplied with lake water and treated water from the distribution network. Skjevrak I, Lund V, Ormerod K, Herikstad H. Water Res. 2005 Oct;39(17):4133-41. Epub 2005 Aug 31.
Polycarbonate containers	Plastic	Determination of potential migrants in polycarbonate containers used for microwave ovens by high-performance liquid chromatography with ultraviolet and fluorescence detection. Nerín C, Fernández C, Domeño C, Salafranca J. J Agric Food Chem. 2003 Sep 10;51(19):5647-53.
Plastic food contact material	Plastic	Non-targeted multi-component analytical surveillance of plastic food contact materials: Identification of substances not included in EU positive lists and their risk assessment. Skjevrak I, Brede C, Steffensen IL, Mikalsen A, Alexander J, Fjeldal P, Herikstad H. Food Addit Contam. 2005 Oct;22(10):1012-22.
Food packages	Plastic	Determination of polymer additives-antioxidants and ultraviolet (UV) absorbers by high-performance liquid chromatography coupled with UV photodiode array detection in food simulants. Gao Y, Gu Y, Wei Y. J Agric Food Chem. 2011 Dec 28;59(24):12982-9.
Wood plastic composites (LDPE)	Plastic	Characterization of wood plastic composites made from landfill-derived plastic and sawdust: volatile compounds and olfactometric analysis. Félix JS, Domeño C, Nerín C. Waste Manag. 2013 Mar;33(3):645-55.
PET bottle	Plastic	Effect of temperature on the release of intentionally and non-intentionally added substances from polyethylene terephthalate (PET) bottles into water: chemical analysis and potential toxicity.

		Bach C, Dauchy X, Severin I, Munoz JF, Etienne S, Chagnon MC. Food Chem. 2013 Aug 15;139(1-4):672-80.
Marine microplastic	Plastic	Identification of polymer types and additives in marine microplastic particles using pyrolysis-GC/MS and scanning electron microscopy. Fries E, Dekiff JH, Willmeyer J, Nuelle MT, Ebert M, Remy D. Environ Sci Process Impacts. 2013 Oct;15(10):1949-56.
Marine microplastics (North sea)	Plastic	Occurrence and spatial distribution of microplastics in sediments from Norderney. Dekiff JH, Remy D, Klasmeier J, Fries E. Environ Pollut. 2014 Mar;186:248-56.
Plastic baby bottles	Plastic	Development and application of a non-targeted extraction method for the analysis of migrating compounds from plastic baby bottles by GC-MS. Onghena M, van Hoeck E, Vervliet P, Scippo ML, Simon C, van Loco J, Covaci A. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2014;31(12):2090-102.
Polypropylene film	Plastic	Effects of Ultraviolet (UV) on Degradation of Irgafos 168 and Migration of Its Degradation Products from Polypropylene Films. Yang Y, Hu C, Zhong H, Chen X, Chen R, Yam KL. J Agric Food Chem. 2016 Oct 5
Consumer electronics plastics	Plastic	Highly Selective Screening of Estrogenic Compounds in Consumer-Electronics Plastics by Liquid Chromatography in Parallel Combined with Nanofractionation-Bioactivity Detection and Mass Spectrometry. Jonker W, Ballesteros-Gómez A, Hamers T, Somsen GW, Lamoree MH, Kool J. Environ Sci Technol. 2016 Nov 15;50(22):12385-12393.
Plastic bags (LDPE)	Plastic	Safety and durability of low-density polyethylene bags in solar water disinfection applications. Danwittayakul S, Songngam S, Fhulua T, Muangkasem P, Sukkasi S. Environ Technol. 2017 Aug;38(16):1987-1996
Rubber	Rubber	Occupational vitiligo due to unsuspected presence of phenolic antioxidant byproducts in commercial bulk rubber. O'Malley MA, Mathias CG, Priddy M, Molina D, Grote AA, Halperin WE. J Occup Med. 1988 Jun;30(6):512-6.
Nitrile-butadiene rubber gloves	Rubber	[Identification of migrants from nitrile-butadiene rubber gloves]. Mutsuga M, Kawamura Y, Wakui C, Maitani T. Shokuhin Eiseigaku Zasshi. 2003 Apr;44(2):103-9.
Urine of wild-derived house mice	Secondary contamination	Are MUPs a Toxic Waste Disposal System? Kwak J, Strasser E, Luzynski K, Thoß M, Penn DJ. PLoS One. 2016 Mar 11;11(3):e0151474.
Fish Seabreams (Diplodus	Secondary contamination	Accumulation of endocrine disrupting chemicals in the liver of Diplodus sargus sargus in Torre Guaceto Natural Reserve. Rizzo D, Pennetta A, De Benedetto GE.

sargus)		Mar Pollut Bull. 2017 Jun 30;119(2):219-222.
Wastewaters (influent and Danube) in Hungary	Water	Multiresidue analysis of pollutants as their trimethylsilyl derivatives, by gas chromatography-mass spectrometry. Sebok A, Vasanits-Zsigrai A, Helenkár A, Záray G, Molnár-Perl I. J Chromatogr A. 2009 Mar 20;1216(12):2288-301.
River sediment (France)	Water	Effect-directed analysis of endocrine-disrupting compounds in multi-contaminated sediment: identification of novel ligands of estrogen and pregnane X receptors. Creusot N, Budzinski H, Balaguer P, Kinani S, Porcher JM, Aït-Aïssa S. Anal Bioanal Chem. 2013 Mar;405(8):2553-66.
Drinking water (Polyethylene plumbing) in USA	Water	Release of drinking water contaminants and odor impacts caused by green building cross-linked polyethylene (PEX) plumbing systems. Kelley KM, Stenson AC, Dey R, Whelton AJ. Water Res. 2014 Dec 15;67:19-32.
Water (source to tap water) in China	Water	Different senescent HDPE pipe-risk: brief field investigation from source water to tap water in China (Changsha City). Tang J, Tang L, Zhang C, Zeng G, Deng Y, Dong H, Wang J, Wu Y. Environ Sci Pollut Res Int. 2015 Oct;22(20):16210-4.
Drinking water in China	Water	Do estrogenic compounds in drinking water migrating from plastic pipe distribution system pose adverse effects to human? An analysis of scientific literature. Liu ZH, Yin H, Dang Z. Environ Sci Pollut Res Int. 2017 Jan;24(2):2126-2134.
River (Romania)	Water	Environmental exposure of anthropogenic micropollutants in the Prut River at the Romanian-Moldavian border: a snapshot in the lower Danube river basin. Moldovan Z, Marincas O, Povar I, Lupascu T, Longree P, Rota JS, Singer H, Alder AC. Environ Sci Pollut Res Int. 2018 Sep 5.

Appendix 2: Procedural history

On the basis of an opinion of the ECHA Member State Committee and due to initial grounds for concern relating to suspected endocrine disruptor, suspected reproductive toxicant, wide dispersive use and consumer use, 2,4-di-tert-butylphenol, CAS No 96-76-4 (EC No 202-532-0) was included in the Community rolling action plan (CoRAP) for substance evaluation to be evaluated in 2017. The updated CoRAP was published on the ECHA website on 21 March 2017. The competent authority of Belgium (hereafter called the evaluating MSCA) was appointed to carry out the evaluation.

In accordance with Article 45(4) of the REACH Regulation, the evaluating MSCA carried out the evaluation of the above substance based on the information in your registration(s) and other relevant and available information.

In the course of the evaluation, the evaluating MSCA identified additional concerns regarding suspected mutagenicity.

The evaluating MSCA considered that further information was required to clarify the abovementioned concerns. Therefore, it prepared a draft decision under Article 46(1) of the REACH Regulation to request further information. It subsequently submitted the draft decision to ECHA on 21 March 2018.

The decision making followed the procedure of Articles 50 and 52 of the REACH Regulation as described below.

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

Registrant(s)' commenting phase

ECHA received comments from you and forwarded them to the evaluating MSCA without delay. The evaluating MSCA took the comments from you, which were sent within the commenting period, into account and they are reflected in the reasons (Appendix 1) for the following information requests:

In vivo mammalian Alkaline – Comet assay (OECD TG 489)
Fish sexual development test (OECD TG 234)

Based on your comments, the following information requests were removed from the initial draft decision:

Extended one-generation reproductive toxicity study (OECD TG 443)
Toxicokinetics study (OECD TG 417)
Exposure data

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

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

Subsequently, the evaluating MSCA received proposals for amendment to the draft decision according to which the decision was amended.

ECHA referred the draft decision, together with your comments, to the Member State Committee.

ECHA invited you to comment on the proposed amendments. Any comments on the proposals for amendment were taken into account by the Member State Committee and are reflected in the Reasons (Appendix 1).

MSC agreement seeking stage

The Member State Committee reached a unanimous agreement on the draft decision during its MSC-65 meeting and ECHA took the decision according to Article 52(2) and 51(6) of the REACH Regulation.

Appendix 3: Further information, observations and technical guidance

1. This decision does not imply that the information provided by you in the registration(s) is in compliance with the REACH requirements. The decision neither prevents ECHA from initiating compliance checks on your dossier(s) at a later stage, nor does it prevent a subsequent decision under the current substance evaluation or a new substance evaluation process once the present substance evaluation has been completed.

2. Failure to comply with the request(s) in this decision, or to otherwise fulfil the information requirement(s) with a valid and documented adaptation, will result in a notification to the enforcement authorities of your Member State.

3. In relation to the required experimental studies, the sample of the substance to be used ('test material') has to have a composition that is within the specifications of the substance composition that are given by all registrant(s). It is the responsibility of all the registrant(s) to agree on the tested material to be subjected to the test(s) subject to this decision and to document the necessary information on the composition of the test material. The substance identity information of the registered substance and of the sample tested must enable the evaluating MSCA and ECHA to confirm the relevance of the testing for the substance subject to substance evaluation.

4. In relation to the experimental studies the legal text foresees the sharing of information and costs between registrant(s) (Article 53 of the REACH Regulation). You are therefore required to make every effort to reach an agreement regarding each experimental study for every endpoint as to who will carry out the study on behalf of the other registrant(s) and to inform ECHA accordingly within 90 days from the date of this decision under Article 53(1) of the REACH Regulation. This information should be submitted to ECHA using the following form stating the decision number above at: https://comments.echa.europa.eu/comments_cms/SEDraftDecisionComments.aspxFurther advice can be found at http://echa.europa.eu/regulations/reach/registration/data-sharing. If ECHA is not informed of such agreement within 90 days, it will designate one of the registrants to perform the stud(y/ies) on behalf of all of them.