Scientific Committee on Consumer Safety

SCCS

OPINION on
Butylparaben
(CAS No. 94-26-8, EC No. 202-318-7)

The SCCS adopted this document
during the plenary meeting on 26 October 2023

ACKNOWLEDGMENTS

SCCS members listed below are acknowledged for their valuable contribution to the finalisation of this Opinion.

For the preliminary and for the final version of the Opinion

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This Opinion has been subject to a commenting period of min eight weeks after its initial publication (from 8 June to 21 August 2023). Comments received during this period were considered by the SCCS. For this Opinion, main changes occurred in the following sections: 3.4.10.1 pages 59-60 (the SCCS used the BMD approach), as well as the reference list.

All Declarations of Working Group members are available on the following webpage:
Register of Commission expert groups and other similar entities (europa.eu)

1. ABSTRACT

The SCCS concludes the following:

1. *In light of the data provided and taking under consideration the concerns related to potential endocrine disrupting properties of Butylparaben, does the SCCS consider Butylparaben safe when used as a preservative in cosmetic products up to a maximum concentration of 0.14 %?*

On the basis of safety assessment considering all available data and the concerns related to endocrine activity, the SCCS is of the opinion that the use of Butylparaben as a preservative in cosmetic products at concentrations of up to 0.14% (expressed as acid) is safe.

2. *Alternatively, what is according to the SCCS the maximum concentration considered safe for use of Butylparaben as a preservative in cosmetic products?*

/

3. *Does the SCCS have any further scientific concerns with regard to the use of Butylparaben in cosmetic products?*

In the absence of exposure data specific for children to Butylparaben in cosmetic products, potential safety concerns cannot be excluded.

The SCCS mandates do not address environmental aspects. Therefore, this assessment did not cover the safety of Butylparaben for the environment.

Keywords: SCCS, scientific opinion, butylparaben, preservative, Regulation 1223/2009, CAS No. 94-26-8, EC No. 202-318-7

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SCCS

The Committee shall provide Opinions on questions concerning health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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2. MANDATE FROM THE EUROPEAN COMMISSION

Background on substances with endocrine disrupting properties

On 7 November 2018, the Commission adopted the review[1] of Regulation (EC) No 1223/2009 on cosmetic products ('Cosmetics Regulation') regarding substances with endocrine disrupting (ED) properties. The review concluded that the Cosmetics Regulation provides the adequate tools to regulate the use of cosmetic substances that present a potential risk for human health, including when displaying ED properties.
The Cosmetics Regulation does not have explicit provisions on EDs. However, it provides a regulatory framework with a view to ensuring a high level of protection of human health. Environmental concerns that substances used in cosmetic products may raise are considered through the application of Regulation (EC) No 1907/2006 ('REACH Regulation'). In the review, the Commission commits to establishing a priority list of potential EDs not already covered by bans or restrictions in the Cosmetics Regulation for their subsequent safety assessment. A priority list of 28 potential EDs in cosmetics was consolidated in early 2019 based on input provided through a stakeholder consultation. The Commission carried out a public call for data in 2019[2] for 14 substances (Group A)[3] and a second call in 2021[4] for 10 substances (Group B)[5] in preparation of the safety assessment of these substances. Butylparaben is one of the above-mentioned substances for which the call for data took place.

Background on Butylparaben

Butylparaben (CAS No. 94-26-8, EC No. 202-318-7) with the chemical name 'Butyl 4-hydroxybenzoate' is currently regulated as a preservative (Annex V entry 12a) in a concentration up to 0.14 % (as acid) when used on its own or for the sum of its combined use with propyl paraben and its salts (Annex V, entry 12a, column g).

Butylparaben has been subject to different safety evaluations by the SCCP in 2005 (SCCP/0874/05)[6], 2006 (SCCP/1017/06)[7] and 2008 (SCCP/1183/08)[8] and by the SCCS in 2010 (SCCS/1348/10)[9], 2011 (SCCS/1446/11)[10] and 2013 (SCCS/1514/13)[11]. In particular, the last SCCS opinion from 2013 states that '*The additional submitted data does not remove the concern expressed in the previous opinions on the relevance of the rat model for the risk assessment of parabens. Although much toxicological data on parabens in rodents exists, adequate evidence has not been provided for the safe use of propyl- or butylparaben in cosmetics'*.

[1] https://ec.europa.eu/transparency/regdoc/rep/1/2018/EN/COM-2018-739-F1-EN-MAIN-PART-1.PDF
[2] https://ec.europa.eu/growth/content/call-data-ingredients-potential-endocrine-disrupting-properties-used-cosmetic%20products_en
[3] Benzophenone-3, kojic acid, 4-methylbenzylidene camphor, propylparaben, triclosan, Homosalate, octocrylene, triclocarban, butylated hydroxytoluene (BHT), benzophenone, homosalate, benzyl salicylate, genistein and daidzein
[4] https://ec.europa.eu/growth/content/call-data-ingredients-potential-endocrine-disrupting-properties-used-cosmetic-products-0_en
[5] Butylparaben, Methylparaben, Ethylhexyl Methoxycinnamate (EHMC)/Octylmethoxycinnamate (OMC)/ Octinoxate, Benzophenone-1 (BP-1), Benzophenone-2 (BP-2), Benzophenone-4 (BP-4), Benzophenone-5 (BP-5), BHA/Butylated hydroxyanisole/tert-butyl-4-hydroxyanisole, Triphenyl Phosphate and Salicylic Acid
[6] https://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_00d.pdf and https://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_019.pdf
[7] https://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_074.pdf
[8] https://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_138.pdf
[9] https://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_041.pdf
[10] https://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_069.pdf
[11] https://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_132.pdf

During the call for data, stakeholders submitted scientific evidence to demonstrate the safety of Butylparaben as a preservative in cosmetic products. The Commission requests the SCCS to carry out a safety assessment on Butylparaben in view of the information provided.

Terms of reference

1. *In light of the data provided and taking under consideration the concerns related to potential endocrine disrupting properties of Butylparaben, does the SCCS consider Butylparaben safe when used as a preservative in cosmetic products up to a maximum concentration of 0.14 %?*

2. *Alternatively, what is according to the SCCS the maximum concentration considered safe for use of Butylparaben as a preservative in cosmetic products?*

3. *Does the SCCS have any further scientific concerns with regard to the use of Butylparaben in cosmetic products?*

3. OPINION

3.1 CHEMICAL AND PHYSICAL SPECIFICATIONS

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

Butylparaben

3.1.1.2 Chemical names

IUPAC: Butyl p-hydroxybenzoate

EC name: Butyl 4-hydroxybenzoate

(ECHA Brief Profile Butyl 4-hyroxybenzoate, 2022)

3.1.1.3 Trade names and abbreviations

Depository supplied synonyms: (n-)butyl paraben, butyl parahydroxybenzoate; 4-Hydroxybenzoic acid n-butyl ester

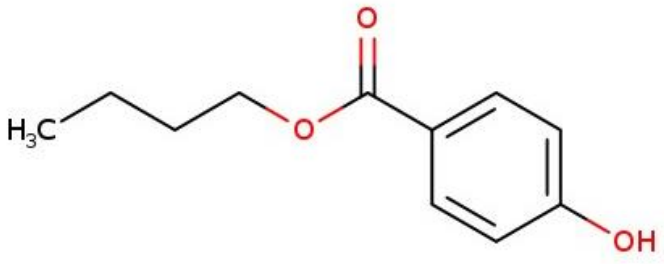
Additional depository supplied synonyms can be found at the link provided below:

PubChem:
https://pubchem.ncbi.nlm.nih.gov/compound/Butylparaben#section=Depositor-Supplied-Synonyms

3.1.1.4 CAS / EC number

CAS No. 94-26-8, EC No. 202-318-7

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

$C_{11}H_{14}O_3$

3.1.2 Physical form

Solid: white particulate/powder

(ECHA Brief Profile Butyl 4-hyroxybenzoate, 2022)

3.1.3 Molecular weight

194.2286 g/mol
(ChemIDplus)

3.1.4 Purity, composition and substance codes

>99%

(PubChem)

SCCS comment
The analytical methods used for the determination of purity of the test substance should be provided, according to the SCCS Notes of Guidance.

3.1.5 Impurities / accompanying contaminants

SCCS comment
Data on impurities of the test substance must be provided. The analytical methods used for the determination of impurities along with the results of these studies should be provided, according to the SCCS Notes of Guidance.

3.1.6 Solubility

In water: 207 mg/L at 20°C (pH not specified)

(Yalkowsky & He, 2003)

Freely soluble in acetone, ethanol, ether, chloroform, propylene glycol.
Very slightly soluble in glycerin.

(PubChem)

3.1.7 Partition coefficient (Log Pow)

Computed Log P_{ow} =3.57 (pH and temperature not reported)

(Hansch *et al.*, 1995)

3.1.8 Additional physical and chemical specifications

Boiling point (°C): 369°C at 77 mmHg (ChemSpider)
330 – 337 °C at 102.4 kPa

(ECHA Brief Profile Butyl 4-hyroxybenzoate, 2022)

Melting point (°C): 68-69°C

(PubChem)

Vapour pressure: 0.002 Pa at 20°C, 0.005 Pa at 25°C, 0.113 Pa at 50°C

(ECHA Brief Profile Butyl 4-hyroxybenzoate, 2022)

2.51x10-4 mm Hg at 25 °C. With very feint phenolic odour

(PubChem)

pKa: 8.47

(PubChem)

Density: 1.2365 g/cm3 at 20.0 °C

(ECHA Brief Profile Butyl 4-hyroxybenzoate, 2022)

Surface tension: ca. 44.5 mN/m at 20 °C at 90% of the saturation

(ECHA Brief Profile Butyl 4-hyroxybenzoate, 2022)

3.1.9 Homogeneity and Stability

Stable in air and does not hydrolyse in hot or cold water or in acidic conditions. Above pH 7, considerable hydrolysis occurs. Shelf life 24 months or longer if stored properly.

(PubChem)

3.2 TOXICOKINETICS

3.2.1 Dermal / percutaneous absorption

Dermal absorption studies present in previous opinions

Dermal absorption studies have been extensively reviewed and evaluated in previous opinions (summarised in SCCS 1348/10, section 3.3.1). The SCCS noted several shortcomings in the data provided and based upon a combination of the three Fasano (2004a, 2004b and 2005) studies, the SCCS derived the value of 3.7% as a worst-case assumption for the dermal absorption of unmetabolised butylparaben. This percentage originated from the mean dermal absorption of 37% measured in split-thickness skin (Fasano 2004b), using a correction factor of 10 to account for skin metabolism as seen in the full thickness skin experiments (Fasano 2004a, 2005). The factor of 10 was considered to be a conservative value as in these studies the measured butylparaben concentration in the receptor fluid was not 10, but 65 to 150 times lower than the metabolite parahydroxy benzoic acid (PHBA) concentration, meaning that butylparaben undergoes extensive metabolism in human skin.
The conclusion was: '*Until a properly conducted dermal absorption and toxicokinetic study in humans will allow the assignment of a more scientifically solid value, the SCCS will use a dermal absorption value of 3.7% in its MoS safety calculations'*.

(SCCS/1514/13)

Dermal absorption studies submitted by applicant

Re-analysis of Fasano (2005) study

The applicant performed a re-analysis of the OECD 428 Test Guideline study by Fasano (2005) and came to the conclusion that the total amount of radioactivity considered absorbable at 24 hours was 30.1%. Given the skin was clearly metabolically competent from the receptor fluid analysis, and esterase metabolism is rapid in skin, it was assumed that at least 90% of the test substance had been converted to the primary metabolite PHBA. Therefore, the dermal absorption was estimated to be, not 3.7% as used by the SCCS in the 2013 opinion of butylparaben, but **3% (*i.e.* 30.1/10)** for parent butyl paraben absorption through human skin. In humans, dermal absorption was said to be likely even lower than this in reality.

SCCS comment
There is no dermal absorption study available which was done according to the SCCS Notes of Guidance (SCCS/1628/21), although requested on several occasions. The SCCS is of the opinion that a value of 3% is not acceptable.

Newly submitted data: In vivo rodent dermal absorption

Mathews *et al.* (2013) performed an *in vivo* 14C ring-labeled dermal dosing study in adult HSD male and female Sprague Dawley rats (203–260 g, and 181–193 g, respectively, and 8–10 weeks old at dosing)
The radiolabeled dermal doses (10 and 100 mg/kg) were applied onto 4 cm^2 skin on the backs of the rats.
The treated skin was excised and washed with a series of water-wetted gauzes; protective appliance, skin samples, skin rinses and gauzes were stored at -20°C prior to analysis. Background radioactivity was about 25 dpm, and the limit of detection was twice background. Results showed that of the 10 mg/kg and 100 mg/kg butylparaben applied for 72 hours, about 52% and 8% of the test dose was absorbed, respectively. Urine was the primary route of elimination with a very small amount present in faeces. On a mass basis, the total absorbed dose was comparable (5.2 mg and 8 mg for 10 and 100 mg/kg, respectively). Butylparaben was not readily absorbed and the observed differences in absorption with increasing dose indicated a saturation of the capacity for dermal absorption over this dose range. At 100 mg/kg, less than 3% and 8% of the dose had penetrated and was excreted at 24 hours and 72 hours, respectively. Overall recovery of the dermally applied dose was about 90%.
The applicant stated that this study supports an estimate of 3% dermal absorption of parent paraben.

SCCS comment
As the amount of product applied on the limited skin surface of 4cm^2 is too high, the study cannot be used to decide or support on a dermal absorption of 3%.

Aubert *et al.* (2009, published in 2012):
This dermal toxicokinetic and mass balance study in rats is described further below in the section on toxicokinetics. In this study, a total absorption value of 32.7% (males) and 33.1% (females) total radioactivity (excreted and within the skin) was observed. Using the approach described above, the applicant is of the opinion that 32.7/10 (males) and 33.1/10 (females) can account for the fact that the majority of butylparaben will be metabolised to PHBA by esterases. This is said to also support a value of 3% dermal absorption for use in the safety assessment.

SCCS comment
The study by Aubert *et al.* (2009) shows a dermal absorption of 33% in rat. In the previous Opinion, a factor of 10 was used for the safety assessment in humans. This was an approach proposed in the 2013 Opinion as long as there was no properly conducted dermal absorption and toxicokinetic study.

Review provided by the applicant on metabolism in the skin

The potential for carboxylesterases to be metabolically active and perform first pass effective clearance for parabens in the skin, has been investigated in multiple species *in vitro and ex vivo*, including human, rabbit, rat and pig (Williams, 2008). Lobemeier *et al.* (1996) showed that both the epidermal and dermal layers of human skin have the capacity to hydrolyse all parabens, extensively though not completely.
Another study showed that all parabens are metabolised by human and rat skin (Harville *et al.*, 2007). However, in that study, human and rat skin were found to have different rates of paraben hydrolysis to yield PHBA, with human skin esterases appearing less metabolically active in producing PHBA than rat skin. Rates of hydrolysis were seen to be more similar between human and minipig (Jewell *et al.*, 2007). In the Fasano study (2005), there was substantial metabolism of butylparaben to PHBA in metabolically competent human skin *in vitro* such that virtually no parent butylparaben was measurable in the receptor fluid. Skin esterases act as effective first pass metabolism for all parabens in the skin (Williams *et al.*, 2008), and if any small amount of parent parabens enters the blood, this would be rapidly metabolised (as evidenced from intravenous dosing studies (Mathews *et al.*, 2013). Based on these studies, the applicant concluded that a 3% dermal absorption value for parent butylparaben may be used in risk assessment, with the recognition that this value remains conservative for humans *in vivo*.

SCCS comment
None of the dermal absorption data provided is in line with the guidance given by the SCCS in the NoG (SCCS/ 1628/21). The SCCS is of the opinion that a value of 3% is not acceptable.

3.2.2 Other studies on toxicokinetics

Toxicokinetic data present in previous opinions

Free parabens are considered as the toxicologically active form and this in turn is determined by the efficiency of the drug metabolising enzymes involved in the metabolism of parabens in humans (carboxylesterases, UDP-glucuronosyltransferases and sulfotransferases). It is generally recognised that UDP-glucuronosyltransferase enzymes are not fully developed until the age of 6 months and data suggests a reduced carboxylesterase expression in children below 1 year of age. Therefore, dermal exposure to parabens of newborns and infants up to 6 months of age may result in a higher internal dose and the half-life of the unmetabolised parabens may be longer when compared to adults. Data regarding parabens metabolism in adult humans, neonates/newborns and early infants is missing and this requires particular consideration in the risk assessment. The unborn foetus will be better protected by the relatively efficient systemic parabens inactivation by the mother than the neonate/newborn or early infant dermally exposed to parabens.

SCCS comment
New and not previously evaluated toxicokinetic data (Mathews *et al.*, 2013; Campbell *et al.*, 2015; Moos *et al.*, 2016) have been submitted and reviewed by the Applicant.

These studies will be taken into account when performing the final safety evaluation. However, no new data has been submitted regarding metabolism in the above-mentioned young age groups and therefore the relevant human data required for reducing uncertainties in the risk assessment of butylparaben in younger age, is still missing.

Review of toxicokinetic data provided by the applicant

3.2.2.1 *Oral Toxicokinetics studies*

3.2.2.1.1 *In vitro metabolism*

Mathews *et al.* (2013)
Comparative metabolism was investigated using cryopreserved hepatocytes from rats (male and female Harlan Sprague Dawley) and humans (59-year Caucasian female non-smoker; 45-year Caucasian male non-smoker) (Mathews *et al.*, 2013). Incubations contained 0.93–0.98 million cells/mL for all except human male hepatocytes, which contained 0.65 million cells/mL. A final concentration of 1 µM butylparaben for clearance studies and 10 µM [^{14}C] BPB (0.5 µCi) for metabolism studies was used. Aliquots of 50µL were removed at different time points to estimate the clearance. Intrinsic clearance (Cl_{int}) and half-lives (T1/2) of butylparaben in hepatocytes were determined, and metabolism was further investigated.

Figure 1 below shows rapid and complete butylparaben clearance in female human hepatocytes. There was no sex difference in either human or rat hepatocytes. Butylparaben was extensively hydrolysed to yield PHBA as the major primary metabolite for both sexes and species (92–100% in rat, 78–84% in human) after 5 hours of incubation. In human hepatocytes p-hydroxyhippuric acid (the glycine conjugate of PHBA) was also observed (16–22%). Both of these metabolites are non-toxic to mammals and even though there is a rat vs human difference in the extent of PHBA measured, the overall outcome of rapid and complete clearance of butylparaben is the same.

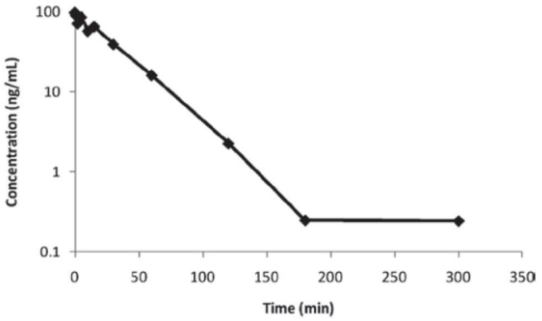


Figure 1: Concentration of butylparaben vs time in female human hepatocytes *in vitro*

The half-life of butylparaben in female and male rat hepatocytes was 3.8 ± 0.3 and 3.3 ± 0.1 min, respectively, corresponding to Cl_{int} of 811 ± 53 and 903 ± 28 mL/min · kg. The half-life estimated for female and male human hepatocytes was 23.9 ± 1.3 and 29.6 ± 5.2 min, respectively, corresponding to Cl_{int} of 92 ± 5 and 111 ± 22 mL/min · kg.

SCCS comment
Some shortcomings were observed: human half-live values are provided with SD, these are SD of replicates, not of different samples as for humans; there was only one sample per sex available; and for rodents the number of males and females was not indicated. The

number of hepatocytes used in the incubations were different for the human and rodent experiment, which makes conclusions difficult to interpret.

3.2.2.1.2 *In vivo* rat-oral kinetics

Aubert *et al.* (2009, published in 2012)

This study was already evaluated in SCCS/1514/13. The SCCS concluded that butylparaben is rapidly metabolised (C_{max} at 0.5 hrs) to PHBA. Plasma metabolite characterisation revealed only one metabolite, namely PHBA, independent of time of collection, paraben type and route of administration. The study revealed that the principal route of excretion was via the urine and that no selective organ / tissue storage was observed.

Mathews *et al.* 2013/NTP (2012) Study Report M88007 - Rat – oral kinetics

Adult HSD male and female rats (203–260 g, and 181–193 g, respectively, and 8–10 weeks old at dosing) were used. Single oral doses contained [^{14}C]BPB (50 µCi/animal in all studies) an appropriate amount of non-radiolabelled butylparaben and Cremophor® EL in a dose volume of 5 mL/kg. Oral doses (10, 100 and 1000 mg/kg) were administered by intragastric gavage via a syringe equipped with a ball-tipped 16G gavage needle.
Urine and faeces of rats were collected separately (up to 72 hours). At the end of the final excreta collection, the cages were rinsed with water and ethanol. Samples were stored at −20˚C in the dark until analysed. At the end of studies, the animals were euthanized by asphyxiation with carbon dioxide and blood was collected via cardiac puncture with a heparinised syringe. Plasma was prepared from blood by centrifugation for 10 min at 3000 g and 4 °C. The following tissues were excised and weighed: liver, kidney, brain, muscle (hind leg), abdominal skin, adipose (perirenal), spleen, heart, lung, ovaries, uterus and testes. Gastrointestinal tract tissues were freed of contents prior to weighing. All samples were stored at −20 ˚C prior to analysis.
Background radioactivity was about 25 dpm, and the limit of detection was twice the background. The excretion of radioactivity following single oral dosing of 10, 100 and 1000 mg/kg bw/day butyl paraben in male rats at 72 hours showed that the extent of excretion is similar at all 3 doses in urine and faeces. Urine is the main route of excretion, with only a small amount in faeces. Besides radioactivity measurement, also metabolites were identified as shown in **Figure 2.**

SCCS comment

As mentioned in SCCS/1514/13, the study by Mathews *et al.* (2013) confirms the previous conclusion that the main route of excretion appears via the urine.

The excretion at 24 hours and the tissue distribution of the dose of 100 mg/kg oral dose of butylparaben in male and female rats show that the excretion is rapid and extensive within 24 hours. The highest levels of the residual amounts were found in the liver and kidneys
From the urinary metabolite analyses in the rat experiments, Mathews *et al.* (2013) observed the metabolites as shown in **Figure 2** below.

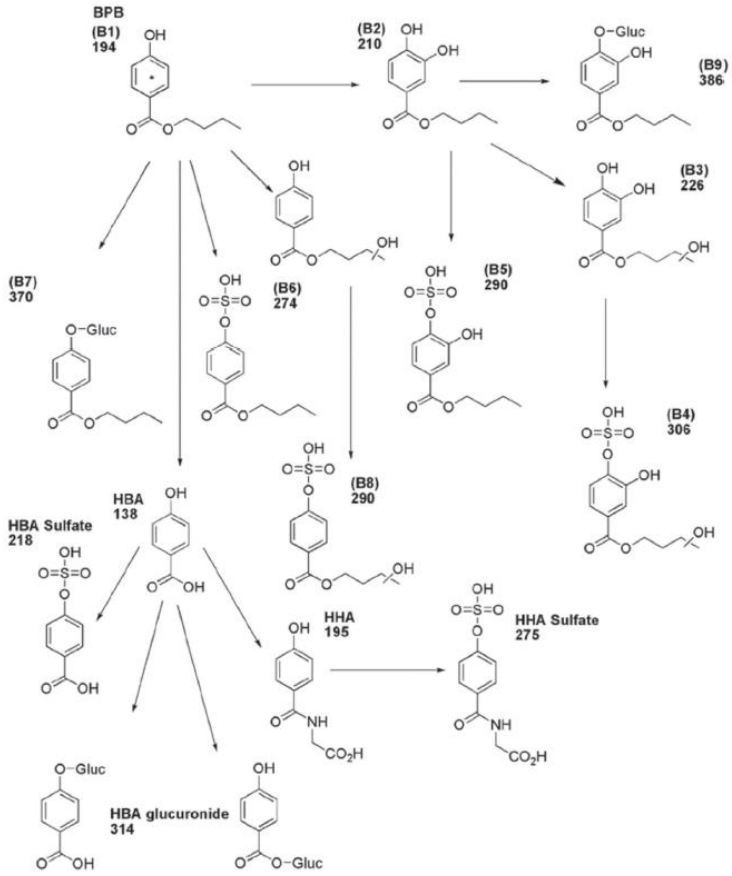


Figure 2: Proposed pathways for the metabolism of butylparaben following oral administration in Sprague Dawley rats according to the observations in rat urine in Mathews *et al.* (2013). The molecular weight (g/mol) is given next to each metabolite. HBA (p-hydroxy benzoic acid (or PHBA); HHA phydroxyhippuric acid (or PHHA); glucuronide conjugates abbreviated as "-Gluc."

3.2.2.1.3 *In vivo human – oral kinetics*

Moos *et al.* (2016) investigated metabolism and urinary excretion of butylparaben in 3 healthy, human 31-year-old volunteers (1 female, 2 males) after an oral dose of deuterium-labelled analogues (10 mg). Each volunteer received two single oral doses at least 2 weeks apart. Consecutive urine samples were collected over 48 hours after each dose. 80.5% of the oral dose was excreted in the first 24 hours. The excretion profile is shown in **Figure 3.**

A mean total of 5.6% of the administered dose was present as butylparaben in urine after 48 hours. In all cases, p-hydroxyhippuric acid (PHHA) was identified as the major metabolite (57.2-63.8%). PHBA) represented 3.0-7.2%. PHBA and PHHA are both non-toxic metabolites and both effect clearance; PHHA is the further secondary metabolite of PHBA.

The applicant argued that this shows that glycine conjugation of the PHBA is in humans more effective than in rats and that this mechanism adds another route in humans generating even more effective clearance. A new metabolite, 3 OH-n-butyl paraben, was observed together with various hydroxylations on the aromatic ring (r-OH, 0.3% of the dose). It is possible that these hydroxylated metabolites also exist in rodents but have never been analysed.

The applicant concludes that from both Moos *et al.* (2016) and Mathews *et al.* (2013), qualitatively, the same metabolites are present in both rat and human urine. The overall outcome of rapid and extensive clearance of butylparaben in both rat and human is similar. Qualitatively, Phase 2 glucuronides and sulphates are produced in rat and humans. The main difference in metabolism is a greater amount of glycine conjugation produced in humans, but this also leads to more effective and rapid clearance over 24-48 hours (**Figure 4**).

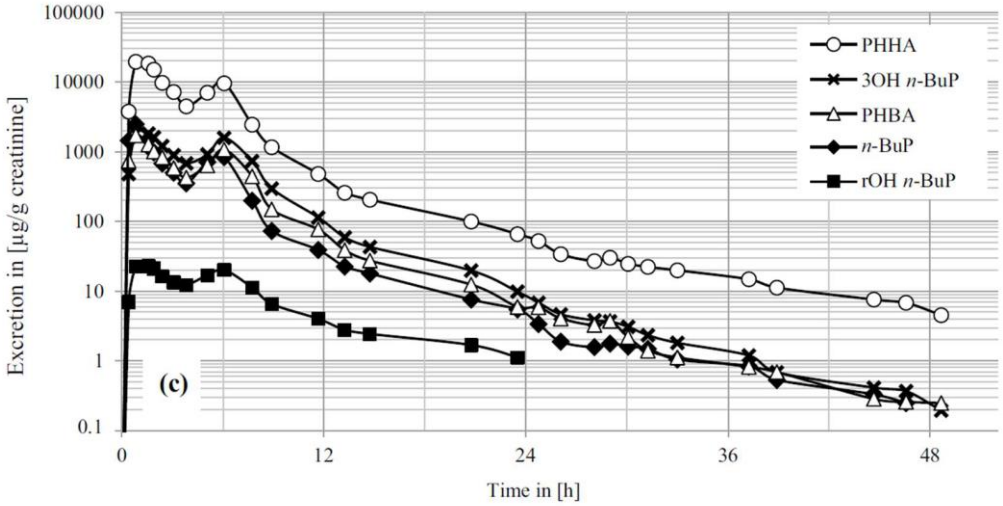


Figure 3: Creatinine-corrected metabolite concentrations in urine after oral dosage, shown in semilogarithmic scale (continuous data from one volunteer; profiles were similar for the other two volunteers) (Moos *et al.* 2016).

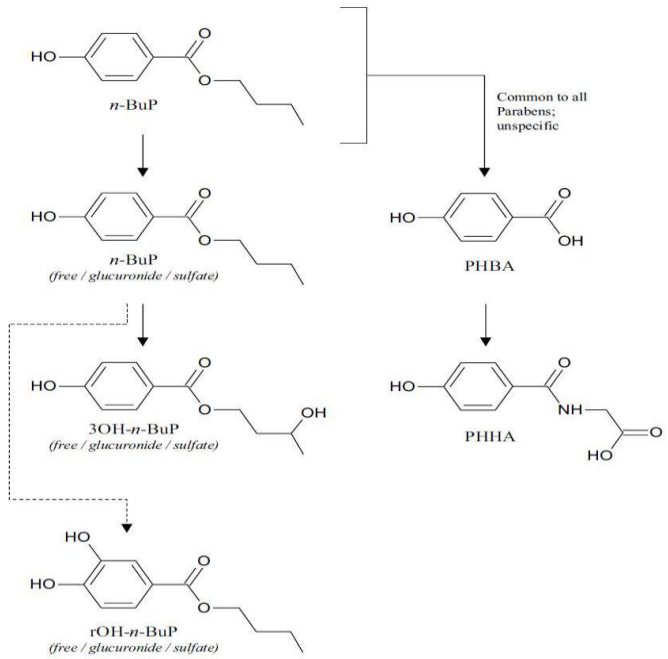


Figure 4: Proposed metabolism of butylparaben (BuP) in humans following single oral doses. Dashed line = very minor metabolite. Ring hydroxylation could occur on any carbon in the benzene ring. (Moos *et al.* 2016).

SCCS comment
The qualitative metabolism in rats and humans after oral administration of butylparaben shows a number of common metabolites. Quantitative results are not available. The study from Moos *et al.* 2016 confirms a high level of oral absorption in humans (80.5% of the oral dose was excreted in the first 24 hours).

3.2.2.2 Dermal toxicokinetics studies

Data on the dermal kinetics of butylparaben in the rat (Aubert *et al.* 2009, 2012; Mathews *et al.* 2013) and in humans (Janjua *et al.* 2008) were submitted.

3.2.2.2.1 *In vivo rat – dermal kinetics*

Aubert *et al.* (2009; published in 2012) estimated toxicokinetics in rats after dermal exposure to compare with the oral exposure (section 3.2.2.1). This study has been previously evaluated by the SCCS (SCCS/1514/13): dermally administered butylparaben showed a relatively low and slower (C_{max} at 8 hrs) uptake in serum. Elimination was complete after 12-22 hrs via the dermal route. In general, very similar pharmacokinetic profiles were found in the blood of male and female rats.

Mathews *et al.* (2013)
See also section 3.2.1 on dermal absorption, where the mass balance data from this *in vivo* rat study is used to corroborate a dermal absorption value of 3% for the safety assessment. In addition, tissue distribution data were available. In comparison to the oral route in rat, there is ~1.5 fold more in the kidney from the dermal route.

3.2.2.2.2 *Human in vivo – dermal kinetics*

Janjua *et al.* (2008)
In a 2-week single-blinded study, 26 healthy Caucasian male subjects were given a whole body topical application of basic cream 2 mg /cm^2 (control week) and then cream containing 2% (w/w) of diethylphthalate (DEP), dibutylphthalate (DBP) and butylparaben, each daily for 1 week. Urinary samples were analysed by LC-MS/MS. Extremely low amounts of free butylparaben in urine following dermal exposure were observed; the majority of applied substance that had penetrated the skin and was cleared in urine was either PHBA or a conjugated form (**Figure 5**).

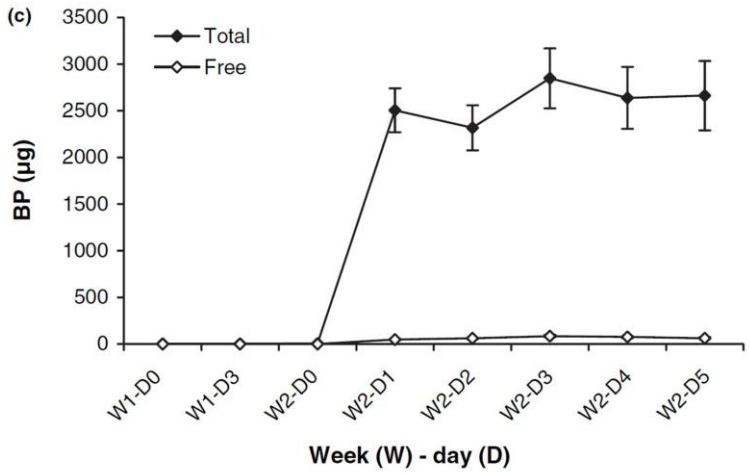


Figure 5: Total '24- hour urine' excretion of free (unconjugated) and total (free plus glucuronidated) butylparaben through the control week (Week 1) and treatment week (Week 2). The values are mean ± SEM of 24- hour urine samples, N = 26.

SCCS comment
The study by Janjua *et al.* (2008) was previously evaluated by the SCCS (SCCS/1514/13). It was noted that the exposure to 2% butylparaben is higher than the average dermal exposure of consumers. Furthermore, PHBA and butylparaben sulphate were not determined, which may lead to an underestimation of total butylparaben (free + conjugated). Exposure was performed together with two phthalates, which is not an ideal test condition to investigate butylparaben in specifics.

3.2.2.3 *Subcutaneous toxicokinetics studies*

Aubert *et al.* (2009, 2012)
The SCCS concluded earlier that the uptake of radioactivity in serum after subcutaneous application of butylparaben was high and relatively rapid (Cmax at 2-4hrs). Elimination was complete after 12-22 hrs following administration.

3.2.2.4 *Intravenous toxicokinetics studies*

Mathews *et al.* (2013)
The intravenous route of butylparaben administration in rats was studied by single intravenous dose formulations containing [^{14}C]butylparaben (50 µCi/animal), an appropriate amount of non-radiolabelled butylparaben and propylene glycol: 0.9% saline:ethanol (60:30:10; v:v:v) in a dose volume of 1 mL/kg. Intravenous doses (10 mg/kg) were administered via a lateral tail vein using a syringe with a 27G needle.
As with the oral, subcutaneous and dermal routes, rapid clearance and excretion is observed, and the same broad spectrum of metabolites. Metabolic excretion appears to be more extensive following intravenous dosing (80% complete) with the same 10 mg/kg/ dose than with the oral route (63.5%). This provides further evidence that butylparaben, penetrated through the skin into the blood stream, would be rapidly and extensively metabolised, more than by the oral route.

3.2.2.5 *Lung toxicokinetics studies*

There are no toxicokinetics studies via the inhalation route.

3.2.2.6 *Oral mucosa*

Kurosaki *et al.* (1997)
Regional differences in permeability of human oral mucosa were studied. Newly designed perfusion cells were applied to five different sites *i.e.*, dorsum of tongue, ventral surface of tongue, labial mucosa, floor of mouth and buccal mucosa of human volunteers. Absorption rates of four parabens, methyl, ethyl-, propyl- and butylparaben were correlated to lipophilicities, with the most lipophilic absorption less than the least. The absorption rate constants in buccal mucosa were approximately one-half of those estimated in other oral mucosa.

3.2.3 Pharmacokinetic modelling for a novel IVIVE approach to risk assessment

Campbell *et al.* (2015)
A pharmacokinetic model for oral and dermal exposure to parabens was developed to explore a different way of performing risk assessment using *in vitro* data and human biomonitoring data, in particular, in relation to situations where endocrine disruption effects had been measured during *in vitro* assays. In this case, the authors propose that *in vitro* to *in vivo* extrapolations (IVIVE) can be performed taking dose-response data from *in vitro* tests and, if also possible, existing *in vivo* endpoint assays and comparing effects data (at known internal doses) to internal dose metrics (from PBK modelling estimations) and measures in blood/plasma from human biomonitoring data. This method may also provide a useful solution to the main problem for classical risk assessment for parabens which is that oral metabolism in rat (the main route of choice for *in vivo* animal toxicology studies for parabens) is quantitatively different from human systemic exposure via dermal exposure and metabolism.

Campbell *et al.* (2015) used the oral and dermal toxicokinetic data from Aubert *et al.* (2009) for butylparaben in rat to build a rat-specific PBK model. Ye *et al.* (2006) had generated data on butylparaben via the oral route in humans. There is also the study by Janjua *et al.* (2008) (discussed previously in SCCS 2010) for dermally applied butylparaben in humans that can be used to build a human PBK model. The generic model structure is shown in **Figure 6**.

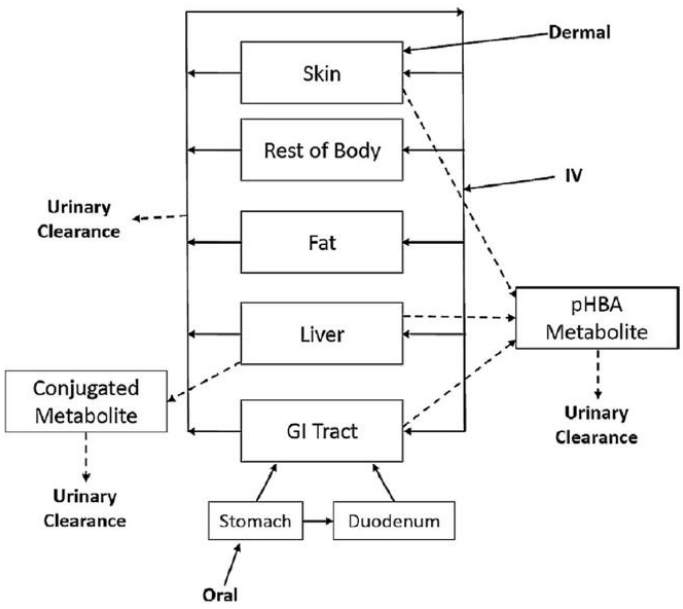


Figure 6: Physiologically-based kinetic (PBK) model structure for butylparaben as developed by Campbell *et al.* (2015).

Campbell *et al.* (2015) used chemical specific parameters for butylparaben. The *in vitro* to *in vivo* extrapolation for butylparaben provided good fits to the measured total butylparaben in plasma after a single oral bolus of 3, 10 or 100 mg/kg (**Figure 6**). The prediction of total butylparaben (free + conjugate) in plasma is within a factor of 2 of all the data. While the model does overpredict the Aubert *et al.* (2009) plasma data up to 4 hours after dosing, the simulation was within a factor of 4 of all the time-points and within a factor of 1 for all measured concentrations from 8 to 24 hours. Similarly, a human PBK model was fit to the Janjua *et al.* (2008) rat oral data (Figure 7).

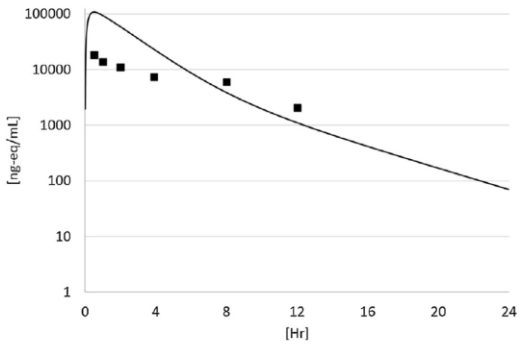


Figure 7: PBK model prediction of total radioactivity in plasma after a single 100 mg/kg oral bolus dose of butylparaben to Sprague Dawley rats using data from Aubert *et al.* 2009, 2012 (reproduced from Campbell *et al.* 2015).

In the human, the only controlled dermal study was a 5-day dermal exposure to butylparaben in ointment (Janjua *et al.*, 2008) at 40 µg/cm². The simulation (**Figure 8)** provides an exceptional fit to both the serum concentration of free butylparaben (top panel) and the cumulative excretion of free and total (free plus glucuronide conjugate) butyl-paraben. Based on the authors' estimation, approximately 16% of the applied butylparaben dose was absorbed (as paraben and metabolites) into skin.

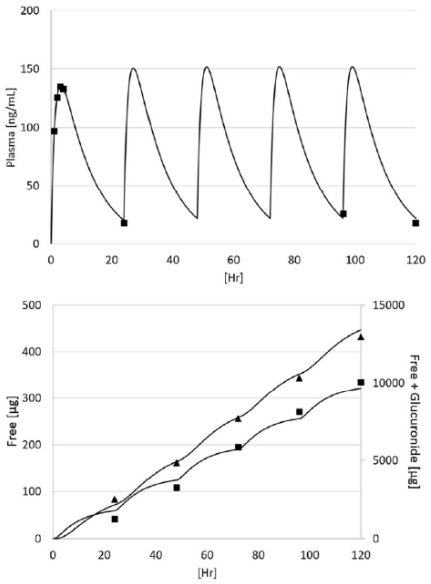


Figure 8: Prediction of the human serum concentration of free butylparaben (top) and cumulative excretion in urine (bottom) of free butylparaben (square) and free plus glucuronide metabolite (triangle) after once daily dermal exposure to 40 µg/cm² butylparaben in ointment (including two other substances diethylphthalate and dibutylphthalate) as applied to the whole body except genitals and scalp, over the course of 5 days (data as per Campbell *et al.* 2015 using data from Janjua *et al.* 2008).

Toxicokinetics data of Matthews *et al.* (2013) in the rat (studies performed before March 2013) and Moos *et al.* (2016) in humans and other new data have been used to improve and refine the Campbell model (report in PBPK Annex). The structure of the PBK model for butylparaben via oral, dermal and subcutaneous routes of exposure, is shown in **Figure 9.**

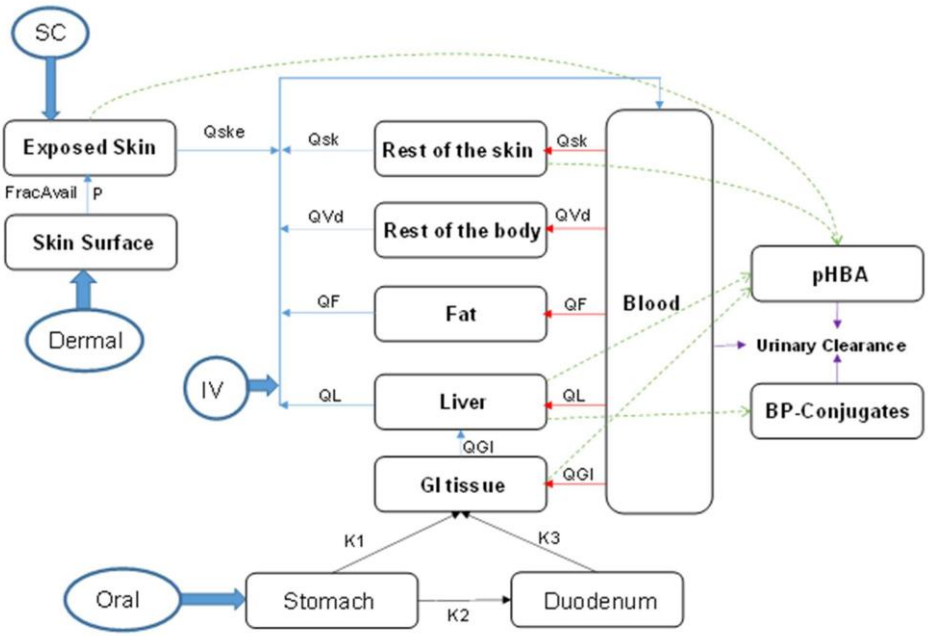


Figure 9: Structure of the butylparaben PBK model. QGI, QL, QF, QVd, Qsk, Qske refer to blood flow to each tissue compartment. All tissues are described as flow limited. K1, K2

and K3 represent first-order absorption occurring in the stomach and duodenum. P and FracAvail represent the permeability and fraction available for absorption through the skin.

The new human data for butylparaben exposure via the oral route from Moos *et al.* (2016) was interrogated and the PBK model simulation for these data in **Figure 10** show that the model performs well.

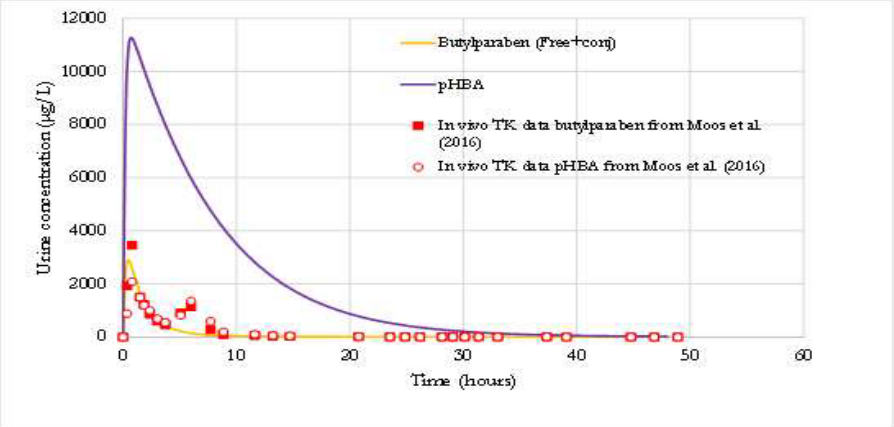


Figure 10: Butylparaben and PHBA concentrations in urine in adult humans following oral dosing-simulations using the *in vivo* PK data from Moos *et al.* (2016).

The oral and the dermal models for both rat and human were considered to be acceptable for using the quantitative output in a conservative risk assessment. Estimates of internal exposures to parent paraben ester were conservative overpredictions of what occurs in reality, as the complete aspects of metabolic clearance via Phase 2 metabolism could not be factored fully into the models due to a lack of data on the full range of metabolites. **Tables 1 and 2** show the PBK model estimates for blood C_{max} and AUC values and urine C_{max} following a range of simulated doses via the dermal and oral routes in rats and humans.

Table1: Summary of rat and human dose metrics in blood and urine after dermal exposure simulations to butylparaben ester

	Dose mg/kg/day	BP Blood Cmax* (µg/L)	Blood AUC** (µg*h/L)	BP + BP-Conjugates Urine Cmax (µg/L)
Rats	2	17.3	133.4	20.6
	100	863.3	6668.9	1030.2
	325	2805.6	21,674	3348.3
	1000	8632.7	66,689	10,302
Humans	2	20.9	260.8	1730
	100	1045.0	13,040	86,500
	325	3396.2	42,379	281,124
	1000	10,449	130,396	864,998

*Cmax for the last simulated day of exposure; **AUC – area under the curve during last simulated day of exposure.

Table 2: Summary of rat and human dose metrics in blood and urine after oral exposure simulations of butylparaben ester at selected doses

	Doses mg/kg/day	BP Blood Cmax* (µg/L)	Blood AUC** (µg*h/L)	BP + BP-Conjugates Blood Cmax (mg/L)
Rats	2	9.8	8.9	0.2
	100	488.7	447.2	8.7
	325	1588.1	1453.5	28.4
	1000	4886.5	4472.4	87.4
Humans	2	19.9	22.8	50.5
	100	994.3	1141.1	2525.0
	325	3231.5	3708.7	8206.3
	1000	9943.1	11,411	25,250

*Cmax for the last simulated day of exposure; *AUC: area under the curve during last simulated day of exposure

SCCS comments

It is noted that the rat model using data by Aubert *et al.* (2009, 2012) overpredicts the peak concentration of radioactivity **by a factor of 4.** According to the IPCS-WHO guidance (2010) on PBPK models in risk assessment the Cmax must be within a factor of 2 of the experimental data. Furthermore, **the rat model sensitivity/uncertainty analysis is missing**.

For the human PBK model, both oral and dermal absorption-related parameters were calibrated using the values by Janjua *et al.* (2007). For the dermal route, the dose metric provides the plasma concentration, this is correct.

The parameter with high uncertainty and sensitivity is the dermal absorption (estimated Janjua *et al.* 2007). Importantly, **the rat and human models were validated using the same data as used for the model calibration.** However, it is crucial that external data is used to validate the model.

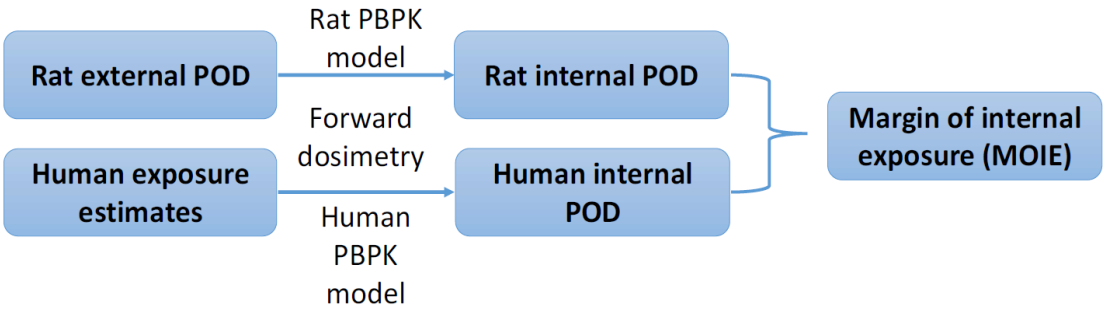
Applicants' conclusions on toxicokinetics

There is good evidence to suggest that only a low level of butylparaben parent ester is absorbed systemically via the dermal route and the oral route. Parabens are rapidly metabolised by esterases in man and do not accumulate in human tissue (Abbas *et al.*, 2010). The use of data via the subcutaneous route is problematic for risk assessment as this route does neither account for metabolic clearance by the skin as the dermal route nor for a rapid first-pass effect as the oral route. There are some differences in kinetics between the dermal and oral routes, and between rat and human, that can be adequately investigated, described and incorporated into PBK modelling investigations using the available data. Metabolism of butylparaben is effective and butylparaben is rapidly hydrolysed, conjugated, and excreted in urine in both rat and human albeit there are some qualitative differences in phase 2 metabolites. Understanding these differences enables a confident risk assessment to be performed using a margin of **internal exposure (MOIE) approach**. Systemic butylparaben ester is effectively converted and cleared via the formation of its main acid metabolite PHBA. PHBA is converted to Phase 2 clearance metabolites *i.e.* the glucuronide, sulphate and glycine (PHHA) metabolites.

SCCS conclusion on toxicokinetics

The concept of **the MOIE approach** has been proposed for route-to-route extrapolation. It is an **extension of the Margin of Exposure (MOE) approach** for cosmetics in the EU. It is based on the comparison of internal dose metrics (C_{max}, AUC conc/time). As such, the individual assessment factor 4 that covers the interspecies differences in toxicokinetics can be left out as these differences are taken into account using a PBK approach (animal PBK model and human PBK-model) (Bessems *et al.* 2017).

A general scheme of the MOIE concept is shown here:



PBPK models must be built for rat and humans and need to be calibrated and validated. Validation must be done using external data.
In the case of butylparaben, the models built for rat and humans, are parameterised with physiological parameters (ADME) and physico-chemical parameters. This is done as follows:

Physiological parameters:
1. Flow, volume of organs, etc. based on literature
2. Skin absorption
   In rat: 34.3% of the applied dose was absorbed into the skin at a rate of 0.0005/h/cm² (Aubert *et al.*, 2009,2012). This value was determined (calibration) by curve fitting
   In human: 16% of the applied dose was absorbed into the skin at a rate of $8.8x10^{-6}$/h/cm² , data from Janjua *et al.* (2008). This value was determined (calibration) by curve fitting
3. Metabolism: the hydrolysis rates of butylparaben have been examined in microsomal systems of liver and skin and were done for rat and human (Jewell *et al.*, 2007a; Ozaki *et al.*, 2013). This value was determined by *in vitro* to *in vivo* extrapolation
4. Elimination: human data from Janjua *et al.* (2008) and Moos *et al.* (2016). This value was determined by curve fitting
5. Other:
   - Volume of distribution (Vd) for PHBA: based on Moos *et al.* (2016). This value was determined by curve fitting
   - Oral uptake into duodenum from stomach, into GI from stomach. These values were estimated from Moos *et al.* (2016) for human and Aubert *et al.* (2012) for rat

Physicochemical parameters; partition coefficient
Calibration with a combination of quantitative structure activity (QSAR) and *in vitro* to *in vivo* extrapolation (IVIVE).

-**For the rat model:**
The simulations according to the oral study of Aubert *et al.* (2012) are overpredicting the peak concentration of radioactivity bya factor of 4, whereas according to the WHO/SCCS predictions of maximal concentration (Cmax) must be within a factor of 2 of the experimental data. This is also the case for the dermal rat data.
Parameters with high or low uncertainty (=level of confidence in model predictions) and sensitivity (=overall importance of a parameter) must be determined and lacking are the oral/dermal absorption parameters, where the estimated dermal absorption is derived from the Aubert study, the same study that was used for calibration and validation, which is not acceptable.

- **For the human PBPK model**:
The oral and dermal absorption-related parameters were calibrated from Janjua *et al.* 2007
The dermal route dose metric provides the plasma concentration, which is correct.
Parameter analysis reveals that the parameter with high uncertainty and sensitivity is the dermal absorption (estimated from Janjua *et al.* 2007), meaning that the same study was used for calibrations and validations, which is not acceptable.
Therefore, the MOIE approach is here **not applicable**. This would additionally mean that in the MOIE scenario, proposed by the Applicant, **the dermal absorption would have been 16%** instead of 3 or 3.7 % as proposed by the Applicant.

Much uncertainty exists with respect to the dermal absorption of butylparaben and in fact none of the studies meets the quality criteria as indicated in the Notes of Guidance, 11th Revision. In the absence of appropriate quantitative data for the dermal absorption of butylparaben, a 50% default value will be used for the dermal absorption of butylparaben.

3.3 EXPOSURE ASSESSMENT

3.3.1 Function and uses

Butylparaben has been used widely and safely as a preservative in cosmetics and pharmaceutical preparations around the world for more than 70 years.

3.3.1.1 Cosmetics use

The use of butylparaben as a preservative in cosmetics is regulated in Annex V to Regulation EC N°1223/2009. The latest update to Annex V relating to the co-use of butyl-paraben and/or propylparaben was published on 5 August 2019.
https://ec.europa.eu/growth/tools-databases/cosing/pdf/COSING_Annex%20V_v2.pdf
Butylparaben can maximally be used in any cosmetic product up to 0.14% (alone, as acid) or up to a combined maximum of 0.14% (as acid) as the sum of the individual concentrations of butylparaben, propylparaben and their salts, when used together as a mixture of ingredients in the same product. The maximum total paraben concentration in the context of combined paraben use with those paraben ingredients listed in entry 12 (methyl-, ethylparaben and their salts) is 0.8% (as acid), but butylparaben in that mixture must not exceed 0.14% (as acid).
Given the concentration in the regulation is cited 'as acid', molecular weight conversions are needed to convert this value to the % inclusion level of butylparaben ester as follows:

- Molecular weight of p-hydroxybenzoic acid is 138.111 g/mol
- Molecular weight of butylparaben is 194.23 g/mol
- The maximum value of butylparaben ester is 0.14% x (194.23/138.111) = 0.197%

Therefore, technically, the current regulatory restriction translates to a maximum concentration of 0.197% butylparaben ester in all cosmetic product types, except leave-on products for the nappy area in children under the age of 3 years, which is not allowed. **The value of 0.197% butylparaben ester as maximal inclusion in finished cosmetic products has been used in the exposure assessments to calculate an aggregate systemic exposure dose (SED)** in section 3.3.2.

3.3.1.2 Food use

Under US FDA regulation, butylparaben is generally recognised as safe (GRAS) when used as a chemical preservative in foods, with a use limit of 0.1%. Butylparaben is not approved for use as an additive or preservative in EU foods (EFSA 2004; Directive 2006/52/EC). In EFSA (2004) the opinion was given that there was not sufficient data to set an acceptable daily intake (ADI). There is a lack of interest in the use of butylparaben as a preservative in foods and it has not been formally approved for use.

3.3.1.3 Pharmaceutical use

Butylparaben is rarely used in Europe as a preservative of choice in pharmaceutical products (EMA, 2015). RIVM (2018) found that in the Netherlands only 9 medicinal products containing butylparaben could be found on the market and there was no cause for concern regarding its use.

3.3.2 Calculation of SED/LED

Applicant exposure scenarios: an explanation of the different exposure scenarios is presented
Scenario A:

- Tier 1 – maximum % inclusion level of 0.197% for butylparaben ester as per the 11th SCCS Notes of Guidance (2021) deterministic method, covering a highly worst -case aggregate exposure calculation
- Tier 2 - as per A1 using regulatory maxima with product habits and practices data included using the Creme Care and Exposure model (probabilistic person-oriented approach)
- Tier 3 - as per A2 using regulatory maxima with product habits and practices data plus product occurrence data included using the Creme Care and Exposure model (probabilistic person-oriented approach)

Scenario B exposure assessment using Cosmetics Europe 2016 survey data:

- Tier 1 - % inclusion levels for butylparaben in individual product types as per the 2016 Cosmetics Europe Survey. The P90 values are presented (NB. the P95 values were not significantly different (see Annex 2) in a deterministic additive approach as per the SCCS Notes of Guidance (2021) method, covering a high-end aggregate exposure calculation derived using the Creme Care and Exposure model
- Tier 2 - as per B1 P90 values (as above) with product habits and practices data included using the Creme Care and Exposure model
- Tier 3 - as per B2 P90 values (as above) with product habits and practices data plus product occurrence data included using the Creme Care and Exposure model

According to the Applicant, Tiers 2 & 3 probabilistic exposure assessments present a scientifically robust approach for safety evaluation, bringing all the evidence and data into the evaluation and tending towards a more realistic exposure assessment.

Exposure scenarios according to SCCS

Using the data on external dermal dose for adults, one can incorporate a dermal absorption value into the modelling to generate a systemic exposure dose (SED) of butylparaben in each scenario, which can be taken forward into the final safety evaluation.
In this case, a value of 50% dermal absorption of butylparaben ester was used.

Table 3: scenario A with Tiers 1, 2 and 3
Scenario A – Tier 1 (Maximum inclusion, deterministic approach)

Product	maximum use (w/w%) in the finished product (as esters)	Calculated relative daily exposure to product [1] (mg/kg bw/day)	Total dermal external exposure to butylparaben (µg/kg bw/day)*	Calculated SED [2] (µg/kg bw/day)
Shower gel	0.197	2.79	5.5	2.75
Hand wash	0.197	3.33	6.56	3.28
Shampoo	0.197	1.51	2.97	1.485
Hair conditioner	0.197	0.67	1.32	0.66
Hair Styling	0.197	5.74	11.31	5.655
Body lotion	0.197	123.2	242.7	121.35
Face cream	0.197	24.14	47.56	23.78
Hand cream	0.197	32.7	64.42	32.21
Liquid foundation	0.197	7.9	15.56	7.78
Lipstick, lip salve [3]	**0.197**	**0.9**	**1.77**	**1.77**
Make-up remover	0.197	8.33	16.41	8.205
Eye shadow	0.197	0.33	0.65	0.325
Mascara	0.197	0.42	0.83	0.415
Eyeliner	0.197	0.08	0.16	0.08
Non-spray	0.197	22.08	43.5	21.75
Toothpaste [3]	**0.197**	**2.16**	**4.26**	**4.26**
Mouthwash [3]	**0.197**	**32.54**	**64.1**	**64.1**
Aggregate			**529.58**	**299.855**

[1] According to values in Table 3A and 3B on page 24-25 of the SCCS Notes of Guidance (11th revision) (2021) [= $E_{product}$]
[2] Total dermal external exposure **x 50% dermal absorption** (see section 3.2.1)
[3] SCCS default 100% dermal absorption.

Scenario A - Tier 2 (probabilistic person-oriented approach)

Product	P95 dermal [1] external exposure to butylparaben (µg/kg bw/day)	calculated SED (µg/kg bw/day) with 50% Dermal absorption [2]
Shower gel	5.8703	2.9352
Hand wash	0.5735	0.2868
Bar soap	2.9512	1.4756
Shampoo	2.8944	1.4472
Hair conditioner	1.7724	0.8862
Hair Styling	4.6331	2.3166
Body lotion [4]	**0.0000**	**0.0000**
Face cream	27.6033	13.8017
Hand cream	15.8301	7.9151
Liquid foundation	7.2183	3.6092
Lipstick, lip salve [3]	**0.2016**	**0.2016**
Make-up remover	0.0000	0.0000
Eye shadow	0.1108	0.0554
Mascara	0.4033	0.2017
Eyeliner	0.0133	0.0067
Non-spray Deo	20.8388	10.4194
Toothpaste [3]	**4.2695**	**4.2695**
Mouthwash [3]	**64.0939**	**64.0939**
ALL PRODUCTS [5]	**125.8400**	**113.9**

[1] According to values from models 1b and 1c, respectively from Tables 48 and 49 in Annex 2 Creme report
[2] Total dermal external exposure x 50% dermal absorption (see section 3.2.1)
[3] SCCS default 100% absorption.
[4] Using standard mass body lotion data, which was higher value than prestige products.
[5] The P95 value for 'all products' is not additive of all 18 products in the table. It is the output of probabilistic modelling.

Scenario A – Tier 3 (probabilistic person-oriented approach + Mintel occurrence data)

Product	P95 dermal [1] external exposure to butylparaben (µg/kg bw/day)	calculated SED (µg/kg bw/day) with 50% Dermal absorption [2]
Shower gel	2.586	1.293
Hand wash	0.000	0.000
Bar soap	0.000	0.000
Shampoo	1.368	0.684
Hair conditioner	0.000	0.000
Hair Styling	0.000	0.000
Body lotion [4]	**0.000**	**0.000**
Face cream	19.857	9.929

Hand cream	0.000	0.000
Liquid foundation	3.162	1.581
Lipstick, lip salve [3]	**0.000**	**0.000**
Make-up remover	0.000	0.000
Eye shadow	0.007	0.003
Mascara	0.153	0.077
Eyeliner	0.000	0.000
Non-spray Deo	0.000	0.000
Toothpaste [3]	**0.000**	**0.000**
Mouthwash [3]	**0.000**	**0.000**
ALL PRODUCTS [5]	**31.357**	**13.5667**

[1] According to values from models 1b and 1c, respectively from Tables 48 and 49 in Annex 2 Creme report
[2] Total dermal external exposure x 50% dermal absorption (see section 3.2.1)
[3] SCCS default 100% absorption.
[4] Using standard mass body lotion data, which was higher value than prestige products.
[5] The P95 value for 'all products' is not additive of all products in the table. It is the output of probabilistic modelling.

Table 4: Scenario B with tiers 1,2 and 3

Scenario B – Tier 1 (deterministic additive approach using Cosmetics Europe 2016 survey)

Product	P90 use levels (w/w%) in the finished product	calculated relative daily exposure to product [1] (mg/kg bw/day)	calculated SED (µg/kg bw/day) with 50% Dermal absorption [2]
Shower gel	0.04	2.79	0.52
Hand wash	0.20	3.33	3.28
Shampoo	0.00	1.51	0.00
Hair conditioner	0.00	0.67	0.00
Hair Styling	0.00	5.74	0.00
Body lotion	0.15	123.20	92.40
Face cream	0.15	24.14	18.11
Hand cream	0.20	32.70	32.21
Liquid foundation	0.15	7.90	5.93
Lipstick, lip salve [3]	**0.10**	**0.90**	**0.90**
Make-up remover	0.01	8.33	0.43
Eye shadow	0.04	0.33	1.51
Mascara	0.06	0.42	0.13
Eyeliner	0.05	0.08	0.02
Non-spray	0.10	22.08	11.04
Toothpaste [3]	**0.20**	**2.16**	**4.26**
Mouthwash [3]	**0.20**	**32.54**	**64.10**
Aggregate			**170.72**

[1] According to values in Table 3A and 3B on page 21-22 of the SCCS notes of guidance (11th revision) (2021)
[2] Total dermal external exposure x 50% dermal absorption (see section 3.2.1): Table 27 Annex 2 Creme report.
[3] SCCS default 100% absorption.
***the P90 values were not significantly different from the P95 values and were used as conservative estimates.**

Scenario B – Tier 2 (probabilistic person-oriented approach)

Product	P95 dermal external exposure to butylparaben (µg/kg bw/day) [1]	calculated SED (µg/kg bw/day) with 50% Dermal absorption [2]
Shower gel	1.106	0.553
Hand wash	0.574	0.287
Bar soap	2.951	1.476
Shampoo	0.003	0.001
Hair conditioner	0.001	0.000
Hair Styling	0.002	0.001
Body lotion [4]	**0.000**	**0.000**
Face cream	21.018	10.509
Hand cream	15.830	7.915
Liquid foundation	5.496	2.748
Lipstick, lip salve [3]	**0.102**	**0.102**
Make-up remover	0.000	0.000
Eye shadow	0.020	0.010
Mascara	0.123	0.061
Eyeliner	0.003	0.002
Non-spray Deo	10.578	5.289
Toothpaste [3]	**4.270**	**4.270**
Mouthwash [3]	**64.094**	**64.094**
ALL PRODUCTS [5]	**126.171**	**97.318**

[1] According to values from models 1b and 1c, respectively from Tables 50 and 52 in Annex 2 Creme report
[2] Total dermal external exposure x 50% dermal absorption (see section 3.2.1)
[3] SCCS default 100% absorption.
[4] Using standard mass body lotion data, which was higher value than prestige products.
[5] The P95 value for all products is not additive of all 18 products in the table. It is the output of probabilistic modelling.

Scenario B – Tier 3 (probabilistic person-oriented approach + Mintel occurrence data)

Product	P95 dermal external exposure to butylparaben (µg/kg bw/day) [1]	calculated SED (µg/kg bw/day) with 50% Dermal absorption) [2]
Shower gel	0.487	0.243
Hand wash	0.000	0.000
Bar soap	0.000	0.000
Shampoo	0.001	0.001
Hair conditioner	0.000	0.000
Hair Styling	**0.000**	**0.000**
Body lotion [4]	0.000	0.000
Face cream	15.120	7.560

Hand cream	0.000	0.000
Liquid foundation	2.408	1.204
Lipstick, lip salve [3]	**0.000**	**0.000**
Make-up remover	0.000	0.000
Eye shadow	0.001	0.001
Mascara	0.047	0.023
Eyeliner	0.000	0.000
Non-spray Deo	0.000	0.000
Toothpaste [3]	**0.000**	**0.000**
Mouthwash [3]	**0.000**	**0.000**
ALL PRODUCTS [5]	**18.064**	**9.032**

[1] According to values from models 1b and 1c, respectively from Tables 50 and 52 in Annex 2 Creme report
[2] Total dermal external exposure x 50% dermal absorption (see section 3.2.1)
[3] SCCS default 100% absorption.
[4] Using standard mass body lotion data, which was higher value than prestige products.
[5] The P95 value for all products is not additive of all 18 products in the table. It is the output of probabilistic modelling.

3.4. TOXICOLOGICAL EVALUATION

The Applicant provided the following information: Parabens, and specifically butylparaben, have been used in cosmetics for more than 70 years, and their safety has been reviewed progressively over the decades as new information has arisen. Comprehensive reviews providing evidence to assure safety for parabens and specifically for n-butylparaben have been published previously:

- Cosmetic Ingredient Review in 1984
- European Food Safety Authority (EFSA) (2004) Opinion on the safety of parabens in foods
- Soni *et al.* (2005) – scientific review of parabens data
- Golden *et al.* (2005) – scientific review of parabens data
- US National Toxicology Program (NTP) (2005) safety data review for butylparaben (gaps triggered the need for the NTPs subsequent safety programme on butyl-paraben – live phases completed before March 2013). See data at https://ntp.niehs.nih.gov/data/index.html
- The SCCS have periodically reviewed the safety of parabens as new information has arisen *e.g.* in 2005 (SCCP/0874/05), 2006 (SCCP/1017/06), 2008 (SCCP/1183/08), 2010 (SCCS/1348/10), 2011 (SCCS/1446/11) and 2013 (SCCS/1514/13). The last of these reviews in 2013 was specifically focused on butylparaben and propyl-paraben.
- RIVM (2018) – review of butyl paraben data for consumer use in the Netherlands
- The Cosmetics Ingredient Review (CIR) 2008/2012 and an amended safety report with new data was published in October 2019 (CIR, 2019) https://online.personalcarecouncil.org/ctfastatic/online/lists/cir-pdfs/FR746.pdf).
- Danish Environmental Protection Agency (2020) Annex XV report
- Health Canada (2020) Draft screening assessment for parabens (available online at https://www.canada.ca/content/dam/eccc/documents/pdf/pded/parabens/Draft-screeningassessment-parabens-group.pdf)

3.4.1. Irritation and corrosivity

3.4.1.1 Skin irritation

Two *in vivo* studies were reported by the Cosmetic, Toiletry and Fragrance Association (CTFA) as reviewed in the Cosmetic Ingredients Review (CIR) for butylparaben, originally performed in 1984. 0.3% butylparaben was applied to the backs of six rabbits for 3 consecutive days; almost all rabbits showed mild irritation (CTFA, 1976 as reported in CIR 2008). No signs of irritation were observed when a product formulation containing 0.2% propylparaben and 0.1% butylparaben was applied to the genital mucosa of six albino rabbits. The single 0.1 ml application of the undiluted product produced no evidence of mucosal irritation during the 7-day observation period; a concentration of 0.2% butylparaben showed mild irritation (CTFA, 1980a as reported in CIR 2008). Butylparaben (5%) was a mild irritant when applied to the skin of guinea pigs for 48 hours (NTP, 2005).

Applicants' conclusion on skin irritation
There is no evidence to suggest from animal studies that butylparaben is a skin irritant and decades of human use in cosmetics have not revealed any issues relating to skin irritation. Moreover, considering that butylparaben ester is used in cosmetic products only at concentrations up to 0.197%, it can be concluded that there is no risk of skin irritation for the consumer.

SCCS comment
Butylparaben shows mild irritant properties when dermally applied to guinea pigs (5%) and rabbits (0.3% in product formulation). Moderate irritation was indicated when applied to the skin of rabbits (0.2% in product formulation) (NTP, 2005). A 2005 review by the NTP furthermore concluded that butylparaben may cause skin irritation in humans (NTP, 2005).

A recent *in vitro* study showed no skin irritation (Svobodova *et al.*, 2023).

3.4.1.2 Mucous membrane irritation / eye irritation

In vitro study
Sivasegaran *et al.* (2007) investigated the response of cultured bovine lenses over time to butylparaben. The focusing ability of the lens was measured with an automated laser scanner over a period of 96h. At 120h post-treatment, the lenses were analysed by using a confocal laser scanning microscope to determine the characteristics of nuclei, and the morphology and distribution of mitochondria within the lenses. Irritancy was investigated at both an optical and cellular level. Butylparaben was tested at 0.002% and 0.2%; at 0.2% it was found to be mildly irritating.

In vivo study
Two studies in rabbits have investigated the eye irritation effects of products containing butylparaben at concentrations of 0.1–0.8%. No eye irritation was seen.

CTFA (1980b); CTFA (1981) as reported in CIR (2008)

Applicants' conclusion on eye irritation
There is no evidence to suggest from animal studies that butylparaben is an eye irritant and decades of human use in cosmetics have not revealed any issues relating to eye irritation, particularly when considering that butylparaben ester is used in cosmetic products only at concentrations up to 0.197%.

SCCS comment
Products containing 0.1-0.8% butylparaben do not cause eye irritation in rabbits (CIR, 2008). However, according to the review by NTP (2005), butylparaben may cause eye irritation in humans. A recent *in vitro* study reported no eye irritation (Svobodova *et al.*, 2023).

3.4.2 Skin sensitisation

Animal data
Butylparaben (0.1%) was injected intracutaneously three times per week at random sites on the back and upper flanks of guinea pigs for a total of ten injections. No reactions were reported 24 hours after the initial injection. A challenge dose given two weeks later also failed to produce sensitisation 24 or 48 hours later (Matthews *et al.*, 1956; Sokol, 1952 [cited by CIR, 1984]). The same results were obtained in a similar experiment using the sodium salt of butylparaben (5%) (Matthews *et al.*, 1956).
In a study by Brulos *et al.* (1977) (as cited in CIR 2008), 20 albino guinea-pigs were given intradermal injections of Freund's complete adjuvant on days 0 and 9, and then 5% butyl-paraben was applied under 48-hour occlusive patches to the clipped dorsal skin, every other day for 3 weeks. This was 10 applications in total. Twelve days after administration of the last inductive patch, a challenge patch was applied for 48 hours to a different skin site. The skin site was scored for evidence of sensitisation after 1, 7, 24 and 48 hours from removal of the patch. Six of the twenty animals reacted to the challenge patch containing 5% butyl paraben in olive oil. The mean erythema score was 1.7 (maximum score of 4) and there were pathological allergic lesions in two of the six positive animals.

Human data
Despite the fact that parabens have been used widely for decades, contact allergy to parabens is relatively rare (Lundov *et al.*, 2009). In the USA, the prevalence of positive reactions to parabens in patch-tested individuals has decreased from 1.7% in 1996–1998 to 0.6% in 2001–2002 (Marks *et al.*, 2000; Pratt *et al.*, 2004). In Europe, a 10-year multicentre analysis from 1991 to 2000 showed stable prevalence of positive parabens patch tests between 0.5 - 1.0% (Wilkinson *et al.*, 2002). In 2019, the parabens were selected as contact non-allergens of the year with a prevalence rate of below 1% in Europe (Fransway *et al.*, 2019).

Applicants' conclusion on sensitisation
From decades of safe use, parabens are not of concern with respect to the endpoint of sensitisation and butylparaben is not classified under CLP regulation as a skin sensitiser.

SCCS comment
Animal tests indicate that butylparaben is non-sensitising. The NTP review on butyl-paraben (2005) showed that human studies indicate a low sensitisation potential when applied up to 15%. A recent publication, using NAMs, showed limited sensitisation but suggested that the concentration used in cosmetic products would be too low for that (Svobodova *et al.*, 2023).

3.4.3 Acute toxicity

3.4.3.1 Acute oral toxicity

In mice, oral administration (gastric intubation) of 5 g/kg did not lead to deaths; similarly, no deaths were seen in rats orally administered with 25 g/kg butylparaben (CTFA, 1976;

CTFA 1980; as cited in CIR 2008). This observation was in agreement with Sado (1973), who calculated an LD50 of 13,200 g/kg in dd-strain mice for butylparaben.
The sodium salt of butylparaben was tested for acute toxicity in mice by Matthews *et al.* (1956) and the LD50 was found to be 950 mg/kg.

3.4.3.2 Acute dermal toxicity

In rabbits, the acute dermal toxicity of 0.2% butylparaben ester was tested; the dermal LD50 was >2 g/kg

(CTFA 1980; as cited in CIR 2008).

3.4.3.3 Acute inhalation toxicity

There are no animal studies covering the acute inhalation toxicity of butylparaben.

3.4.3.4 Acute subcutaneous toxicity

The sodium salt of butylparaben ester was administered subcutaneously to groups of five mice. The reported LD50 was 2.5 g/kg

(Adler-Hradecky & Kelentey, 1960).

3.4.3.5. Acute intraperitoneal toxicity

The intraperitoneal (i.p.) LD50 of the sodium salt of butylparaben was 230 mg/kg bw in mice and lacrimation was seen in the eyes of mice (Matthews *et al.*, 1956).

SCCS overall conclusion on acute toxicity
The SCCS is of the opinion that butylparaben has no acute toxicity.

3.4.4 Repeated dose toxicity

In the former SCCS Opinion on parabens (SCCS/1514/13), no adequate NO(A)EL-value for the paraben esters under consideration could be retrieved from the studies listed in Appendix 1 of SCCS/1514/13. Consequently, the NOEL value of 2 mg/kg bw/day, based on Fisher *et al.* (1999) was determined to be a conservative choice for the calculation of the MoS of propyl- and butylparaben. The Committee acknowledged the fact that the Fisher *et al.* (1999) study involves subcutaneous instead of oral administration but emphasized that **2 mg/kg bw/day** clearly represents a NOEL instead of a NOAEL.

The Applicant argued that from the general reviews of paraben safety over the past 5 decades of use, there have been no concerns expressed about the general toxicity per se of parabens (Soni *et al.* (2001, 2005), CIR 2008/2012, and CIR 2019). As stated in CIR 2008, "subchronic and chronic oral studies indicate that [all] parabens are practically non-toxic". It is furthermore noted that in more recent years, there has been more focus on reproductive and developmental studies, which are discussed in detail in section 3.4.5. The general toxicity repeat-dose studies that are available for butylparaben are discussed below.

3.4.4.1 Repeated dose (28 days) oral / dermal / inhalation toxicity

Oral - rats

The effects of a formulation containing 0.2% propylparaben ester and 0.1% butylparaben ester were tested by oral administration in male and female rats for one month. No signs of toxicity were noted. Food consumption, body weight gain and haematological values were similar for both the treated and control groups. Minor changes noted in blood chemistry and organ weights were of no toxicological significance. Histological examination of the tissues revealed no treatment-related changes (CTFA, 1980 as cited in CIR 2008).

In white Wistar rats (n=12 males; n=12 female per dose group), the sodium salt of butylparaben was given at 2% or 8% orally in the diet. Intakes for animals on the 2% diets averaged from 0.9 to 1.2g/kg/day. While the intake of rats on the 8% diets averaged from 5.5 to 5.9 g/kg/day. 8% in the diet for 12 weeks resulted in 100% mortality before the end of the treatment period in males given such a high dose. Females also had many early deaths and showed myocardial depression. The high-dose butylparaben diet also produced a significant decrease in body weight for all animals, while the lower 2% dose produced no toxic effects. The **NOEL** from this study was **in the range 900-1200 mg/kg bw/day** (Matthews *et al.*, 1956).

In Fisher-344 weanling rats (n=5 male per dose), a diet of 4% butylparaben for nine days acted entirely on the pre-fundic region of the forestomach epithelium adjacent to the fundic mucosa, while oral intubation of butylparaben (0.25 or 50 mg/kg) daily for 13-15 weeks produced no toxic effects (Rodrigues *et al.*, 1986).

DNA methylation was investigated within the context of an OECD 407 Test Guideline 28-day study. Male Sprague Dawley rats (7-week-old, n=5/group, 4 groups) were dosed with 0, 10, 100 and 1000 mg/kg in corn oil (vehicle), by oral gavage. 24 hours after the last dose, testes, tails and epididymal spermatozoa samples were collected, DNA was extracted, and the DNA samples from each group were pooled, digested (methylation-specific restricted restriction digestion), and analysed by differential display random amplification of polymorphic DNA (RAPD). Among 57 RAPD amplicons, six were methylation specific. Densitometric analysis of stained agarose gels revealed that five of these amplicons were elevated 1.4- to 3.8-fold in epididymal sperm DNA in treated vs. control animals, indicating a potential effect on spermatogenic germ cells in adult rats (Park *et al.*, 2012)

Oral - mice

8-week-old ICR/Jcl mice (10 male and 10 female) were provided with a diet of pellets containing butylparaben (0.6, 1.25, 2.5, 5, or 10% equivalent to 900, 1900, 3800, 7500 and 15,000 mg/kg bw/day) for six weeks. A group of n=20 males and n=20 females acted as control groups. Deaths occurred within the first two weeks in those given the two highest doses (5 or 10%; >7500 mg/kg bw/day). Body weight gain was approximately the same as controls at a dose of 900 mg/kg bw/day. At levels greater than 900 mg/kg bw/day, there was significant atrophy of lymphoid tissue in the spleen, thymus, and lymph nodes and multifocal degeneration and necrosis in the liver parenchyma. No significant lesions or adverse effects were seen at a dose of 900 mg/kg bw/day butylparaben, therefore the **NOEL in this study was 900 mg/kg bw/day** (Inai *et al.*, 1985)

Fifty healthy female Swiss strain albino mice weighing 30-35g were divided equally in five different groups (n = 10). Animals received three different doses of butylparaben (13.33 (low dose LD), 20 (mid dose (MD) and 40 (high dose HD) mg/kg/day) in 200µl of olive oil. Oral treatments were given to all the animals for 30 days using a feeding tube attached to hypodermic syringe. Animals were sacrificed on Day 31 by cervical dislocation and the liver was quickly isolated and blotted free of the blood. Effects were analysed on lipid peroxidation, gluthathione levels, enzymes (superoxide dismutase (SOD), catalase (CAT),

glutathione peroxidase (GPx), gluthathione reductase (GR), gluthathione S-transferase (GST) involved in redox mechanisms. Oral administration of BP for 30 days resulted in significantly ($p < 0.05$) reduced levels of SOD (LD -17.93%, MD -35.86%, HD -66.84%), CAT (LD-22.24%, MD -39.43%, HD -59.25%), GPx (LD -16.07%, MD -38.36%, HD -59.34), GR (LD -12.26%, MD -33.02%, HD -48.58%) and GST (LD -14.03%, MD -27.06%, HD -49.32%) as compared to control. Reductions in antioxidant enzyme activity were highly dose-dependent (SOD r = -0.907, CAT r = -0.948, GPx r = -0.969, GR r = -0.980, GST r = -0.915). In this study, treatment of butylparaben for 30 days causes alteration in antioxidative systems as well as increases lipid peroxidation ultimately causing oxidative stress in experimental animals. The impact of this observation on liver toxicity was not identified (Shah *et al.*, 2011).

3.4.4.2 Sub-chronic (90 days) oral / dermal / inhalation toxicity

No studies submitted.

3.4.4.3 Chronic (> 12 months) toxicity

Rat study:
A 96-week study was performed to investigate the effects of butylparaben. Rats (n=24/sex/group) were fed diets containing 2% (900 to 1200 mg/kg bw/day) or 8% (5500 to 5900 mg/kg bw/day) butylparaben in the diet for 12 weeks. Negative controls were included in the study. Food intake and the body weights of animals were recorded every other week. Based on the food intake, biweekly butylparaben consumption was determined. Food and butylparaben intake remained fairly constant throughout the course of experiment. At the end of the experiments, animals that survived were killed and kidney, liver, heart, lung, spleen and pancreas were removed for microscopic examinations. All male animals died before 12 weeks at the 8% dose of butyl paraben. Females also showed signs of toxicity at this dose but details were not specified. There were no toxic effects at the 2% diet 900-1200 mg/kg bw/day of butylparaben. The **NOEL in this study was therefore 900-1200 mg/kg** bw/day (Matthews *et al.*,1956).

Mouse study:
Eight-week-old ICR/Jcl mice (n=50 male; n=50 female) were given butylparaben orally (0.15, 0.3, or 0.6%) in the diet for 102 weeks. 0.6% butylparaben was defined as the maximum tolerated dose. N=50 males and n=50 females were also used in control groups with a basal diet. Body weights were measured once a week for the first 6 weeks, once every other week for the next 24 weeks and once every 4 weeks to the end of the study. Food consumption was measured every week for the first 30 weeks, once every other week for the next 20 weeks and once every 4 weeks to the end of the study. There was no significant difference in the food consumed in treated groups vs control animals. Data were analysed from those animals surviving for 78 weeks. A high incidence of amyloidosis affecting the spleen, liver, kidney, and/or adrenal gland was observed. These occurred in 45% and 27% of males and females, respectively, that survived for >78 weeks or died with tumours during the experimental period. Tumours were present in treated and control animals alike and there were no significant differences in the treated animals. It has been reported that spontaneous amyloidosis is common in aged mice (Soret *et al.* 1977). The maximum ingested dose of butylparaben that was considered to be non-tumourigenic was approximately 40 mg/mouse, which was equivalent to 65.8g/day in a human.
For a 70 kg adult, this would **suggest a NOEL of 940 mg/kg bw/day.** Inai *et al.* (1985)

Rat study - Liver effects: as observed in a multigenerational continuous breeding study. See study description in section 3.4.5 below**. A NOAEL of 325 mg/kg bw/day was established in adult female rats of the F1 generation** that showed some signs of

adaptive liver effects in this study (NTP, 2011; published subsequently in Hubbard *et al.*, 2020).

Conclusion by the applicant
Many of the studies in this section were dosed at very high levels. Based on the results from these studies investigating repeat-dose toxicity, the NOAEL from general repeat-dose toxicity studies of butylparaben was observed to be ≥900 mg/kg bw/day. In a multi-generation continuous breeding study, performed by the US NTP in 2011 (and published later in Hubbard *et al.* 2020), adult rats were seen to display adaptive effects in the liver, hence the liver was regarded as a target organ in this study and **a conservative NOAEL was established at 325 mg/kg bw/day.**

3.4.5 Reproductive toxicity

In vivo animal reproductive and developmental studies that are available for use in a cosmetic safety assessment for butylparaben are summarised in **Table 5**

Table 5: summary of DART (developmental and reproductive toxicology) studies on butylparaben

Test substances	Test system	Test principle(s)	Result(s) and conclusion(s)	Reference
A) ***in vivo* experiments -** female effect				
Butylparaben	Sprague Dawley rats, F0 (aged 11 weeks, n=22/sex/group) and F1c parental (F1cP) animals (aged 12-13 weeks, n =26-40)	RACB[*)] study to GLP: supplementation in NIH-07 powdered feed at levels of 0, 5000, 15000, or 40000 ppm.	**No female reproductive or developmental effects observed at any dose in any generation**. Increases in liver weights, and some incidences of non-neoplastic liver lesions suggest the liver is a target organ. No findings were observed that would support any mechanism of BP-induced endocrine disruption **NOAEL = 5000 ppm, equivalent to 325 – 740 mg/kg/day** (observations in liver in adult F1 females only)	NTP (live phase completed 2011); Hubbard *et al.*, 2020
Butylparaben	Sprague Dawley rats, F0 (aged 11 weeks, n=22/sex/group) and F1c parental (F1cP) animals	RACB study to GLP: supplementation in NIH-07 powdered feed at levels of 0,	**No female reproductive or developmental effects.** Showed exposure to the test article to	NTP (live phase completed 2012);

	(aged 12-13wks, n =26-40)	5000, 15000, or 40000 ppm.	support the NTP RACB study.	Roberts *et al.*, 2016
Butylparaben 17β-oestradiol	CF-1 and CD-1 female mice Non-GLP, No guideline, No mention of group size	Evaluation of the effects of butyl-paraben on success of implantation in fertilised mice; **subcutaneous injection** of 0, 1.4, 14, 271,407, 542, 813, 949 mg BP/kg/day, on day 1 to 4 of gestation. Additional uterotrophic assay with BP at 0, 20, 200, 949 mg/kg bw/day in two different mice strains. 14 mg/kg bw/day 17β-oestradiol was administered as positive control in both assays.	Butylparaben had **no impact on the number of implantation sites and measured parameters,** *e.g.* number of pups born, litter weights, pup weight and survival, number of intrauterine blastocyst implantation sites. 17β-oestradiol terminated all pregnancies. A uterotrophic assay was conducted to re-evaluate *in vivo* data	Shaw and de Catanzaro, 2009
Butylparaben	Sprague Dawley rats	Developmental study according to OECD test guideline and GLP. Oral gavage, 0, 10, 100 and 1000 mg/kg bw/day on gestation days 6-19. Foetuses examination on gestational day 20; developmental parameters measured	At the highest dose, maternal food consumption reduced during exposure time, weight gain reduced on days 18-20. No developmental parameters changed. Developmental (oral) **NOAEL: 1000 mg/kg bw/day.**	Daston, 2004
B) *In vivo* experiments: male effects				
Butylparaben	Sprague Dawley rats, F0 (aged 11 weeks, n=22/sex/group) and F1c parental (F1cP) animals (aged12-13 wks) n = 26-40)	RACB study to GLP: supplementation in NIH-07 powdered feed at levels of 0, 5000, 15000, or 40000 ppm.	**No male reproductive or developmental effects observed at any dose.**	NTP (live phase completed 2011); Hubbard *et al.* 2020.
Butylparaben	Sprague Dawley rats, F0 (aged 11 weeks, n=22/sex/group)	RACB study to GLP: supplementation in NIH-07 powdered feed at levels of	**No male reproductive or developmental** effects observed	NTP (live phase completed 2012);

	and F1c parental (F1cP) animals (aged 12-13 weeks, n =26-40)	0, 5000, 15000, or 40000 ppm.	at any dose. Showed exposure to the test article to support the NTP RACB study.	Roberts *et al.* 2016
Butylparaben	Wistar rat GLP Non guideline	Repetition of the Oishi study (2001) under GLP with MeP or Butylparaben using the same strain of rats but 16 instead of 8 animals per dose group, same **oral route dosage** levels of 0, 100, 1000 and 10,000 ppm in food. blood samples were weekly taken for the analysis of LH, FSH and testosterone	There were **no** treatment related effects on testes, ventral prostates and preputial glands in any of the groups. Unlike Oishi (2001), sperm parameters were found unaffected. With both MeP and Butylparaben, the highest dose level in food corresponds to a **NOAEL of 10,000 ppm, NOAEL=1100 mg/kgbw/day**	Hoberman *et al.* 2008
Butylparaben	Sprague Dawley rats Non GLP Non guideline	Study of the effect of butyl- paraben on the development of the reproductive organs of F1 offspring when pregnant rats are **subcutaneously** injected with 100 or 200 mg butyl-paraben/kg/day from gestation day 6 to postnatal day 20 (lactation period).	At both dosage levels, the weights of testes, seminal vesicles and prostate glands were decreased, together with the sperm count and the sperm motile activity in the epididymis. Testicular expression of estrogen receptor (ER)-α and ER-β mRNA was significantly increased at the highest dosage level.	Kang *et al.* 2002
Butylparaben	CD-1 ICR mice Non GLP Non guideline	Study of the effects of butyl- paraben on general function of the male mouse reproductive system. Mice (25-27 days old) received butyl-paraben through the oral route for 10 weeks at dosage levels of	Administration of butylparaben at 146 and 1504 mg/kg bw/day caused an increase in epididymal weights, a decrease in testis spermatid count and in serum testosterone concentration.	Oishi 2002

		14.4, 146, 1504 mg/kg bw/day.	**NOAEL =14.4 mg/kgbw/day.**	
Butylparaben	Wistar rat Non GLP Non guideline	Study of the potential reproductive effects of butyl- paraben on male rats (19-21 days old), receiving butyl-paraben through the oral route for 8 weeks at dosage levels of 10.4, 103, 1026 mg/kg bw/day.	There were no treatment related effects on testes, ventral prostates and preputial glands in any of the groups. Decreases in cauda epididymal sperm reserve, sperm count, daily sperm production and in serum **testosterone concentration were observed from 10.4 mg/kg bw/day onwards (LOAEL).**	Oishi 2001
Butylparaben	Wistar rat Non GLP Non guideline	Effects of neonatal exposure to butylparaben on development of rat testis after a single **subcutaneous** administration of 2 mg butyl-paraben/kg/day for 17 days (postnatal days 2-18). Other substances tested were diethylstilbestrol ethinyloestradiol bisphenol A, genistein, octylphenol.	Effects of neonatal exposure to butylparaben on development of rat testis after a single subcutaneous administration of 2 mg butyl-paraben/kg/day for 17 days (postnatal days 2-18). Also tested were diethylstilbestrol ethinyloestradiol bisphenol A, genistein, octylphenol **NOEL = 2 mg/kg bw/day**	Fisher *et al.* 1999

*) reproductive assessment by continuous breeding

The Applicant provided extensive argumentation against the use of the PoD selected in the former SCCS opinion on parabens (SCCS/1514/13). The PoD was based on the study by Fisher *et al.* (1999). The arguments were taken from the 2019 CIR report on the safety of parabens and were summarised as follows:
"i) this study involves a subcutaneous route of exposure, which results in chemicals circumventing the physiological barriers and bypassing the portal of entry metabolism, and therefore this route is not relevant to real life cosmetics use;
ii) this study is not an OECD Test Guideline study (eg, the butylparaben-treated group contained only 3 rats and the control group contained only 5 rats);
iii) only one postpartum dose at 2 mg/kg bw/day was tested;

iv) male rats were exposed to butylparaben postnatally, which did not examine the inter-generation toxicity (eg, a more robust study design should involve gestational exposure of paraben to pregnant rats while examining toxicity in the male offspring); and
v) typical DART end points were not covered, such as AGD, PPS (preputional separation), weight of the epididymis and seminal vesicle, sperm counts, reproductive hormone levels, and so on."

SCCS comment
The SCCS agrees with the provided limitations of the Fisher *et al.* (1999) study for use in the risk assessment of cosmetics, taken from the CIR (2019) report. The former SCCS opinion on parabens (SCCS/1514/13) could not determine an adequate NO(A)EL-value for the paraben esters under consideration from the studies listed in Appendix 1 of SCCS/1514/13. Consequently, the NOEL value of 2 mg/kg bw/day, based on Fisher *et al.* (1999), was determined to be a conservative choice for the calculation of the MoS of propyl- and butylparaben. The Committee acknowledged the fact that the Fisher *et al.* (1999) study involves subcutaneous instead of oral administration but emphasised that 2 mg/kg bw/day clearly represents a NOEL instead of a NOAEL.

Further reasoning by the Applicant:
Further argumentation was provided by the Applicant for each study included in **Table 5.**
An academic study in mice by Kang *et al.* (2002) investigated the effects of butylparaben in F1 male offspring after pregnant females were subcutaneously injected with 100 or 200 mg/kg/day. This too was not an OECD Test Guideline study nor a comprehensive assessment of reproductive and developmental effects. As noted above, some observations relating to sperm effects and male reproductive organ weights were noted at both doses but without evidence of a clear dose-response, and a conclusive PoD could not be obtained.

Preliminary academic studies by Oishi (2001) in rats and Oishi (2002) in mice noted above, initially indicated the possibility of effects on male sperm, and were non-GLP and non OECD Test Guideline studies. These early research studies were the stimulus for further comprehensive investigations on reproductive and developmental toxicity performed by Daston (2004), Hoberman *et al.* (2008) and Shaw and deCantazaro *et al.* (2009).

Daston (2004) performed a quality reproductive and developmental study in rats. Sprague-Dawley rats were given butylparaben in 0.5% carboxymethylcellulose by oral gavage at dose levels of 0, 10, 100, or 1,000 mg/kg bw/day on gestation days (GD) 6–19 (sperm positive day GD 0). A range of parameters for female reproductive effects and developmental effects in males and females were investigated. There were no reproductive or developmental effects observed up to 1000 mg/kg bw/day. The highest dose level of 1000 mg/kg bw/day produced decreases in maternal weight gain during some of the treatment intervals (reaching statistical significance during the gestation day 18–20 interval), as well as a significant decrease in food consumption measured over the entire 15-day treatment period. However, the observed decreases in maternal weight gain did not follow a dose response. A benchmark dose model was further submitted by the applicant, using the data to illustrate this point. **The NOAEL for adult females from this study was taken as 1000 mg/kg/day and the developmental NOAEL was also 1000 mg/kg/day.** The observations in Daston (2004) were confirmed in mice by Shaw and deCantanzaro (2009), who saw no effects on female reproductive parameters at subcutaneous doses of up to 949 mg/kg bw/day and no effects in an uterotrophic assay conducted in two different strains of mice.

Hoberman *et al.* (2008) specifically investigated the putative effects on male reproduction and sperm in rats, in a study designed with the aim of reproducing the observations seen by Oishi (2001). The dosing regimen, dosing period and diet were replicated, as the dosing period represents a critical window of development. The robustness of the study was

improved in comparison to Oishi (2001) by performing the study to GLP, improving the statistical analysis and by including additional reproductive endpoints that would be informative about mode of action. Rats were observed for mortality at least twice a day, a full range of clinical and general observations were made daily. Blood samples were taken weekly for the assessment of hormones and haematological parameters. Gross necropsy was performed at termination and male organs and reproductive glands (liver, adrenal glands, thyroid, pituitary, right and left testes, right and left epididymis, seminal vesicles, and prostate) were weighed and retained for histology. Sperm concentration and motility was evaluated. There were no effects seen up to the highest dose tested of 1000 mg/kg bw/day in any of the parameters measured. Body weight increased as per the control group over the duration of the study. There were no effects on male sperm motility, count or daily sperm production. There were no adverse histological findings in any of the organs and glands tested. **The NOEL in this study was 1000 mg/kg bw/day.** To add further confidence that there are no reproductive or developmental effects for butylparaben, a large multigenerational reproductive assessment by continuous breeding study was performed by the US NTP (live phase completed in 2011), as published by Hubbard *et al.* (2020). The Applicant considered this the pivotal study to derive the POD for the safety evaluation and is detailed below.

Pivotal Study – Hubbard *et al.* 2020

A multigenerational reproductive assessment by continuous breeding (RACB) study design using a multiple breeding approach was performed (according to the methods described in Chapin & Sloane, 1996). Sprague Dawley rats, F0 (aged 11 weeks, n =22/sex/group) and F1c parental (F1cP) animals (aged 12-13 weeks, n =26-40) were dosed with ≥99.7% pure butylparaben (CAS 94-26-8) in feed daily. Animals were exposed to butylparaben via supplementation in NIH-07 powdered feed at levels of 0, 5000, 15000, or 40000 ppm (Zeigler Brothers, Inc., Gardners, PA). Exposure started with the F0 generation and continued through the F1 and F2 generations. F0 adults were exposed to butylparaben during a 2-week pre-breed exposure period, during cohabitation, and gestation and lactation for the F1a, F1b, and F1c generations, until necropsy. The F1c generation was exposed throughout life. The F2c generation was exposed to butylparaben via the mother during gestation and lactation until study completion on PND 21. Multiple successive pairings (3 per generation) in both the F0 and F1 generations are conducted to evaluate the potential for any butylparaben-induced reproductive toxicity. In this design, the successive number of matings and evaluation of offspring provides increased statistical power to identify test article related toxicities compared to standard multigeneration studies. Additionally, maturation of F1c offspring to adulthood allows for the evaluation of the potential attenuation (or enhancement of) test article related effects on fertility and fecundity. Body weights and feed consumption were measured throughout the study (pre-cohabitation, cohabitation, gestation, lactation) and used to calculate chemical consumption (mg/kg/day). All assessed sperm parameters, including testicular spermatid count, motility, and caudal sperm count were unaffected by dietary BP at daily exposures in excess of 300 mg BP/kg/day.

No histological findings and only sporadic weight effects were noted in assessed male reproductive organs in exposed groups. No effects on reproductive performance (*e.g.* mating or litter parameters) of the F0 or F1c were associated with butylparaben-exposure. Following necropsy, the liver was identified as the primary target organ of butylparaben toxicity. Increased incidence of mononuclear cell infiltration was the only dose related microscopic finding identified in F1cNP interim animals, suggesting onset of other hepatic lesions may require a longer duration of exposure.

There was no evidence of butylparaben-induced endocrine activity-related developmental or reproductive toxicity following dietary exposure up to 40,000 ppm (approximately 3,000-7,000 mg/kg/day). Butylparaben-exposure was not associated with adverse alterations of fertility, fecundity, pubertal attainment, or reproductive parameters in F0, F1, or F2 generations. No findings were observed that would support the purported

mechanism of butylparaben-induced endocrine disruption in perinatally-exposed rodents. Following necropsy, the liver was identified as the primary target organ of butylparaben toxicity due to dose related increases in relative liver weight and increased incidences of non-neoplastic liver lesions, which may be considered secondary to sustained adaptive liver responses as a result of developmental long-term exposure to butylparaben. In F0 rats, minimal liver effects including minimal evidence of inflammatory mononuclear cell infiltrates and minimal hypertrophy of the periportal hepatocytes started at 15,000 ppm (equivalent to intakes ranging from 1000 – 2000 mg/kg/d), no effects were seen at 5,000 ppm. In F1 female rats, minimal periportal hepatocyte hypertrophy started at 5,000 ppm (equivalent to intakes ranging from 325-740 mg/kg/day), no increased incidence was seen in males at this dose.

The observed minimal periportal hepatocyte hypertrophy at 5,000 ppm in F1 animals in one sex only (females) without any relevant increase of liver weight is not considered adverse at this stage but a typical adaptive effect secondary to a sustained adaptive liver response as a result of developmental long-term exposure and increased metabolism. This is supported by numerous publications such as by the European Society of Toxicologic Pathology (ESTP), stating that hepatocellular hypertrophy without histologic or clinical pathology alterations indicative of liver toxicity is considered an adaptive and a non-adverse change (in the absence of overt adverse changes such as inflammation, necrosis, or degeneration) (Hall *et al.* 2012). The adaptive liver response is directed toward maintaining homeostasis through modulation of various cellular and extracellular functions. At all levels of organisation, these adaptive responses are beneficial in that they enhance the capacity of all units to respond to chemical induced stress, are reversible and preserve viability (Williams & Iatropoulos, 2002). In addition, experts of the EU Human Health Working Group agreed that hepatocellular hypertrophy leading to less than 15% increased mean absolute or relative liver weight, should not be regarded as adverse, and should not be used for the purpose of defining the LOAEL for that specific study, in the demonstrated absence of other histopathological findings such as necrosis, inflammation, fibrosis, vacuolation, pigmentation, degeneration, hyperplasia, or other effects that are indicative of specific liver toxicity. The highest dose at which only such non-adverse changes occur should be identified as the NOAEL.

Therefore, the NOAEL from this study, based on liver effects in adult females, is defined at 5,000 ppm (325 – 740 mg/kg/day). The lower value of 325 mg/kg bw/day was selected as a conservative PoD.

To ensure that the lack of any adverse findings in offpring in the RACB study was not due to insufficient test article exposure, a study was performed (live phase completed in 2012) to assess exposure and effects in pups.

Roberts *et al.* (2016) – A Supporting study to Hubbard *et al.* 2020

The objective of this investigation was to elucidate the extent to which butylparaben can be transferred to offspring during gestation and lactation as well as understand the development of the F1 metabolic capabilities as they relate to dietary butylparaben exposure. Overall, this study provides preliminary data that ensures satisfactory internal exposures of test material would be achieved in pups during critical windows of development in Hubbard *et al.*, 2020.

The dosing regimen and test conditions were identical to those described above for Hubbard *et al.* (2020). Total butylparaben exposure to offspring via placental and lactational transfer was low compared to maternal levels. However, the percent of free butylparaben was higher in fetuses and pups compared to dams, with the level of free butylparaben in pup plasma exceeding that of dams at most time points during lactation. During lactation, prior to direct pup feeding, total butylparaben exposure in pups was very low. However, poor conjugation of butylparaben in pups resulted in higher exposure to free butylparaben compared to that in dams. This was attributed to differential conjugation via the differential expression of UGT and SULT enzymes in pups vs dams. The average percent

of free butylparaben in dam plasma at all time-points and exposure concentrations was less than 1%, with more than 99% conjugated.
This study confirms that both dams and pups in Hubbard *et al.* 2020 were exposed to butyl- paraben and 3-hydroxy butylparaben (3-OH BP), albeit at low levels, at the measured time points. Pups were more exposed to free unmetabolised butylparaben than F0 animals, according to plasma measures but this did not lead to any adverse effects. This study did not provide mass balance information or absolute quantitative data on the amount of test material absorbed but was confirmatory that internal exposures had been affected. Moreover, despite the higher internal exposure to free butylparaben in the pups compared to that in the dams there was no evidence of greater vulnerability of the pups with respect to eliciting any adverse effects and there were no effects related to any endocrine mechanism of action.

Conclusions from reproductive and developmental studies by the Applicant
A Reproductive Assessment by Continuous Breeding (RACB) study performed to GLP acts as **the pivotal study for butylparaben safety evaluation** (Hubbard *et al.* (2020) and confirms that butylparaben is not a reproductive or developmental toxicant, supporting the previous findings of Daston (2004), Hoberman *et al.* (2008) and Shaw & deCantazaro *et al.* (2009). A supporting study in Roberts *et al.* (2016) confirms that pups and dams in Hubbard *et al.* (2020) were sufficiently exposed to parent butylparaben in the RACB study and there were no effects in pups. Some adaptive effects in the liver were seen in female adults, and some non-neoplastic lesions, suggesting the liver as a potential target organ of toxicity at high dose. Based on the observed effects in liver in Hubbard *et al.* (2020), **a conservative NOAEL of 325 mg/kg/day can be used in a safety evaluation.**

SCCS comment
The SCCS has closely looked to the argumentation of the applicant with respect to the *in vivo* reproductive and developmental studies available to determine a suitable NOAEL value and agrees with a conservative NOAEL value of 325 mg/kg bw/d.

3.4.6 Mutagenicity / genotoxicity

3.4.6.1 Mutagenicity / genotoxicity *in vitro*

The applicant provided an overview of the available *in vitro* mutagenicity/genotoxicity studies on butylparaben, see **Table 7.**

Table 7: *In vitro* assays for butylparaben

Methods	Test Article	Results	Reference
Bacterial gene mutation assays			
S.typhimurium TA97, TA98, TA100, TA1535; OECD Test Guidelines Ames Test	1, 3, 10, 33, 100, 166, 333, 1000, 3333 µg BP/plate	Non-mutagenic (with and without S9)	NTP (2018)

S. typhimurium TA92, TA94, TA98, TA100, TA1535, TA 1537, TA2637; reverse mutation	1000 mg BP/plate (5.148 mmol/plate)	Non-mutagenic (-S9)	Ishidate *et al.* (1984); as cited by WHO JECFA 2001.
S. typhimurium TA98, TA100; reverse mutation	1000 mg BP/plate (5.148 mmol/plate)	Non-mutagenic (-S9)	Haresaku *et al.* (1985); as cited by WHO JECFA 2001.
Mammalian cell assays			
In vitro chromosome aberration assay; Chinese hamster cells	0.06 mg BP/ml (308 µM – maximum tolerated dose)	Mutagenic (1–3% increases in polyploid cells)	Ishidate *et al.* (1978); as cited by CIR 2008.
In vitro chromosome aberration assay; Chinese hamster ovary cells	60 mg BP/ml (308 mM)	Non-mutagenic	Ishidate *et al.* (1984); as cited by WHO JECFA 2001.
In vitro chromosome aberration assay; Chinese hamster cells	0.75 mM BP (equivalent to 0.146 mg/ml)	Mutagenic	Tayama *et al.* (2007)

3.4.6.2 Mutagenicity / genotoxicity *in vivo*

The Applicant provided an overview of the available *in vivo* mutagenicity/genotoxicity studies on butylparaben, see **Table 8.**

Table 8: *In vivo* assays for butylparaben

Methods	Test Article	Results	Reference
DNA migration; Mouse ddY n=4 per dose (Comet Assay)	Oral dose: 2000 mg BP/kg (10.3 mmol/kg); >98% pure	Negative: No DNA damage was seen in stomach, colon, liver, kidney, bladder, lung, brain and bone marrow 3 and 24 hours after treatment. There were no deaths, morbidity or adverse clinical signs.	Sasaki *et al.* (2002)

Conclusion on genotoxicity by the Applicant:
Based upon the body of evidence, butylparaben (and all parabens) has been considered for many years to be 'not mutagenic' and there is no new evidence to suggest otherwise.

SCCS comments
Based on a systematic study of the scientific literature (see Appendix 2), the SCCS noted that the studies/ references shown by the Applicant did not cover the entire field available in the scientific literature.

The SCCS does not agree with the conclusions made by the applicant. **A complete analysis of the scientific literature was done by the SCCS.**

The conclusions of this analysis were:

1. Butylparaben was tested on *S. typhimurium* TA92, TA94, TA97, TA98, TA100, TA1535, TA1537 strains in 2 studies with negative results. However, the SCCS noted that 1 strain combination recommended by the OECD TG 471 (Adopted: 21 July 1997 Corrected 26 June 2020) has not been represented (E. coli WP2 uvrA, or E. coli WP2 uvrA (pKM101), or S. typhimurium TA102). These are sensitive strains for a variety of oxidative agents and crosslinking agents. As it is known that the *S. typhimurium* strains tested in the available studies may not detect these types of mutagens, **the SCCS is of the opinion that, unless documented negative results are available to the SCCS, a valid Ames test with the previously lacking bacterial strain combination should be provided**.
2. No data on ***in vitro* mammalian gene mutation** tests have been found in the open literature.
3. Butylparaben has been tested using ***in vitro* chromosomal aberration/ micronucleus** tests on human peripheral blood leukocytes in one study of high relevance with a positive result, in two studies of limited relevance on Chinese hamster fibroblast cells with a negative and on human blood leukocytes with an equivocal result, and in four studies of low relevance with inconclusive (MCF-10A (human breast epithelial cells, MCF-7 and MDA-MB-231 human breast cancer cells), an equivocal (human blood leukocytes, CHO-K1 cells) or inconclusive (human blood leukocytes) results. In one study on Chinese hamster cells, important details were not available to the SCCS to assess the study.
   It is not possible to draw firm conclusions from the available study results in the open literature on *in vitro* chromosomal aberrations/*in vitro* micronucleus endpoint with butylparaben. Hence, a valid study on the chromosomal aberration endpoint with butylparaben should be provided.
   This is particularly important considering that no valid *in vivo* micronucleus/ chromosomal aberration study with butylparaben is available.
4. Butylparaben was tested using an ***in* vitro Comet assay** in one study of high relevance with a negative result (HaCaT and SVK14 human keratinocytes); in three studies of limited relevance with a positive result (CHO-K1 cells), weakly positive (human lymphocytes), or a negative result (MCF-10A, MCF-7 and MDA-MB-231 cells); in one study of low relevance which could not be assessed because of insufficient information.
   None of the studies were conducted according to GLP status. The results can only be considered as supportive in the overall WoE; however, they suggest a DNA-damaging potential of butylparaben.
5. Butylparaben was tested using an *in vitro* sister chromatid exchange test in one study of high reliability on human leukocytes with a positive result and in one study of limited reliability on CHO-K1 cells with an equivocal result.
   None of the studies were conducted according to GLP status. The results can only be considered as supportive in the overall WoE; however, they suggest a DNA damaging potential of butylparaben.
6. Butylparaben was tested using *in vitro* human sperm cells with an Oxy-DNA kit designed to detect 8-hydroxy-deoxyguanosine levels. However, because the quality

of the test cannot be assessed, the results have not been taken into consideration during WoE analysis of genotoxicity.

7. Data on ***in vivo*** **chromosome aberrations/ micronucleus** tests with butylparaben have not been found in the open literature.
8. Data on ***in vivo*** **mammalian gene mutation** tests with butylparaben have not **been found in the open literature.**
9. **Butylparaben was tested in Comet assay after oral administration in two studies of limited relevance with positive results (human sperm cells and rat blood leukocytes and hepatocytes); in two studies of low relevance which could not be assessed because of insufficient information or with an inconclusive result (cells from glandular stomach, colon, liver, kidney, urinary bladder, lung, brain, and bone marrow).**
   Based on the available study results on the *in vivo* comet assay with butylparaben (Table 8) a DNA damaging effect cannot be excluded.

In summary, the SCCS was of the opinion that a valid Ames test with the OECD 471 recommended bacterial strain combination should be provided. Furthermore, the available study results in the open literature on *in vitro* chromosomal aberrations/micronucleus endpoint with butylparaben did not allow drawing firm conclusions. Hence, a valid study on chromosomal aberration with butylparaben was requested.

Genotoxicity endpoint	Gene mutations		Micronucleus test	Chromosomal aberration test
	in bacteria	in mammalian cells		
In vitro	Inconclusive One OECD 471 recommended tester strain combination was not used.	/	Inconclusive	Inconclusive
In vivo	/	No relevant data available	No relevant data available	No relevant data available
Overall conclusion on genotoxic hazard	**Unless a documented negative result is available to the SCCS, a valid Ames test with lacking OECD 471 recommended bacterial strain combination should be provided.**		**Hazard cannot be excluded - A valid *in vitro* micronucleus/chromosomal aberration study should be provided**.	

New studies on Ames test and micronucleus test *in vitro* were received from the Applicant (in December 2022/January 2023) in response to the SCCS preliminary conclusion on genotoxicity of butyl 4-hydroxybenzoate (= butylparaben).

Gene mutation assay using bacteria

Guideline:	OECD 471
Test system:	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, *Escherichia coli* WP2 uvrA
Replicates:	Three separate experiments, triplicate plates
Test substance:	Butyl 4-hydroxybenzoate, CAS number 94-26-8
Batch:	BCCF8282
Purity:	The Purity was stated as 100%, but no retest or expiry date was provided for the use of batch BCCF8282 in the study. Therefore, the purity of the test article was determined after the completion of the experimental phase. The purity was determined to be 99.9%. Since there was no significant loss in purity between the quality release date provided by the supplier and the analysis conducted, the test article was considered stable and suitable for use in this study.
Concentrations:	Experiment 1 (range finding) – plate incorportion test: ±S9 mix (β-Naphthoflavone/Phenobarbital-induced rat liver post-mitochondrial fraction): all tester strains: 5, 16, 50, 160, 500, 1600 and 5000 µg/plate Mutation Experiment 2– pre-incubation step: -S9: all strains: 75 - 1500 µg/plate +S9 (): all strains: 75 - 1500 µg/plate Mutation Experiment 3 – pre-incubation step: -S9: TA98: 5-150 µg/plate; TA100, TA1535, TA1537, WP2 uvrA: 5-300 µg/plate +S9: TA98, TA100 and WP2 *uvrA*: 5-600 µg/plate; TA1535 and TA1537: 5-300 µg/plate
Vehicles:	stock solutions were prepared by formulating Butyl 4-hydroxybenzoate under subdued lighting in DMSO (dimethyl sulphoxide), with the aid of vortex mixing, to give the maximum required treatment concentration. Subsequent dilutions were made using DMSO.
Positive Controls:	-S9 mix: 2-nitrofluorene (2NF): 5 µg/plate for TA98; sodium azide (NaN_3): 2 µg/plate for TA100, TA1535; 9-aminoacridine (AAC): 50 µg/plate for TA1537; 4-nitroquinoline 1-oxide (NQO) 2 µg/plate for WP2 *uvrA* +S9 mix: 2-Aminoanthracene (AAN): 5 µg/plate for TA98, TA100 and TA1535 or 15 µg/plate for WP2 *uvrA*; benzo[a]pyrene (B[a]P): 10 µg/plate for TA98
Negative controls:	Vehicle control with DMSO
GLP:	In compliance
Study period:	Study Initiation Date: 19 October 2022; Study Completion Date: 13 December 2022

Material and methods

Butyl 4-hydroxybenzoate was assayed for mutation in four histidine-requiring strains (TA98, TA100, TA1535 and TA1537) of *Salmonella typhimurium*, and one tryptophan-requiring strain (WP2 uvrA) of *Escherichia coli*, both in the absence and in the presence of metabolic activation by a β-Naphthoflavone/Phenobarbital-induced rat liver post-mitochondrial fraction (S-9), in three separate experiments.

All Butyl 4-hydroxybenzoate treatments in this study were performed using formulations prepared in anhydrous analytical grade DMSO.

Results
Mutation Experiment 1 treatments of all the tester strains were performed in the absence and in the presence of S-9, using a plate-incorporation method at final Butyl 4-hydroxybenzoate concentrations of 5, 16, 50, 160, 500, 1600 and 5000 µg/plate, plus vehicle and positive controls. Following these treatments, evidence of toxicity was observed at 1600 µg/plate and above in all strains in the absence and presence of S-9.
Mutation Experiment 2 treatments of all the tester strains were performed in the absence and in the presence of S-9. The maximum test concentration was reduced to 1500 µg/plate based on toxicity observed in Experiment 1. Narrowed concentration intervals were employed covering the range 75-1500 µg/plate, in order to examine more closely those concentrations of Butyl 4-hydroxybenzoate approaching the maximum test concentration and considered therefore most likely to provide evidence of any mutagenic activity. In addition, all treatments were further modified by the inclusion of a pre-incubation step. Following these treatments, evidence of toxicity ranging was observed at 150 or 300 µg/plate and above in all strains in the absence of S-9 and at 300 µg/plate and above in all strains in the presence of S-9.
Due to excessive toxicity resulting in insufficient analysable concentrations for all strain treatments in Mutation Experiment 2, a third experiment (Mutation Experiment 3) using pre incubation methodology was performed. Treatment concentrations of 5-150 µg/plate (strain TA98 in the absence of S-9), 5-300 µg/plate (strains TA100 and WP2 uvrA in the absence of S-9 and strains TA1535 and TA1537 in the absence and presence of S-9) or 5-600 µg/plate (strains TA98, TA100 and WP2 uvrA in the presence of S-9) were employed. Following these treatments, evidence of toxicity was observed at 150 and/or 300 µg/plate in all strains in the absence of S-9 and in strains TA1535 and TA1537 in the presence of S-9, and at 300 µg/plate and above in strains TA98, TA100 and WP2 uvrA in the presence of S-9.
No precipitation was observed following Mutations Experiments 1, 2 and 3.

Vehicle and positive control treatments were included for all strains in both experiments. The numbers of revertant colonies were comparable with acceptable ranges for vehicle control treatments, and were elevated by positive control treatments.
Following Butyl 4-hydroxybenzoate treatments of all the test strains in the absence and presence of S-9, no increases in revertant numbers were observed that were ≥2 fold (in strains TA98, TA100 and WP2 uvrA or ≥3-fold (in strains TA1535 and TA1537) the concurrent vehicle control. This study was considered therefore to have provided no evidence of any Butyl 4-hydroxybenzoate mutagenic activity in this assay system.

Conclusion by the Applicant
It was concluded that Butyl 4-hydroxybenzoate did not induce mutation in four histidine-requiring strains of Salmonella typhimurium (TA98, TA100, TA1535 and TA1537) and one tryptophan-requiring strain of Escherichia coli (WP2 uvrA) when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines and a toxic concentration), in the absence and in the presence of a rat liver metabolic activation system (S-9).

SCCS comment
The results of the study indicate no mutagenic effect of butylparaben in the bacterial gene mutation endpoint.
Labcorp Early Development Laboratories Ltd., Butyl 4-hydroxybenzoate: Bacterial Reverse Mutation Assay, December 2022

***In vitro* Cytokinesis-block Micronucleus Test in human lymphocytes**

Guideline:	OECD 487 (draft approved April 2014)
Species/strain:	Cultured human peripheral blood lymphocytes pooled from two donors (F/M)
Replicates:	Duplicate cultures, one experiment
Test substance:	Butyl 4-hydroxybenzoate CAS number 94-26-8
Batch:	BCCF8282
Purity:	The purity was stated by the Applicant to be 100%; Abatch BCCF8282 was stored at 15 to 25°C, protected from light. As no retest or expiry date was provided, the purity of the test article was determined after the completion of the experimental phase of thie study. The purity was determined to be 99.9%. Since there was no significant loss in purity between the quality release date provided by the supplier and the analysis conducted, the test article was considered stable and suitable for use
Concentrations:	Preliminary test (range-finder): ±S9 (β-Naphthoflavone/Phenobarbital-induced rat liver post-mitochondrial fraction): mix (3 h exposure + 17 h): 3.79 to 1942.3 µg/mL -S9 mix (20 h exposure): 3.79 to 1942.3 µg/mL Micronucleus Experiment: ±S9 mix (3 h exposure + 17 h): 47.83 to 200 µg/mL -S9 mix (24 h exposure): 1.27 to 47.83 µg/mL Additional Micronucleus Experiment: ±S9 mix (3 h exposure + 17 h): 30.5 to 150 µg/mL
Solvent/negative control:	culture medium
Positive Controls:	-S9 mix: Mitomycin C (MMC, 0.3 and 0.1 µg/mL); Colchicine (COL, 0.06, 0.07, 0.015, 0.02 µg/mL) +S9 mix: Cyclophosphamide (CP, 10 µg/mL)
Vehicle:	Test article stock solutions were prepared by formulating Butyl 4-hydroxybenzoate (butyl paraben) in DMSO to give the maximum required treatment concentration. Subsequent dilutions were made using DMSO.
GLP:	In compliance
Study period:	Study Initiation Date: 19 October 2022; Study Completion Date: January 2023

Material and methods

Butylparaben was tested in an *in vitro* micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two adult donors in a single experiment. Treatments covering a broad range of concentrations, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S-9) from β Naphthoflavone/Phenobarbital-induced rats. The test article was formulated in dimethyl sulphoxide (DMSO) and the highest concentrations tested in the Micronucleus Experiment (limited by toxicity), were determined following a preliminary cytotoxicity Range-Finder Experiment. For each treatment, three concentrations were selected for

micronucleus analysis, such that a range of cytotoxicity from maximum (55±5%) to little or none was covered.
Treatments were conducted (as detailed in the following summary table) 44-48 hours following mitogen stimulation by phytohaemagglutinin (PHA). Cytochalasin B, formulated in DMSO was added directly (0.05 mL per culture) to all continuous cultures at the time of treatment to give a final concentration of 6 µg/mL per culture. The test article concentrations for micronucleus analysis were selected by evaluating the effect of butyl paraben on the cytokinesis-block proliferation index (CBPI). A minimum of one thousand binucleate cells from each culture (2000 per concentration, 4000 for the vehicle control) were analysed for micronuclei.

Results
Micronuclei were analysed at three concentrations and a summary of the data is presented in the following table:

Treatment	Concentration (µg/mL)	Cytotoxicity (%) $	Mean MN Cell Frequency (%)	Historical Control Range (%) #	Statistical Significance
3+17 –S-9	Vehicle (a)	-	0.55	0.20 to 1.00	-
	61	6	0.35		NS
	126	33	0.50		NS
	142	52	0.25		NS
	*MMC, 0.3	52	2.55	1.50 to 6.08	p≤0.001
	*COL, 0.07	39	2.45	1.48 to 3.70	p≤0.001
3+17 +S-9	Vehicle (a)	-	0.28	0.20 to 1.10	-
	61	0	0.35		NS
	130	27	0.30		NS
	150	50	0.20		NS
	*CPA, 10	33	1.75	1.21 to 2.59	p≤0.001
20+0 –S-9	Vehicle (a)	-	0.93	0.20 to 1.00	-
	1.27	0	0.70		NS
	25.42	35	0.75		NS
	28.24	51	0.75		NS
	*MMC, 0.1	13	2.80	1.35 to 3.65	p≤0.001
	*COL, 0.02	16	1.65	1.20 to 2.36	p≤0.01

a	Vehicle control was DMSO
*	Positive control
#	95th percentile of the observed range
$	Based on CBPI
MN	Micronucleated
NS	Not significant

Appropriate negative (vehicle) control cultures were included in the test system under each treatment condition. The proportion of micronucleated binucleate (MNBN) cells in the vehicle cultures fell within the 95th percentile of the current observed historical vehicle control (normal) ranges. In the Micronucleus Experiment Mitomycin C (MMC) and Colchicine (COL) were employed as clastogenic and aneugenic positive control chemicals, respectively, in the absence of rat liver S-9. Cyclophosphamide (CPA) was employed as a clastogenic positive control chemical in the presence of rat liver S-9. Cells receiving these were sampled in the Micronucleus Experiment at 20 hours after the start of treatment. All positive control compounds induced statistically significant increases in the proportion of cells with micronuclei.
All acceptance criteria were considered met and the study was therefore accepted as valid.
Treatment of cells with butylparaben in the absence and presence of S-9 resulted in frequencies of MNBN, which were similar to and not significantly (p≤0.05) higher than those observed in concurrent vehicle controls for all concentrations analysed (all treatments) with no indication of any concentration related effect (non-significant linear

trend tests). The MNBN cell frequency of all butylparaben treated cultures fell within normal ranges.

CONCLUSION by the Applicant
It is concluded that butylparaben did not induce micronuclei in cultured human peripheral blood lymphocytes when tested up to its limit of cytotoxicity, in both the absence and presence of S-9.

SCCS comment
The SCCS noted rather poor response of the test system after exposure to Colchicine in the Micronucleus test 20+0 h treatment –S9 (the MN frequency was below 2-fold increase). The SCCS is the opinion that butylparaben does not induce mutations in micronucleus test in human lymphocytes when tested under the conditions of this study (Labcorp Early Development Laboratories Ltd.,2023).

Based on the analysis of available data of genotoxicity and mutagenicity of butylparaben, the SCCS is of the opinion that it can be considered to have no genotoxic potential.

3.4.7 Carcinogenicity

Review provided by the Applicant
The applicant notes that academic research raised suspicions in the previous decade about the presence of butylparaben in breast tissue and it was further questioned whether parabens had a role in breast cancer (Darbre, 2004). Golden, Gandy & Vollmer (2005) effectively highlighted the limitations in the work. The SCCS (SCCP/0874/05 opinion) addressed parabens and breast cancer "Extended Opinion on parabens, underarm cosmetics and breast cancer" and concluded that 'according to the current knowledge, there is no evidence of a demonstrable risk for the development of breast cancer caused by the use of underarm cosmetics.' No further evidence exists that would lead to the need to review this opinion.

In rats, butylparaben ester (0.6 or 1.2%) in the diet for up to 104 weeks did not produce any carcinogenic effect. Butylparaben also showed no enhancing or inhibitory effects on the development of preneoplastic glutathione S-transferase placental form-positive (GST-P^+) foci in the liver of rats (Matthews *et al.*, 1956).

In eight-week-old female and male ICR/Jcl mice, oral administration of butylparaben (0.15, 0.3, or 0.6%) in the diet for up to 102 weeks produced neoplasms in the hematopoietic system, including thymic lymphoma, non-thymic lymphoid leukemia, and myeloid leukemia. Additionally, a moderately high incidence of lung adenomas and adenocarcinomas and of soft tissue myosarcomas and osteosarcomas were found. Tumor incidences, however, were not significantly different from those of the control group (Inai *et al.*, 1985). EFSA (2004) judged this study to be inadequate due to excessive mortality in both the control and treated groups and high tumour incidences in the control group.

Negative results were also reported in another study in mice using the same doses but for a 106-week treatment time (Odashima, 1980). In the rat, butylparaben (0.6 or 1.2%) in the diet for up to 104 weeks did not produce any carcinogenic effects (Odashima, 1980).

Conclusion on carcinogenicity by the Applicant:
There is no evidence of butylparaben acting as a carcinogen.

SCCS comments
The SCCS carried out an analysis of the data available in the scientific literature (**Appendix 2, Summary Table 2.7**). Apart from some limited data, no solid evidence of butylparaben acting as a carcinogen was found.

3.4.8 Photo-induced toxicity

Photo-contact sensitisation and phototoxicity tests on product formulations containing 0.1 to 0.8% methylparaben, propylparaben, and/or butylparaben gave no evidence for significant photoreactivity (CIR, 2019).

Conclusion on phototoxicity by the Applicant:
Butylparaben is not phototoxic.

3.4.9 Human data

The applicant notes that human biomonitoring data are potentially useful in understanding whether exposure modelling of substance intake provides overestimates or is realistic. It is further noted that the systemic presence of a particular substance can result to exposure from multiple sources and care must be taken in making direct quantitative comparisons. Such evaluations have been presented and discussed in a paper by Aylward *et al.* (2018), who show that the deterministic approach is typically conservative and overpredicts real-life exposures.

Health Canada have drawn upon human biomonitoring data to calculate estimated daily intakes in their draft safety evaluation for butylparaben (Health Canada, 2020) (see **Table 9**). In comparison to the systemic exposure dose (SED) of 84.4 µg/kg bw/day as calculated in **Table 3** above for butylparaben from 17 cosmetic products according to the 11th SCCS Notes of Guidance, these data from Health Canada suggest that real life exposures fall in the range 0.18 – 4.4 µg/kg bw/day, indicating that the value used in the safety evaluation used in the dossier submitted by the Applicant is conservative.

Table 9: Estimated daily intakes of butylparaben based on biomonitoring data (from Health Canada)

Source, Location	Age (years)[a]	CER (mg/day)[b]	UC_{Cr}, P95 (CI) (µg/g Cr)[c]	FUE[d]	EDI, P95 (CI) (µg/kg bw/day)
CHMS (Cycle 4, 2014-2015)[c], Canada	3–5	130	3.1 (<LOD–5.1)[e]	0.056	0.46 (NC–0.8)
CHMS (Cycle 4, 2014-2015)[c], Canada	6–11	418	0.8 (0.3–1.3)[e]	0.056	0.19 (0.07–0.31)
CHMS (Cycle 4, 2014-2015)[c], Canada	12–19	1182	9.2 (<LOD–15)[f]	0.056	3.3 (NC–5.3)
CHMS (Cycle 4, 2014-2015)[c], Canada	20–59[g]	1248	9.2 (<LOD–15)[f]	0.056	2.9 (NC–4.72)

CHMS (Cycle 4, 2014-2015)[c], Canada	60–79	1017	6.7 (2.1–11)[e]	0.056	1.7 (0.53–2.8)
Fisher et al. 2017, Canada	Pregnant women	-	12.20[h]	0.056	4.4
Kang et al. 2013, Korea	Neonates	9.6	3.4[i]	0.056	0.18

Abbreviations: CER, creatinine excretion rate; UCCr, creatinine-adjusted urinary concentration; P95, 95th percentile; CI, confidence interval; FUE, fractional urinary excretion; EDI, estimated daily intake; NC, not calculated.
a) Age groups are defined based on age groups reported by CHMS (Health Canada 2017a in Health Canada 2020).
b) Creatinine excretion rate was calculated using the Mage equation [0.993*1.64 [140 – Age] (Wt^1.5Ht^0.5)/1000]. See Appendix A in Health Canada 2020 for values used for age weight and height.
c) Health Canada 2017a in Health Canada 2020.
d) Moos *et al.* 2016.
e) These values were associated with high sampling variability (*i.e.*, coefficient of variation between 16.6% and 33.3%). Health Canada recommends that this data be used with caution (Health Canada 2017a in in Health Canada 2020).
f) CHMS data for the 95th percentile in this age stratum was suppressed due to high variability and "females 3 to 79" was used as a surrogate. Although the 95th percentile value for this group is not known, this approach is considered conservative because the value used to estimate daily intake is the highest reported 95th percentile value for ethylparaben.
g) The "20-39" and "40-59" age groups are presented together. When 95th percentile values were reported for both age groups, the higher value is presented here; when one value was suppressed, the value from the other group is presented here.
h) This value is the specific gravity-adjusted urinary paraben concentration (µg/L) at the 95th percentile; creatinine-adjusted values were not reported. Confidence intervals were not reported. EDI was calculated using the following equation: EDI =(UC*UFR)/FUE, where UC is the urinary concentration, UFR is the urinary flow rate (0.20 L/kg bw/day) and FUE is the fractional urinary excretion.
i) This value is the 75th percentile creatinine-adjusted urinary paraben concentration; the 95th percentile was not reported. Confidence intervals were not reported.

Recently, the Human Biomonitoring (HBM) Commission in Germany has defined 'reference values' for parabens (Apel *et al.*, 2017). They state: "In order to be able to describe the background exposure of the population and its temporal development, the German HBM Commission derives reference values by means of statistical methods. These reference values are based on the 95% confidence interval of the 95th percentile of the concentration of a chemical substance in the matrix obtained from a reference population. Preferably, reference values are derived from data obtained from a representative population sample in the context of the German Environmental Survey, GerES. They allow a uniform assessment of the body burden at the German national level, and are indispensable to demonstrate whether a certain exposure level exceeds the background exposure level, *e.g.* accident-related exposures. Because of their statistical nature, reference values cannot serve to assess health risks.

Reference values are checked continuously and are updated if new information becomes available." Therefore, a reference value is not regarded as a safe value in urine, but as a measure to enable human biomonitoring of a substance over time to see how it may change with exposure pattern changes. For butylparaben, the provisional reference value set by the German HBM Commission is 20 µg/L for women and 10 µg/L for men (Apel *et al.*, 2017), reflecting the general difference between men and women in the use of greater personal care products in the latter. Further evidence is detailed in the CIR, 2019), drawn upon the US NHANES program (the Fourth National Report) which provides a large dataset for human spot urine levels of butylparaben, collected from 2005 to 2014, with 2013 - 2014 being the most recent collection period. The US NHANES data also suggests that real-life human exposure to butylparaben is very low; the median concentration in urine was

below the limit of detection (LOD, 0.1 µg/L) for all groups (age, gender, and race/ethnicity) in the 2011 - 2014 reporting period (CIR 2019).

SCCS comment
Biomonitoring data are gaining interest as they provide total values of exposure from different sources. These are, however, not always known. In the SCCS Opinions, usually conservative deterministic data are considered for aggregate MoS calculations.

3.4.10 Special investigations

3.4.10.1 Potential endocrine activity for butylparaben

The Applicant provided the following information:

A few reviews exist in the literature relating to parabens that discuss the potential of the parent paraben substance to be endocrine active (Golden *et al.*, 2005; Boberg *et al.*, 2010; Nowak *et al.*, 2018). A number of *in vitro* and *in vivo* studies have been performed to investigate endocrine activity, explained in more detail below.

i) Endocrine activity *in vitro*
One of the initial ways to begin assessing potential endocrine activity is to test the potential binding of the parent substance with either the estrogen or androgen receptors. These do not include effective metabolism as would be the case *in vivo*, but they can provide an earlier screening indicator as to whether the parent substance could initially bind to a hormone receptor and provide a relative potency measure of binding or substrate inhibition/competition of butyl paraben vs natural substrates.

Overall evaluation of Level 2 studies:
Butylparaben has been further investigated for estrogenic activity in several subsequent non-guideline in vitro studies in investigative research with a range of inconsistent findings. These studies are listed in **Appendix 1,** in **Table 1.1**

The Applicant is of the opinion that paramount in interpreting the relevance of these *in vitro* data, is that no adverse CMR effects in the intact organism have been seen in a range of GLP *in vivo* studies, where metabolism is functional. Therefore, whilst some *in vitro* assays may show evidence of parent butylparaben binding weakly to the estrogen receptor, no relevant effects in the intact organism *in vivo* arise from an endocrine mode of action.

Observations in studies by Routledge *et al.* (1998) first initiated concerns around the potential for butylparaben to possess endocrine activity. Routledge *et al.* (1998) showed that butylparaben could bind to the estrogen receptor in a yeast-based system but was 8-10,000-fold less potent than the natural endogenous substrate for the ER, 17β-estradiol. Similarly, in a rat estrogen receptor *in vitro* assay, butylparaben showed an affinity that was 5 orders of magnitude lower than the substrate diethylstilboestrol. When following up these *in vitro* observations, with uterotrophic assays, butyl paraben was found to be inactive via the oral route. When injected subcutaneously, bypassing esterase metabolism in the skin to pHBA (Jewell *et al.*, 2007; Hoberman *et al.*, 2008), butylparaben produced a weak response in a uterotrophic assay but was 100,000-fold less potent than 17β-estradiol.

ii) Endocrine activity *in vivo*
All *in vivo* endocrine activity studies in animals are presented in **Appendix 1**, **Table 1.2**
All studies that followed *in vivo* (see **Table 1.2**) confirm the fact that weak estrogenic effects can be seen when butylparaben is injected subcutaneously, but no effects are seen

following oral administration. The subcutaneous route does not reflect real life human exposures orally or dermally, where significant metabolism and clearance of butylparaben is affected via these routes (see section 3.2 on toxicokinetics).

If endocrine activity was a significant mode of action for butylparaben, one would expect to see as a consequence adverse carcinogenic, reproductive or developmental effect in sub-chronic and chronic toxicology studies. As detailed in section 3.4.5, there are robust studies performed to GLP by Daston (2004), Hoberman *et al.* (2008) and the US NTP in 2011 (Hubbard *et al.*, 2020) that show there are no such adverse effects observed *in vivo*. Butylparaben did not cause any reproductive or developmental effects in female or male rats up to a top dose tested of 1000 mg/kg bw/day. Any weak observations via the subcutaneous route in the *in vivo r*odent assays are not relevant to the real-life human exposure situation (as evidenced by data in Janjua *et al.*, 2007).

iii) Observations by Applicant in humans

In a study by Janjua *et al.* (2007), 26 healthy male Caucasian volunteers (21-36 years old; mean = 26 years) had 2% w/v butylparaben applied dermally in a cream. The cream also contained 2% diethyl phthalate and 2% dibutyl phthalate. Topical application of the cream formulation without test substances was performed daily to the whole body at 2mg/cm2 for a week. Cream including the test substances was then applied at the same mass/cm2 for the following week. Concentrations of hormones (FSH, LH, testosterone, estradiol, inhibin B, TSH, FT4, T3 and T4) were measured in the blood. Cream application and blood sampling were done at 0, 24, 96 and 120 hours. There were very minor differences at some time points in serum inhibin B, LH, E2, T4, FT4 and TSH concentrations during the treatment week versus control week. However, they were not treatment related as differences were also seen at t=0 when the treatment had not started and were not statistically or biologically meaningful. This study provides good evidence that hormone levels were not adversely affected by the test substance.

iv) Applicant Conclusions on Endocrine Activity:

The OECD evaluated endocrine activity evidence applying the OECD conceptual framework for endocrine disruptors as follows:

Level 1: Existing data and Non-Test Information (*e.g.*, PC, QSAR, read across)

Level 2: *In vitro* mechanistic assays – *e.g.* receptor binding assays

Level 3: *In vivo* mechanistic assays – *e.g.* uterotrophic assays

Level 4: *In vivo* assays providing data on ED adverse effects in intact organisms

Level 5: *In vivo* assays providing more comprehensive data on ED adverse effects in intact organisms over more extensive parts of the life cycle of the organism

For butylparaben:

Level 1: The chemical structure of butyl paraben **alerts as a phenolic compound** that may theoretically interact with the estrogen receptor in QSAR predictions.

Level 2: Some investigative *in vitro* assays have shown **weak activity for butylparaben** at 10,000 – 100,000-fold lower potency than endogenous substrates such as 17β-estradiol

Level 3**: Some positive responses** have been observed in non GLP uteroptrophic assays in the literature, but only when butylparaben is administered subcutaneously. The subcutaneous route of administration is less relevant to the real biological situation of cosmetic application routes where butylparaben is extensively metabolised orally and dermally.

Level 4: Studies by Daston (2004) and Hoberman *et al.* (2008), whilst showing some non-endocrine mediated general toxicity, showed no adverse effects with respect to carcinogencity, reproductive or developmental toxicity effects. However, these studies did not cover all potential effects, and hence a Level 5 study was ultimately performed as a gold standard evaluation by the NTP (as published in Hubbard *et al.*, 2020).
Level 5: In a well-powered state-of-the-art **GLP** multigeneration study (RACB design) in rats following long-term dietary exposure up to 40,000 ppm (approximately 3,000-7,000 mg/kg/day) (Hubbard *et al.* (2020); studies performed before March 2013), **no evidence of butyl paraben ester induced endocrine disruption or endocrine-mediated adverse effects in the intact organism** was observed.

SCCS comments
An extensive literature search was carried out by the SCCS, and all studies related to the topic and not included yet in the applicant dossier were added to the references list and the **Tables 1.1 ad 1.2.** A number of these were '*in vivo* after deadline' and could as such not be taken up by the Applicant to defend the substance under consideration. These could still be used by the SCCS in safety assessments. In addition, the references that were included in the ECHA SVHC Annex XV report on butylparaben were added to the tables and have also been taken up in the references list. These were also extensively discussed in Annex 3 of Boberg (2020).

Boberg *et al.* (2016) studied the effect of butylparaben on the development of the Wistar rat reproductive system. Rat dams were orally exposed (gavage) to 10, 100 and 500 mg/kg bw/d of butyl paraben (purity > 99%) from gestational day 7 to 21 and from postnatal day 1 to 16 (male offspring) and 17 (female offspring), at a constant administration corn oil volume (2 mL/kg bw/d). The period of exposure was chosen to cover the sensitive window of reproductive development in rat offsprings. The animal strain used was relevant for toxicology purposes and 4 groups of 18 animals were studied. Statistics were well described. The study scored 2 on the Klimisch classification, indicating that the study was of acceptable quality. A number of changes occurred that could be linked to endocrine activity: the **anogenital distance (AGD) was decreased in male rats and the number of sperm in cauda was reduced in all groups.** A dose-response was present. The gene expression analysis showed a down regulation of CYP19a1, but only on D16. Hormone levels remained unchanged (see **Table 1.2**).
The decrease of AGD was considered as the decisive parameter to determine the PoD. Sperm count is seen as a more variable parameter.

Maske *et al.* (2018) reported the results of a study performed on Holtzman male and female rats. Pregnant dams were exposed subcutaneously to 10, 100 and 1000 mg butyl-paraben/kg bw/d from GD6 to the weaning of their pups (PND21). Significant results were observed in the F1 generation in the 10 mg/kg bw group such as a decrease of the pituitary gland (PND30) and hypothalamus weight (PND45), an increase of seminal vesicle weight (PND45), a decrease of the estradiol concentrations in males. The number of seminiferous tubules/testes was also significantly decreased at this dose. A delayed preputial separation was also observed at 10mg/kg bw/d in male rats. In female, these authors reported adverse effects on body weight, adrenal gland weight, hypothalamus weight, pituitary weight, ovary weight, uterus weight, fertility (reduced estrous cycle length), more pronounced at the two highest doses (Maske *et al.*, 2018).

As reported in Boberg *et al.* (2020), the obtained data overview provides useful information for risk assessment purposes. No safe dose (concentration) can be derived from the available data on adverse reproductive effects via endocrine MoA. Two of the available studies show reduced sperm count or quality in perinatally-exposed rats at the lowest

tested dose of 10 mg/kg bw/day with oral and subcutaneous exposure, respectively (Boberg *et al.*, 2016; Guerra *et al.*, 2017b).

Guerra *et al.*(2017a) published results obtained from male Wistar rats exposed to 10, 100, and 200 mg/kg/day subcutaneously. Effects on hormone levels (increase of testosterone level, decrease in FSH and LH), sperm parameters (decrease of motile sperm) and protein levels of receptors in testis (ER and AR) suggested a LOAEL of 10 mg/kg bw/d. In females, effects on FSH levels and sexual behavior were also reported.

Goswami *et al.* (2016) reported effects in Swiss albino mice subcutaneously exposed to 10, 50, 100 mg butylparaben/kg bw such as increased number of uterine glands, increased uterine weight, histological alterations as well as increased endometrial and myometrium thickness and total tissue protein at the highest dose. Those authors concluded to a LOEL of 10 mg butylparaben/kg bw.

Besides the observations done *in vivo* using experimental animals*,* in **Table 1.1** the *in vitro* effects reported for butyl paraben are summarized.
Pop *et al.* (2016) reported an IC50 at 58.5µM using the AR transfected MDA-kb2 cell line showing an anti-androgenic effect of butylparaben. Chen *et al.* (2007) reported also an anti-androgenic activity of a lower butylparaben concentration of 10 µM. Khanna & Darbre (2013) showed proliferation of MCF-7 cells at even lower concentration (10 µM) after a 17-d exposure. Gonzalez *et al.* (2018) observed proliferation of MCF-7 cells and T47D cells after exposure to butylparaben and its hydroxylated metabolite at a range of concentration of 10 pM to 30 µM, suggesting a potential estrogenic effect of butylparaben.

Some *in vivo* human observations are available in the Janjua *et al.* (2007) study. These are supportive for the safety of butylparaben use by consumers and point to the high conservatism in the risk assessment of butylparaben. The results of that study were obtained from a combined test of butylparaben with two phthalates, which does not represent ideal test conditions to investigate the specific parabens concerned.

For the determination of the POD, the SCCS used the BMD approach, according to the new BMD guidance from EFSA (2022).

- BMD modelling

The SCCS performed benchmark dose (BMD) modelling according to the 2022 EFSA Guidance on the use of the BMD approach in risk assessment (EFSA Scientific Committee, 2022). More guidance is included on criteria for acceptability of the results of the modelling than in previous versions (EFSA Scientific Committee, 2019).

Benchmark dose (BMD) analysis was carried out according to the latest EFSA guidance (EFSA Scientific Committee, 2022). To perform the BMD modelling, the SCCS used the Bayesian BMD Modelling web-app (https://zenodo.org/record/7334435#.Y5osYXbMLD4) available at the EFSA R4EU platform (https://efsa.openanalytics.eu/).

The reports of the modelling are shown **in appendix 3**.

- Choice of the BMR:

The EFSA guidance on BMD (2017) recommends for continuous data that the BMR could be defined in various ways. The way recommended here, according to BMD (2022) is to define it as a percent change in the average magnitude of the response variable as compared to the predicted background response. The recommended default value was therefore a BMR of 5%.

The EFSA guidance on BMD (2022) recommends defining the BMR as a percent change in the response relative to the control group (background response):"*in the absence of an already established BMR, experts should consider whether it is possible to define quantitatively 'biologically relevant' to inform the selection of a BMR for the endpoint considered. The BMR may be defined using any of the methods that are available in the literature (e.g. expert knowledge elicitation (EKE), which could be informed by, e.g. the effect size theory (Slob, 2017), 1SD of the background response (US-EPA, 2012), hybrid approach or other definitions), taking biological relevance into account.*"

In conclusion, the SCCS considers that:

-the BMDL established on AGD effects full fill the criteria of acceptability according the EFSA guidance on BMD (2022).

-For the AGD effects, the SCCS selected a default value of 5% (BMR), considering also that a BMR, based on the standard deviation of the control group is around 5%.

Results of BMD modelling:

The modeling of the decrease in AGD in males is presented below and fulfils the EFSA criteria of acceptance. The default BMR of 5% was chosen.

Model	Type	BMDL	BMD	BMDU
Model Averaged	BS	24.503	85.512	370.808

BMD analysis results from Boberg *et al.* (2016) study (BMR=5%)

In conclusion, the SCCS considers the decrease of AGD in males as observed in the Boberg *et al.* (2016) oral study as the critical endpoint leading to a $BMDL_5$ of 24mg/kg bw/day.

In conclusion, the SCCS considers the BMDL of 24 mg/kg bw/day (derived from male rat) as the POD for MOS calculations.

3.5 SAFETY EVALUATION (INCLUDING CALCULATION OF THE MoS)

MARGIN OF SAFETY CALCULATIONS	
Scenario A, Tier 1 (worst case)	
Systemic exposure dose (µg/kg bw/day)	299.9
$BMDL_5$ (mg/kg bw/day)	24.5
MOS	81.7
MARGIN OF SAFETY CALCULATIONS	
Scenario A, TIER 2	
Systemic exposure dose (µg/kg bw/day)	113.9
$BMDL_5$ (mg/kg bw/day)	24.5
MOS	**215.1**
MARGIN OF SAFETY CALCULATIONS	
Scenario A, Tier 3	
Systemic exposure dose (µg/kg bw/day)	13.6
$BMDL_5$ (mg/kg bw/day)	24.5
MOS	1801.5
MARGIN OF SAFETY CALCULATIONS	
Scenario B, Tier 1	
Systemic exposure dose (µg/kg bw/day)	170.7
$BMDL_5$ (mg/kg bw/day)	24.5
MOS	143.5
MARGIN OF SAFETY CALCULATIONS	
Scenario B, Tier 2	-
Systemic exposure dose (µg/kg bw/day)	97.3
$BMDL_5$ (mg/kg bw/day)	24.5
MOS	251.8
MARGIN OF SAFETY CALCULATIONS	
Scenario B, Tier 3	
Systemic exposure dose (µg/kg bw/day)	9.0
$BMDL_5$ (mg/kg bw/day)	24.5
MOS	2722.2

Scenario A – Tier 1 (Maximum inclusion, deterministic approach)
Scenario A - Tier 2 (probabilistic person-oriented approach)
Scenario A – Tier 3 (probabilistic person-oriented approach + **Mintel** occurrence data)
Scenario B – Tier 1 (deterministic additive approach using Cosmetics Europe 2016 survey)
Scenario B – Tier 2 (probabilistic person-oriented approach)
Scenario B – Tier 3 (probabilistic person-oriented approach + **Mintel** occurrence data)

Tier 1 - % inclusion levels for butyl paraben in individual product types as per the 2016 Cosmetics Europe Survey. The P90 values are presented (NB. the P95 values were not significantly different (see Annex 2)) in a deterministic additive approach as per the SCCS Notes of Guidance (2021) method, covering a high-end aggregate exposure calculation derived using the Creme Care and Exposure model
Tier 2 - as per B1 P90 values (as above) with product habits and practices data included using the Creme Care and Exposure model
Tier 3 - as per B2 P90 values with product habits and practices data plus product occurrence data **included using the Creme Care and Exposure model**

In the absence of a well-carried out dermal absorption study, the SCCS is of the opinion that a MOS below 100 for aggregate exposure could present a risk for consumer safety.
Deterministic exposure (scenario A Tier 1) is highly conservative. Scenario A, Tier 2 represents a more realistic scenario and has therefore been used.

3.6 DISCUSSION

Physicochemical properties

The analytical methods used for the determination of the purity of the test substance should be provided, according to the SCCS Notes of Guidance.

Toxicokinetics

-No available *in vitro* dermal absorption study has been done according to the SCCS Notes of Guidance (SCCS/1628/21), although one has been requested on several occasions. The SCCS is of the opinion that a value of 3%, proposed by the Applicant is not acceptable.

-The remarks, made earlier with respect to the dermal exposure of newborns and infants up to 6 months of age and the possibility of exposure to a higher internal dose and potential differences in the half-life of the unmetabolised parabens compared to adults, have not been taken up in the newly submitted data. Additional toxicokinetic data (Mathews *et al.*, 2013; Campbell *et al.*, 2015; Moos *et al.*, 2016) were submitted and reviewed, but these did not bring new data with respect to the above-mentioned young age groups.

-An overview of the oral *in vitro* and *in vivo* toxicokinetic studies showed mainly qualitative data, indicating high oral absorption, extensive clearance and major excretion via the urine and a number of common metabolites in rat and human urine. The main difference in metabolism was described as the appearance of a new metabolite (3OH-n-butylparaben) in humans and a greater amount of glycine conjugation.
The available dermal toxicokinetic studies (Aubert *et al.*, 2009 and Janjua *et al.*, 2008) were discussed in previous SCCS Opinion (SCCS/1514/13). The *in vivo* rat study of Mathews *et al.*, 2013 was used in the argumentation for a dermal absorption value of 3%, which is not accepted.
Intravenous toxicokinetic studies (Mathews *et al.*, 2013) showed, as also seen for the oral, subcutaneous and dermal routes, a rapid clearance and excretion, and the same broad spectrum of metabolites.

- The SCCS appreciates the efforts of the Applicant in proposing an alternative approach, including PBPK modelling and the calculation of MOIE. For the reasons explained and summarised hereunder, it was not possible to apply this approach. The MOIE approach applied is an extension of the Margin of Exposure (MOE) approach for cosmetics in the EU.

It is based on the comparison of internal dose metrics (C_{max}, AUC conc/time). As such, the individual assessment factor 4 that covers the interspecies differences in toxicokinetics can be left out, as these differences are taken into account using a PBK approach (animal PBK model and human PBK-model) (Bessems *et al.* 2017).
Campbell used rat data obtained by Aubert *et al.* (2012). This model was further refined by using the rat study of Mathews *et al.* (2013) and the human study by Moos *et al.* (2016).
SCCS noted that:
(i) for the rat model the peak concentration of radioactivity in the Campbell model was overpredicted by a factor of 4. According to the IPCS-WHO guidance (2010) on PBPK models in risk assessment the Cmax must be within a factor of 2 of the experimental data. Furthermore, the rat model sensitivity/uncertainty analysis was missing.
(ii) for the human PBK model, both oral and dermal absorption-related parameters were calibrated using the values by Janjua *et al.* (2007). The parameter, however, with high uncertainty and sensitivity is the dermal absorption.
(iii) PBPK models must be built for rat and humans and need to be calibrated and validated. Validation must be done using external data. Here, the rat and human models were validated using the same data as used for the model calibration.
The SCCS came to the conclusion that, given the problems identified and the absence of a quality *in vitro/in vivo* dermal absorption study in humans**, the dermal absorption for butylparaben for the calculation of the SED will be the default value of 50%.**

Exposure

For the calculation of the SED, the Applicant proposed two different exposure scenarios (A and B), each with three different tiers.
Scenario A: Tier 1 represents the deterministic method as described in the Notes of Guidance, 11th Revision, which covers a worst-case aggregate exposure calculation; Tier 2 represents a probabilistic person-oriented approach; Tier 3 takes additionally Mintel occurrence data into consideration, a methodology not used by the SCCS.
Scenario B uses the same tiers but uses exposure assessment data obtained in a Cosmetics Europe 2016 survey. As the latter data have not been evaluated by the SCCS, these will not be used in this study.

Toxicological Evaluation

Irritation and corrosivity
Considering that butylparaben ester is used in cosmetic products only at concentrations up to 0.197%, the SCCS is of the opinion that there is no risk of skin irritation for the consumer.

Skin sensitisation
Butylparaben is not sensitising in animals and has in humans only a mild sensitising potential.

Acute toxicity
The SCCS is of the opinion that butylparaben has no acute toxicity.

Repeated dose toxicity

The SCCS agrees with the Applicant that the target organ is the liver and the NOAEL from repeated dose toxicity study is 325 mg/kgbw/d.

Reproductive toxicity
The SCCS has carefully considered and agrees with the Applicant's argumentation with respect to the available *in vivo* reproductive and developmental studies to determine a NOAEL value of 325 mg/kg bw/d.

Mutagenicity / genotoxicity
The SCCS did not agree with the Applicant's conclusions of 'no mutagenicity' and 'no genotoxicity' because an Ames test according to OECD 471 recommended bacterial strain combination was not included and a valid *in vitro* micronucleus/ chromosomal aberration study was not provided. Both tests were subsequently requested (in the presence and absence of S9) and were delivered.
The SCCS carried out a systematic literature search with respect to mutagenicity/ genotoxicity assays of butylparaben (**Appendix 2**)
Based on the analysis of all available data of genotoxicity and mutagenicity of butyl-paraben, the SCCS is of the opinion that butylparaben has no mutagenic/genotoxic potential.

Carcinogenicity

The SCCS carried out an analysis of the data available in the scientific literature with respect to potential carcinogenicity of butylparaben **(Appendix 2, summary Table 2.7).** Because the available evidence shows that butylparaben is not mutagenic/genotoxic **(Appendix 2, Tables 2.1 -2.6),** and that there are no indications of carcinogenicity in the available literature **(Appendix2, Table 2.7),** the SCCS considers that further testing for carcinogenicity is not necessary.

Photo-induced toxicity
Butylparaben is not phototoxic.

Human data
Health Canada have drawn upon human biomonitoring (HBM) data to calculate estimated daily intakes in their draft safety evaluation for butylparaben (Health Canada, 2020). These data suggest that real life exposures would fall in the range 0.18 – 4.4 µg/kg bw/day.
Furthermore, the provisional reference value (a measure to enable HBM of a substance over time to see how it may change with exposure pattern changes), set by the German HBM Commission, is 20 µg/L for women and 10 µg/L for men (Apel *et al.*, 2017).
Both observations indicate that the deterministic aggregate values used in the safety evaluation of butylparaben in this dossier is highly conservative.

Special investigation
Butylparaben displays endocrine activity as shown in a number of *in vitro* and *in vivo* assays (Appendix 1, Tables 1.1 and 1.2). The PoD for calculating the MoS is taken from the oral

rat study of Boberg *et al.* (2016) and is represented by a **$BMDL_5$ value of 24.5 mg/kg bw/day**. The MoS calculated for deterministic aggregate exposure (scenarios A, Tier 1) with a dermal absorption of 50% in all cosmetic categories results in values lower than 100. This exposure is too conservative. Scenario A, tier 2 is a more realistic scenario. **For this scenario, the MoS > 100. Therefore, the SCCS is of the opinion that the concentration of 0.14% of butylparaben present in the different cosmetic product categories is safe.**
A well-performed absorption study could further support this conclusion.

4. CONCLUSION

1. *In light of the data provided and taking under consideration the concerns related to potential endocrine disrupting properties of Butylparaben, does the SCCS consider Butylparaben safe when used as a preservative in cosmetic products up to a maximum concentration of 0.14 %?*

   On the basis of safety assessment considering all available data and the concerns related to endocrine activity, the SCCS is of the opinion that the use of Butylparaben as a preservative in cosmetic products at concentrations of up to 0.14% (expressed as acid) is safe.

2. *Alternatively, what is according to the SCCS the maximum concentration considered safe for use of Butylparaben as a preservative in cosmetic products?*

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3. *Does the SCCS have any further scientific concerns with regard to the use of Butylparaben in cosmetic products?*

   In the absence of exposure data specific for children to Butylparaben in cosmetic products, potential safety concerns cannot be excluded.

   The SCCS mandates do not address environmental aspects. Therefore, this assessment did not cover the safety of Butylparaben for the environment.

5. MINORITY OPINION

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6. REFERENCES

The references linked to Appendix 2 are present in Appendix 2.

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7. GLOSSARY OF TERMS

See SCCS/1647/22, 12th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – Appendix 15 - from page 158

8. LIST OF ABBREVIATIONS

See SCCS/1647/22, 12th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – Appendix 15 - from page 158

Appendix 1 on endocrine activity

Table 1.1: overview of *in vitro* studies related to the endocrine activity of butyl paraben.

The studies, present in Annex 3 Boberg (2020) and not present as such in the Applicant's dossier have been added here. They have been extensively discussed in Boberg (2020).

Test substances	Test system	Test principle(s)	Result(s) and conclusion(s)	Reference	Source
In vitro Assays					
n-Butyl -paraben	Competitive binding assay and Recombinant yeast assay screen Non GLP	DNA sequence of the human estrogen receptor is integrated into the yeast genome. Substances are compared with the potency of estrogen at its receptor.	Butylparaben showed binding affinity for the rat ER about 10^5 lower than E2 and estrogenic activity (10,000-fold less potent than 17βestradiol) The metabolite pHBA, was 2.5 million-fold less potent and is considered nonestrogenic.	Routledge *et al.*, 1998 Miller *et al.*, 2001	Applicant dossier
n-Butyl-paraben	Estrogen-receptor competitive binding assay Non GLP	Substance competes with estradiol in binding with the ER and Relative Binding Affinity (RBA) compared to E2 (E2=100).	IC_{50} for n-Butyl- paraben 1.05 ± 0.35 x 10^{-4} M, compared with an IC_{50} for 17β-estradiol of 0.0009 µM. RBA of 0.0009%	Blair *et al.*, 2000	Applicant dossier
n-Butyl-paraben	MCF-7 cells (human-breast cancer derived cell line shown to be estrogen responsive). Non GLP	Assaying estrogen receptor (ER) dependent proliferation of MCF-7 cells. Cmax (maximal proliferation). Relative Proliferation Potency (RPP) relative to the Cmax of E2.	EC50 1.6 µM butylparaben 17β-estradiol. Cmax of $2x10^{-5}$ M and RPP of $1.5x10^{-6}$ for butylparaben.	Okubo *et al.*, 2001	Applicant dossier

n-Butyl - paraben	MCF-7 cells (human-breast cancer derived cell line shown to be estrogen responsive). Non GLP	Competitive inhibition of [3H]estradiol binding to MCF7 cell estrogen receptors. Only ERα binding confirmed.	Competitive inhibition of [3H]oestradiol binding to MCF7 cell ERα could be detected at 1,000,000-fold molar excess of n-butylparaben (86%). Increased cell proliferation upon exposure was observed through the ER. No antagonist activity of parabens could be detected on regulation of cell proliferation by 17β-oestradiol at 10^{-10} M	Byford *et al.*, 2002	Applicant dossier
n-Butyl-paraben	MCF-7 cells (human-breast cancer derived cell line shown to be estrogen responsive) Non GLP	Principle of gene expression profiling based on DNA microarray analysis with 120 genes selected as showing greater statistical reliability for estrogen responses	Comparative assessment of butyl paraben vs oestradiol showed some albeit low levels of activity for BP	Terasaka *et al.*, 2006	Applicant dossier
n-Butyl - paraben pHBA	Skin and liver cytosol and human epidermal keratinocytes Non GLP	Parabens elevate estrogen levels by inhibiting estrogen sulfotransferases (SULT) in skin	SULT activity was inhibited in skin cytosol by butyl- paraben, but not by PHBA. IC50 = 37 µM estradiol sulfation was inhibited completely by 1 mM BP; no inhibition of androgen sulfation. In human epidermal keratinocytes, IC50= 12 µM. No positive control included.	Prusakiewicz *et al.*, 2007	Applicant dossier

n-Butyl-paraben pHBA flutamide vinclozolin	A stably transfected human embryonic kidney cell line that lacks critical steroid metabolizing enzymes Non GLP	Investigate antiandrogenic activity by measuring inhibition of 0.1 nM testosterone (T)-induced transcriptional activity	Butylparaben inhibited 0.1 nM T-induced transcriptional activity at concentrations above 10 µM (max. 40% inhibition). pHBA was negative. Pos. controls (flutamide and vinclozolin) inhibited 1nM T-induced signal at concentrations of 0.1 to 10 µM (11 to 90% inhibition).	Chen *et al.*, 2007	Applicant dossier
n-Butyl-paraben PHBA 1-oestradiol	MCF-7 cells (human-breast cancer derived cell line shown to be estrogen responsive) Non GLP	Investigate estrogenic effects of mixtures of parabens on cell proliferation; investigate anti-estrogenic effect through inhibition of aromatase, the enzyme that converts androgens into estrogens	Butylparaben induced cell proliferation with EC50values between 0.5 and 10 µM. PHBA was negative. Potency of parabens remains 5 to 6 orders of magnitude below that of 17β-oestradiol. Typical human parabens concentrations (1080nM) are much lower than EC50 and IC50 values	van Meeuwen *et al.*, 2008	Applicant dossier
n-Butyl-paraben	Human adrenocortical carcinoma cell line rat pituitary GH3cell line Non GLP	H295R assay evaluating the ability to interfere with steroid hormone biosynthesis and T-screen assay to define whether the compound is either a thyroid hormone receptor agonist or antagonist by investigating binding and activation of the thyroid receptor (TR), resulting in GH3 cell proliferation	Progesterone production was increased in H295R assay at 30 µM BP. No effect on testosterone or oestradiol production. No positive control included. In T-screen assay, BP increased cell proliferation in GH3 rat cells from 10nM to ±300%. No positive control included. BP increased the effect of T3 and acted agonistic on its own. Above 10µM BP àsignificant decrease in cell proliferation due to cytotoxicity.	Taxvig *et al.*, 2008	Applicant dossier

n-Butyl-paraben	Recombinant rat androgen receptor (rrAR) assay Non GLP	Determine the ability of probable endocrine disruptors to compete with synthetic androgen methyltrienalone (R1881) for binding to recombinant rat AR.	BP IC50=6.2 10^{-4} M (RBA 0.0029) Dihydrotestosterone IC50=1.8 10^{-8} M	Kim *et al.*, 2010	Applicant dossier
n-Butyl-paraben	Stably transfected human estrogen receptor-α transcriptional activation (STTA) assay (OECD Test Guideline 455)	STTA assay evaluates the ability of chemicals to function as an estrogen receptor alpha (ERα) ligand and activate an ERα agonistic responses. PC_{50}, conc of chemical estimated to cause 50% of activity of positive control (17β-oestradiol) response on a plate-by-plate basis	logRTA BP -1.63752 (PC_{50}= 1.25 10^{-7} M) Butyl paraben was weakly estrogenic by ERα mediated transcriptional activity and was approximately 4,300-fold lower than E2.	Kim *et al.*, 2011	Applicant dossier
n-Butyl-paraben	GH3 rat pituitary cancer cell line Non GLP	Induction of an estrogenic biomarker gene - Calbindin-D(9k) (CaBP-9k), involves an ERα-mediated pathway in GH3 cell line	Following 24-hour treatment, a significant increase in CaBP-9k expression of transcript and protein at 10^{-5} and 10^{-4} M BP CaBP-9k and PR are induced by BP via the ER pathway in GH3 cell line.	Vo *et al.*, 2011	Applicant dossier

n-Butyl-paraben	Mouse and Human adipocytes Non GLP	1) Murine 3T3-L1 fibroblasts 2) hADSC (human adipose-derived multipotent stromal cells) 3) GR-responsive luciferase reporter construct MMTV-Luc 4) PolarScreen GR competitor assay	BP promotes adipocyte differentiation in murine 3T3-L1 cells, as revealed by adipocyte morphology, lipid accumulation, and mRNA expression of adipocyte-specific markers. The potency to enhance differentiation increased with increasing chain lengths of parabens. BP activates GR and/or PPARγ in 3T3-L1 pre-adipocytes; no direct binding to, or modulation of, the ligand binding domain of the glucocorticoid receptor was detected by glucocorticoid receptor competitor assays; BP promotes adipose conversion of hADSC	Hu *et al.*, 2012	Applicant dossier
Butyl - paraben	protocol for obesogen screening based on 3T3-L1 cell line, a well characterised adipogenesis model; direct fluorescent measurement using Nile red lipid staining technique. Also PPARγ activation and antagonist experiments. Non GLP	Positive controls: acknowledged obesogens rosiglitazone and tributyltin. 0.39-200 µM test concentration of butylparaben.	LOECs (3T3-L1 cell line): Rosiglitazone 16nM Tributyltin 6.25nM Butylparaben 50µm LOECs (PPARγ calux): Rosiglitazone 30nM Tributyltin 3nM Butylparaben 10µm	Pereira-Fernandes *et al.*, 2013	Applicant dossier

n-Butyl - paraben	MCF-7 and MCF-10A cells Non GLP	Analysed the dose- 0.2, 2, 20,200, 2000 nM and time- 48, 96, 144 and 196 h dependent activity of a single or repeated exposure of butyl- paraben on the proliferation of MCF-7 human breast cancer cells and MCF-10A human breast epithelial cells. Additionally, the effect on estradiol secretion, gene and protein expression of aromatase (CYP19A1) was investigated	Low doses of BP significantly increased 17b-estradiol (E2) secretion in MCF-7 cells but had the opposite effect on MCF-10A cells. Butylparaben increased MCF-10A cell proliferation after single exposure, but not after repeated exposure. Different mechanisms of proliferative action of BP in these two cell lines.	Wróbel & Gregoraszczuk 2013	Applicant dossier

17 parabens; linear C1 to C12, plus 5 non-linear side chain parabens.	Human estrogen receptor α (hERα), hERβ and androgen receptor (hAR) models Non GLP	Transcriptional activities mediated by human estrogen receptor α (hERα), hERβ and androgen receptor (hAR)	Butylparaben induced ER-mediated gene transcription to a level at least 1.2-fold greater than that of E2 in both ERα and ERβ. Agonistic activity REC_{20}* (M) for butylparaben was 2.9 x10-7 for ERα and 1.5x10-7 in ERβ. REC20 ratio (ERα/ERβ) was 2.9. *20% Relative effective conc.; concentration of the test compound showing agonistic activity equivalent to 20% of that of 109 M E2 towards ERα or ERβ. No parabens showed AR agonistic or antagonistic activity. Activities decreased in a stepwise manner as the alkyl chain was shortened to C_1 or lengthened to C_{12}. Estrogenic activity of butylparaben was markedly decreased by incubation with rat liver microsomes, and the decrease of activity was blocked by a carboxylesterase inhibitor.	Watanabe *et al.* 2013	Applicant dossier
n-Butyl-paraben	*In vitro* nuclear receptor coactivator recruiting assay. Non GLP	Antagonist competitive binding on the human estrogen-related receptor γ (ERRγ)	Butyl paraben possessed inverse antagonist activity on ERRγ, with a lowest observed effect level **(LOEL) of 10($^{-7}$)**M. Relative EC50 value of Butylparaben was 3.09 × 10^{-7}	Zhang *et al.*, 2013	Applicant dossier
n-Butyl-paraben	MCF-7 and MCF10A. Non GLP	Butylparaben (20 nm) or 17βestradiol (10 nm). Cell cycle and apoptotic gene expression were evaluated by real-time polymerase chain reaction and protein expression by Western blot.	Cyclins in MCF-7 cells were not affected by butyl- paraben. In MCF10A, BP increased the expression of G1 /S phase genes, and downregulated cell cycle inhibitors. Butylparaben increased BCL2L1 gene, as did 17β-estradiol.	Wróbel & Gregoraszczuk 2014a	Applicant dossier

n-Butyl -paraben	MCF-7 and MCF10A Non GLP	Butylparaben (20 nm) or 17βestradiol (10 nm). Effects on mRNA and protein expression of estrogen receptor (ER)-α (ESR1) and -β (ESR2) and progesterone receptor (PGR)	Butylparaben stimulated PGR mRNA expression in MCF-7 cells. In MCF-10A cells, and increased only PGR mRNA expression. BP increased ESR1 gene and protein expression in MCF-7, not in MCF-10A cells. BP significantly increased ESR2 mRNA and protein expression in MCF-7 cells, in MCF10A cells only ESR2 protein expression.	Wróbel & Gregoraszczuk 2014b	Applicant dossier
Butyl-paraben	Human MDA-kb2 breast carcinoma cells Non GLP	0.1µm and 1µM test substance dissolved in DMSO (vehicle). Cells stably transformed with MMTV-luciferase, cultured in Leibovitz's L-15 medium with 10% FBS, 100U/ml penicillin, 100 mg/ml streptomycin and pre-treated with androgen antagonist flutamide (5µM) at 37ºC. Cells then incubated 24h with and without test compound and evaluated by means of a cell proliferation assay and an assay for glucocorticoid activity (luciferase reporter gene).	In MDA-kb2 cells, butylparaben reached maximum induction levels at 10 µM (1.85 n-fold), EC50 of 1.75 µM after 24hours. Bp tested alone, induced luciferase activity at 1 µM, and at 10nM BP exerted glucocorticoid-like activity 1.44 times higher than solvent control.	Klopčič *et al.* 2015	Applicant dossier

n-Butyl-paraben	Human MDA-kb2 breast carcinoma cells Non GLP	0 and 25 µM in DMSO. Cells stably transformed with MMTV-luciferase express high levels of functional endogenous AR and GR which both act through MMTV promoter. Cells, cultured in Leibovitz's L-15 medium with 10% FBS, 100U/ml penicillin,100mg/ml streptomycin incubated for 24 hrs with and without test compound, and with or without the AR agonist flutamide (5µM).	BP increased the hydrocortisone induced signal to 185.9 ± 7.5%. BP show glucocorticoid receptor (GR) agonist activity since it increased luciferase activity by over 50%. BP showed AR agonist activity	Kolšek *et al.* 2015	Applicant dossier
Butyl-paraben PHBA	*In vitro* testing of BP for inhibition of 17β-HSD1 and 17 βHSD2 activities. Non GLP	Endogenous 17β-HSD1 activity assays performed in intact COV434 cells. Lysates of HEK-293 cells expressing 17βHSD1 or 17β-HSD2.	Butylparaben but not PHBA, inhibited 17 β-HSD2 at 20µM. BP significantly inhibited 17β-HSD1. Regarding the very rapid metabolism of these compounds to the inactive PHBA by esterases, the *in vivo* relevance remains to be determined.	Engeli *et al.*, 2017	Applicant dossier

Butyl-paraben Purity >90% confirmed	Tox 21 Endocrine screening program assays	Estrogen receptor (ER) assays Androgen receptor (AR) assays Thyroid receptor (TR) assays Steroido-genesis assays	18/35 ER assays positive. 2/18 AR assays positive at high dose above a cytotoxic dose – not a substrate for the AR. No assays positive for the TR or steroidogenesis	US EPA Endocrine Screening program 2019*	Applicant dossier
Butyl-paraben purity>99 %	*In vitro* primary rat Sertoli cell culture	Primary Sertoli Dose: 1, 100, 1000 µM Duration of exposure: 6 and 24 h cell culture	Histological evaluation showed increased level of vacuoles in the cytoplasm Immunohistochemistry showed disruption of vimentin filaments and decreased vimentin protein expression.	Alam & Kurohmaru, 2014	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)
Butyl-paraben 99% purity	*In vitro* AR antagonism	AR reporter gene assay (CHO cells) agonism mode (co-exposure with AR agonist R1881) Dose: 0.03-30 µM Duration of exposure: Not reported	No antagonism reported for BP. Butylparaben inhibited the R1881 induced response, but only at cytotoxic concentrations.	Kjærstad *et al.*, 2010	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)
butylparaben	*In vitro* AR antagonism (transfected MDA-kb2 human breast cancer cells (ATCC CRL-2713)).	AR reporter gene assay. Dose: 0.5-100 µM (estimated from graph) Duration of exposure: 24 h	Anti-androgenic activity at three highest doses (approximately in the interval 50-100 µM, read from graph). IC50 = 58.5 µM. No androgenic activity.	Pop *et al.*, 2016	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)

Butyl-paraben	Estrogen-dependent reporter gene assay in T47D-Kbluc breast cancer cells and an estrogen-dependent proliferation assay in MCF-7 breast cancer cells	T47D-Kbluc and MCF-7 breast cancer cells Dose: 0.3-100 µM Duration of exposure: 24 h (reporter gene assay), 72 h (proliferation)	T47D (reporter gene assay, estrogen sensitive): Low dose ↑ (estrogenic response) High dose ↓ (anti-estrogenic response) T47D (reporter gene assay, antagonist mode by presence of E2): High dose ↓ (anti-estrogenic response) MCF-7 (proliferation): Low dose ↑ (estrogenic response) High dose ↓ (anti-estrogenic response) MCF-7 (proliferation, antagonist mode by presence of E2): High dose ↓ (anti-estrogenic response) **Applicant notes estrogenic activity at lower concentrations and anti-estrogenic at higher concentrations**	Pop *et al.*, 2018	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)
Butyl-paraben	*In vitro* anchorage-independent growth of MCF-10A immortalized but non-transformed human breast epithelial cells	MCF-10A human breast epithelial cells Dose: 10 µM Duration of exposure: 17 days	Increased cell proliferation at 10 µM and number of colonies (range tested) **Applicant says effects to be similar to estradiol (positive control)**	Khanna & Darbre, 2013	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)
alkyl esters 5 parabens tested	proliferation of MCF-7 human breast cancer cells	MCF-7 human breast cancer cells Dose: Not reported Duration of exposure: 7 and 14 days	**Effects on proliferation compared to E2:** **After 7 days** **LOEC 0.7 µM** **NOEC 0.5 µM** **EC50 2 µM** **After 14 days** **LOEC 0.5 µM** **NOEC 0.2 µM** **EC50 1 µM** **Applicant reports effects to be similar to estradiol (positive control)**	Charles & Darbre, 2013	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)

n-butyl-paraben	Migratory and invasive properties using three oestrogen-responsive human breast cancer cell lines (MCF-7, T-47-D, ZR-75-1)	MCF-7 human breast cancer cells, T-47-D human breast cancer cells, ZR-75-1 human breast cancer cells Dose: 10 µM Duration of exposure: 7 days and 20 weeks	MCF-7: Motility:7 days; 20 weeks ↑ (increase greater than with E2) Motility after co-exposure with anti-estrogen ↓ Migration ↑ Matrix degradation ↑ Protein expression of E-cadherin, β-catenin: 7 days; 20 weeks ↓ Protein expression of ERα: 7 days ↓ 20 weeks: lower levels than under E2 deprivation conditions and only slightly higher than when the cells were maintained with E2. T-47-D : Motility:7 days; 20 weeks ↑ ZR-75-1: Motility:7 days ↑;20 weeks ↑ **Applicant reports effects to be similar to estradiol (positive control)**	Khanna *et al.*, 2014	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)
	direct effects on follicle growth and ovarian steroidogenesis	Primary culture of pre-antral mouse follicles and primary human granulosa cell cultures Dose: 0.01, 0.1, 1, 10 µM Duration of exposure: up to 12 h for follicles, up to 96 h for granulosa cells	Morphology/growth/developmental pattern of follicles showed no effect. Estradiol production from follicles were not affected. Progesterone production from granulosa cells was furthermore not affected.	Guerra *et al.*, 2016	
n-butyl-paraben	mechanistic responses of aromatase CYP19A1 mRNA, aromatase activity, estradiol biosynthesis and cellular proliferation	MCF-7 and ZR-75-1 breast cancer cells and HMF3A breast fibroblast (ERα negative)	MCF-7, ZR-75-1, HMF3A: Cyp19a1 gene expression ↑ Aromatase activity ↑ Estradiol ↑ MCF-7, ZR-75-1: Proliferation ↑ Co-exposure with aromatase inhibitor proliferation ↓	Williams *et al.*, 2019	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)

n-butyl-paraben	interference of parabens with the estrogen-activating enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD) 1 and the estrogen-inactivating 17β-HSD2	Lysate of human embryonic kidney cells (HEK-293) Dose: 20 µM Duration of exposure: not reported	Activity of 17β-HSD2 ↓ (estradiol to estrone) Activity of 17β-HSD1 ↓ (estrone to estradiol)	Engeli *et al.*, 2017	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)

*https://comptox.epa.gov/dashboard/dsstoxdb/results?search=DTXSID4022527#invitrodb-bioassays-toxcast-data

Table 1.2: *In vivo* studies on endocrine activity

Test substances	Test system	Test principle(s)	Result(s) and conclusion(s)	Reference	
n-Butyl-paraben	Immature female Alpk:AP rats and ovariectomized (OVX) rats, same strain Non GLP	Uterotrophic assay with immature rats. Butylparaben was administered on PND 21-22 once daily for 3 consecutive days at the following dosage levels: butylparaben **oral and subcutaneous** at 40, 200, 400, 600, 800, 1000 and 1200 mg/kg bw/day Uterotrophic assay with ovariectomized (OVX)rats (8-10 weeks old): butylparaben **oral and subcutaneous** at 800, 1000 and 1200 mg/kg bw/day	Immature rat model: 1) No effects were seen after oral dosing with butylparaben. 2)Subcutaneous administration significantly increased uterus wet weights at dosages ≥ 400 mg/kg bw/day (experiment 3). OVX rat model: increased uterus weights only at ≥ 600 mg/kg (experiment 4) or ≥ 800 (experiment 5) mg/kg butylparaben (sc). The positive control oestradiol exerted its effects at an oral dose of 0.4 mg/kg or 0.04 mg/kg bw/day (sc).	Routledge *et al.*, 1998	Applicant dossier
n-Butyl-paraben	B6D2F1 mice Appears compliant with OECD Test Guideline 440 Non-GLP	Uterotrophic assay, s.c. (3 days administration, PND 18-20 in both species). Dose: 100 mg/kg bw/ day (mice) 400, 600 mg/kg bw/day (rats). Estradiol used as positive control (0.1 mg/kg bw/day) for both species	No effects on utrine weight in mice (s.c.). In rats, 400 mg/kg bw/day increased uterus wet weight but not weight mg/bw. However, 600 mg/kg increased both wet weight and relative weight. Statistically significant increase in uterus weight at a subcutaneous dose of 600 mg/kg. Positive control significantly increased the uterus weights. **NOAEL=100mg/kg bw/day (uterine**	Hossaini *et al.* 2000	Applicant dossier

			weight increased in rats)		
n-Butyl-paraben 17β-oestradiol (E2)	CD1 mice Wistar rats Appears compliant with OECD Test Guideline 440 Non-GLP	Uterotrophic assay with both immature and ovariectomized adult mice and immature rats. Animals were treated **subcutaneously** (sc) for three consecutive days with different molar equivalent doses ranging from 3.62 to 1086 micromol/kg body weight of parabens or E2 (0.036 micromol/kg). Estrogen receptor binding affinities of butylparaben relative to E2 was determined.	Uterine weight increased in all models. In mice, **ED50 of E2 for increase in uterine weight was 7 µg/kg bw**, ED50 of butyl paraben was 18 mg/kg bw. In rats, ED_{50} of butyl paraben was 33 mg/kg bw. From abstract: 'NOELs values for parabens uterotrophic activity in IM were from 0.6 to 6.5 mg/kg per day; and OVX (ovariectomized) from 6 to 55 mg/kg. The NOELs IW ranged from 16.5 to 70 mg/kg indicating that IM were more susceptible than Ovx and IW to these effects'	Lemini *et al.*, 2003	Applicant dossier
n-Butyl-paraben	Adult ovariectomized (Ovx) CD1 mice Appears compliant with OECD Test Guideline 440 Non-GLP	Morphometric analysis of uteri in uterotrophic assay. **Subcutaneously** (sc) treated daily for three days with two different doses of butyl - paraben (70 and 210 mg/kg), E_2 (10 mg/kg; 0.036 mmol/kg), and vehicle (butyleneglycol ; V, 10 mL/kg)	Luminal epithelium heights (LEH), glandular epithelium heights (GEH), and myometrium widths (MW) were measured. Butyl- paraben produced uterotrophic effects. **Absolute uterine weight not affected, relative uterine weight increased. LOEL=70 mg/kg bw/day (relative uterine weight increase in both dose groups)**	Lemini *et al.*, 2004	Applicant dossier

n-Butyl-paraben	Wistar rats Non-GLP	Study of the effect of parabens on steroidogenesis in rats and their offspring when dams are **subcutaneously** exposed to 200 - 400 mg butyl-paraben/kg/day from gestation day 7 to 21.	Butylparaben did not show any treatment related effects on plasma hormone levels (T3, T4, 17α-hydroxyprogesterone) testosterone production, anogenital distance, or testicular histopathology. Butyl-paraben caused a decrease in the mRNA β-ER expression level in fetal ovaries, and in mRNA expression of steroidogenic acute regulatory protein and peripheral benzodiazepine receptor in the adrenal glands. However, these effects show no dose-dependency. **No NOAEL defined.**	Taxvig *et al.*, 2008	Applicant dossier
n-Butylparaben 17β-oestradiol	CF-1 and CD-1 female mice Non-GLP Non OECD Test Guideline No mention of group size	Evaluation of the effects of butyl- paraben on success of implantation in fertilised mice. **Subcutaneous** injection of 0, 1.4, 14, 271, 407, 542, 813, 949 mg/kg/day on day 1 to 4 of gestation. Additional uterotrophic assay with butyl- paraben at 0, 20, 200, 949 mg/kg bw/day in two different mice strains. 14 mg/kg bw/day 17β-oestradiol was administered as positive control in both assays.	Butylparaben had no impact on the number of implantation sites observed and did not affect any of the measured parameters, such as the number of pups born, litter weights, individual pup weight and pup survival, number of intrauterine blastocyst implantation sites. 17β-oestradiol (500 ng/animal/day) terminated all pregnancies. A uterotrophic assay was conducted to re-evaluate the in vivo estrogenicity of butylparabens. BP did not affect uterine wet or dry mass at any dose in either strain. 17βoestradiol consistently increased uterine mass in both strains **NOEL = 35 mg/animal/day or 950 mg/kg bw/day**	Shaw and de Catanzaro 2009	Applicant dossier

			(highest dose; no effect on uterine weight)		
n-Butylparaben 17α-ethinyl oestradiol	Sprague Dawley immature female rats Non GLP Non-OECD test guideline	Uterotrophic assay. **Subcutaneous** injection of 62.5, 250, 1000 mg/kg bw/day of paraben for 3 days. Investigation of Calbindin-D9-k (CaBP-9k), biomarker for estrogenic effects.	Subcutaneous injection of 1000 mg/kg bw/day **(highest dose)** induced increased uterine wet weight for butylparaben (also for pos. control at 1 mg/kg bw/day). The effect was blocked by addition of anti-estrogen fulvestrant, indicating estrogen receptordependent pathway. **NOAEL=250 mg/kg bw/day (increase uterine weight)**	Vo and Jeung 2009	Applicant dossier
n-Butyl-paraben 17α-ethinyl oestradiol	Mated Sprague Dawley female rats; Prepubertal (8week-old) females, N=200, n=10/group, 20 groups. 0, 62.5, 250 or 1000 mg/kgbw/day in corn oil (vehicle), by oral gavage. Non GLP NonOECD test guideline	*In vivo* assay to investigate whether **oral-** subacute exposure to butylparaben may induce suppressive effects on reproductive organs in female rats during the critical juvenile-peri-pubertal stage. **Oral-subacute** administration **by gavage** of paraben from postnatal day 21 to 40. Investigation of Calbindin-D9-k biomarker for estrogenic effects.	No significant changes to estradiol, prolactin and T4 levels. Significant increase in uterus thickness at all doses. Decrease of corpora lutea, increase in the number of cystic follicles (~40%) at **62.5 mg/kg bw/d), not dose dependent. but significant.** No effect on vaginal opening. No significant change to estrous cycle. The highest dosage (1000mg/kg bw/day) of butylparaben significantly increased uterine wet weight. Paraben-induced increases in uterine weights were blocked by the pure antiestrogen fulvestrant. A significant decrease in ER-α mRNA and protein expression was observed in the EE-, isopropyl-, and butylparaben-treated groups,	Vo *et al.*, 2010	Applicant dossier

			LOAEL=62.5mg/kg bw/day (adverse effects on ovary)		
n-Butyl-paraben 17ß-oestradiol (E2)	Neonatal Sprague Dawley female rats (n =5) Non GLP Non-OECD test guideline	Effects of neonatal exposure to butylparaben on development of early follicle stages and ovarian factors regulating follicular development and steroidogenesis after **subcutaneous** administration of Butylparaben at doses of 62.5, 250 or 1000 mg/kg bw/day or 17ß-oestradiol (40 µg/kg/day) once daily on PND 1-7. Relative mRNA expression of the following proteins was determined by quantitative real-time PCR: calbindin-9k (CaBP-9k, indicator of estrogenic activity in rat uterus), ovarian anti-Mullerian hormone (AMH), kit ligand/stem cell factor (KITL) and forkhead box protein I2 transcription factor (FoxI2), steroidogenic acute regulatory transport	**Applicant argumentation: Data do not appear to be consistent and dose response relationships are absent.** 250 mg/kg/day: CaBP9k activity; decreased numbers of early primary follicles; mRNA levels of AMH and FoxI2 increased (both not affected by E2); mRNA level of KITL enhanced; mRNA levels of StAR decreased; mRNA levels of CYP11a. 1000 mg/kg/day: increased ovary weight; increased numbers of primordial follicles. **DK-EPA/DTU defined LOAEL = 62.5 mg/kg bw/day**	Ahn *et al.*, 2012	Applicant dossier

		protein (StAR) and CYP11a1.			
utyl-paraben (purity not reported)	Sprague-Dawley rats	Development of male reproductive system**, s.c.** (GD6-PND20). Dose 110, 200 mg/ kg bw/day, n = 5-7 for organ weight/histology, 5 form sperm parameters and 3 for gene expression	Pups: *Live births* ↓ *Surviving to weaning* ↓ AGD Weight: *Testis* ↓↑ *Prostate* ↓ *Seminal vesicle* ↓ Sperm: *Numbers* ↓ *Motility* ↓ *Morphology* ↓ ERα and ERβ expression in testis ↓↑ **NOAEL=100mg/kg bw/day (Effects on, testes, seminal vesicles, prostate glands sperm count and motility)**	Kang *et al.*, 2002	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)
Butyl-paraben (purity not reported)	Wistar rats	Neonatal repeated, s.c injection (PND 2-18). Dose: 2 mg/ kg bw /day, n= 6.	Testis weight Testis histopathology (no significant effects) **NOEL=2 mg/kg bw/day (no effects)**	Fisher et al., 1999	Boberg et al., 2020 (The Danish Environmental Protection Agency (DK-EPA)

Butyl-paraben	Wistar rats	Development of male reproductive system, **oral** (gavage) (GD7-PND21). Dose: 64, 160, 400, 1000 mg/ kg bw/day, n =7-8.	Effects in pups: decreased AGD, sexual maturation affected (delayed puberty), hormone levels (*Testosterone* ↓ *Estradiol* ↑ *Progesterone* ↑ *LH* ↓↑ *FSH* ↓↑ Sperm numbers and daily sperm production ↓), sperm parameters and testicular health (*Testis* ↓ *Epididymis* ↓ *Seminal vesicle* ↓). Decreased sex ratio in pups & bodyweight. Histopathology testis (affected PND 21 and 90). Effects in dams: FSH and LH ↑ Offspring affected at several ages (for many endpoints PND 21, 35, 49, 90, 180. Male offspring: sex ratio affected (fewer males) Bw decreased from PND 0-49, but not affected PND 90-180. Weight of testis, epididmis and seminal vesicles decreasedàover laps with reduced BW and relative weights not reported. AGD shortened on PND1 and 21 (also coincides with reduced BW). Testis histopathology affected on PND 21 and 90 with, fex, reduced and loosely arranged germ cells, reduced layers of seminiferous tubules, reduced numbers of spermatocytes. No obvious effects on Leydig cells. **NOAEL=160mg/kg bw/day (male reproduction and developmental toxicity in rats**	Zhang *et al.*, 2014	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)

Butyl-paraben	Wistar rats	Mechanisms of ED and reproductive disorders, oral (gavage) (GD7-PND21). Dose: 64, 160, 400, 1000 mg/kg bw/day, n = 7-8.	Body weight ↓ Weight: Testis Epididymis ↓ Seminal vesicle Hormones: Testosterone ↓ Estradiol ↑ Gene expression: Star, P450scc, Sult1e1 (affected) Gene and protein expression: Erα, Erβ, Ar (affected) Methylation of Erα promoter ↓ Histopathology testis (affected) ! may be partially same study as Zhang et al., 2014 **NOAEL = 160 mg/kg bw/day (effects are seen at protein level at this dose)**	Zhang et al., 2016	Boberg et al., 2020 (The Danish Environmental Protection Agency (DK-EPA)

Butyl-paraben	Wistar rats	Development of male reproductive system, **oral (gavage)** (GD7-21 and PD1-22). Dose: 10, 100, 500 mg/kg bw/day, n = 18.	AGD and AGDi shortened in both males and females. Number of sperm in cauda reduced in all dose groups. Genes (cell markers, receptors (Ar, Fshr, Lhr), steroidogenesis) were investigated in testis PD 16 and in adulthood. Down regulation of Cyp19a1 in all exposure groups was seen on PD16. Not other effects seen on gene expression. Hormone levels (estradiol measured PD16 males and PD 22 females): no effect. Mammary gland was investigated in females. PD 22: higher number of TEBs in two highest dose groups (100, 500 mg/kg bw/day). Increased outgrowth towards the lymphnode in 100 mg/kg bw/day. Adult: no clear effects **NOAEL = 10 mg/kg bw/day (decrease in AGD)**	Boberg *et al.*, 2016	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)

Butyl-paraben (purity not reported)	Wistar rats	Male reproductive development, **s.c.** (GD 12 - PND21). Dose: 10, 100, 200 mg/kg/day, n = 8/group.	AGD Nipple retention Puberty Weight: Pituitary *Testis* *Epididymis* *Prostate* *Seminal vesicle* *Vas deferens* Histopathology: *Fetal testis* *PND 110 testis ↓ Leydig cells* Hormones: *Testosterone ↑* *FSH ↓* *LH ↓* Sperm: *Spermatogenesis kinetics ↑↓* *Sperm counts* *Motile sperm ↓* *Non-motile sperm* *Normal morphology ↓* *Abnormal morphology ↑* Testis morphometry (no of cells) ESR1 and AR protein in testis ↓ Sexual behavior Fertility **LOAEL = 10 mg/kg/day (spermatogenesis kinetics, sperm head abnormalities & motility affected)**	Guerra *et al.*, 2017b	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)
Butylparaben	Wistar rats	Repeated dose, **oral** (diet) (8 weeks from PND 19-21). Dose: 10.4 ± 3.07, 103 ± 31.2, 1026 ± 310 mg/kg bw/day, n = 8.	Weight: *Testis* *Epididymis ↓* *Prostate* *Seminal vesicle ↓* *Preputial glands* Sperm numbers (testis and cauda) ↓ Testosterone ↓ **LOAEL = 10.4 mg/kg bw/day (0.01%) (decreased cauda epididymal sperm reserves, daily sperm production)**	Oishi 2001	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)

Butylparaben	CD-1 ICR mice	Repeated dose, oral (diet) (10 weeks from PND 27-29). Dose: 14.4 ± 3.60, 146 ± 35.9, 1504 ± 357 mg/kg bw/day, n = 8	Weight: Testis Epididymis ↓ Prostate Seminal vesicle Preputial glands Sperm morphology: Type and stage (affected) Testosterone ↓ **LOAEL = 14.4 mg/kg bw/day (elongated spermatid counts were significantly lower)**	Oishi 2002	Boberg et al., 2020 (The Danish Environmental Protection Agency (DK-EPA)
Butyl-paraben	Wistar rats	Repeated dose, **oral (diet)** (Start PND22. Continued for 8 weeks). Dose: 10.9 ± 0.4, 109.3 ± 8.2, 1087.6 ± 67.8 mg/kg bw/day, n=8.	**Weight:** ***Testis*** ***Epididymis*** ***Prostate*** ***Seminal vesicle*** **Sperm:** ***Numbers*** ***Motility*** ***Morphology*** **Histopathology:** ***Epididymis*** ***Testis*** ***Prostate*** ***Seminal vesicle*** **Hormones:** ***Testosterone*** ↓ ***FSH*** ↑ ***LH*** ↓ **NOAEL = 1086.6 mg/kg bw/day (10000 ppm) (effects on hormone levels)**	Hoberman *et al.*, 2008	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)
Butyl-paraben	Wistar rats	Repeated dose, **oral** (p.o.) (start PND 19-21, 8 weeks). Dose: 50 mg/kg n = 6.	**Weight:** ***Testis*** ***Prostate*** ***Seminal vesicle*** **Sperm:** ***Sperm numbers*** ↓ ***Sperm motility*** ↓ **Hormones:** ***Testosterone*** ↓ ***Estradiol*** ↑ ***LH*** ↓ ***FSH*** ↓ ***Testosterone/LH*** ↓ ***Testosterone/Estradiol*** ↓ **Testis DNA damage** ↑ **Histopathology testis (affected)**	Riad *et al.*, 2018	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)

			LOAEL = 50 mg/kg (hormone levels, sperm parameters and testis DNA damage)		
Butyl-paraben	Sprague-Dawley rats	Reproductive toxicity**, oral** (single administration) (3-week-old male rats). Dose: 1000 mg/kg bw, n= 8.	Evalution of vimentin filaments, actin and alpha-tubulin (IHC) showed that the Sertoli cell vimentin filaments were affected by exposure, without changes in the microtubule network. Also, histological evaluation (HE) showed detachment and displacement of spermatogenic cells from away from Sertoli cells. Histopathology: *Testis - detachment and displacement of spermatogenic cells from Sertolic cells* IHC: *Testis - vimentin filaments were affected 6 and 24 h after exposure. No effect on the microtubule network.* **LOAEL = 1000 mg/kg bw/day (testicular histology)**	Alam & Kurohmaru 2014	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)
Butyl-paraben	Wistar rats	Female reproductive development and uterotrophic assay**, s.c** (GD12-GD20 and GD12 to end of lactation (PND20)) Dose: 10, 100, or 200 mg/kg (E2 positive control), n =7 (uterotrophic) n = 7-9 (repro dev) Estradiol positive control (10 µg/kg bw)	No effect on uterine weight Positive control (estradiol) ↑ uterine weight. No effect on no of delivered pups, body weight, AGD, nipple retention, VO, first estrous (or BW at VO and first estrous), estrous cycling. FSH increased at 10 mg/kg/day. No effects were seen on organ weights and BW at PND 75. No effects were seen on no of germ cells (PND 20) or follicles	Guerra *et al.*, 2017a	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)

			(adulthoodd). Some effects on sexual behavior, but not statistically significant (200 mg/kg/day). 50% gestational rate (200 mg/kg/day) but data from pregnant animals were comparable to controls. **LOAEL = 10 mg/kg bw/day (FSH and sexual behaviour)**		
Butyl-paraben	Sprague-Dawley rats	Sperm parameters**, s.c.** 57 days, 3 alternating days per week (start 6 weeks old). Dose: 0 (Both naÏve control and vehicle exposed control), 150, 300, 600 mg/kg bw/day, n = 8-10.	***NOTE: naïve control used for stat analysis*** Prostate weight ↑ Sperm numbers (affected, ↓↑) Sperm morphology: *Normal* ↓ *Abnormal* ↑ **LOAEL = 150 mg/kg bw/day (prostate/testis/sperm)**	Garcia *et al.*, 2017	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)
Butyl-paraben	CF1 mice	Pharmacokinetic effects E2, **subcutaneous** (one injection). Dose: 1, 3, 9, mg (35, 103.3, 310 mg/kg, females) (26.9, 79.5, 242.1 mg/kg, males), n = 10/group.	Urinary estradiol concentrations were measured (both sexes) after BP exposure. In males E2 levels were increased after 3 mg exposure at 8 h. In females' estradiol levels were increased after 3 mg exposure at 6, 8, and 10 h. **NOAEL = 1 mg (26.9 mg/kg in males)**	Pollock *et al.*, 2017	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)
Butylparaben	Swiss albino mice	Effects on uterus, **subcutaneous**, 7 days (adult). Dose: 0, 10, 50, 100 mg/kg bw, n ≥ 5. Estradiol used as positive control (0.001mg/kg bw)	Uterine glands ↑ Uterine weight ↑ Endometrial and mycometrium thickness ↑ Total tissue protein ↑ Histological alterations **LOEL = 10 mg/kg bw (uterine effects)**	Goswami & Kalita 2016	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)

			The absolute uterine weight was not affected, however relive uterine weight was increased in both dose groups as well as in the positive control. **LOEL = 70 mg/kg bw/day (relative uterine weight)**	Lemini *et al.*, 2004	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)
Butyl-paraben	Sprague-Dawley rats	Female reproductive endpoints**, oral** (5 weeks, dissolved in corn oil, I assume by gavage) (8 weeks old. Dose: 100 mg/kg/day, n = 6. (An additional group was given VCD (induces POF) + BP, but these results were not included in this evaluation)	Affected estrous cycle length after exposure. Upregulated Amh mRNA levels, but no effect on Foxl2 and Kitlg. Downregulation of Hsd3b1and Cyp19a1. Downregulation of Lhr. Increased FSH levels in serum and decrease in secondary follicles and Graafian follicles. **LOAEL = 100 mg/kg/day**	Lee *et al.*, 2017	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)
Butyl-paraben	Holtzman rats	Fertility study in F1 females**, s.c**. (GD6-PND21). Doses: 10, 100, 1000 mg/kg bw/day, n = 15.	F1 females: Increase bw at all time points from birth to PND75 (10 mg/kg bw/day). Delayed VO (100, 1000 mg/kg bw/day). Reduced estrous cycle length (10, 1000 mg/kg bw/day), E2 level reduced at all age measured (100 mg/kg bw/day). Testosterone and progesterone levels were affected at several ages, and significance only found in some cases. Fertility was affected (increased pre-and post-implantation loss at 100, 1000 mg/kg bw/day). Increased number of days before copulation was noted in all exposed groups. Different ovarian follicle types were affected at different ages and	Maske *et al.*, 2018	Boberg *et al.*, 2020 (The Danish Environmental Protection Agency (DK-EPA)

			effects seen at both 100 and 1000 mg/kg bw/day. Ovarian gene expression of ERα and Star was upregulated (100 mg/kg bw/day. Weight of adrenal gland, hypothalamus, pituitary, ovary, uterus were all affected at different ages. **NOAEL = 10 mg/kg bw/day (estrous cycling)**		

Appendix 2 on Genotoxicity studies

I. APPROACH FOR ASSESSING GENOTOXICITY STUDIES

I.1. Evaluation of reliability and relevance of results of genotoxicity studies – general considerations

Evaluation of data quality for genotoxicity hazard includes evaluation of reliability and relevance (Klimisch *et al.*, 1997; OECD, 2019; ECHA).
The relevance of study results was categorized into high, limited or low relevance. It was based on its reliability and on the relevance of the test system and study design. The SCCS developed a scoring system for reliability based on the scoring system of Klimisch *et al.* (1997) in line with recommendations in the 11th Revision of the SCCS Notes of Guidance (SCCS/1628/21) and the EFSA Scientific Committee Guidance on genotoxicity testing strategies (EFSA, 2011).

The reliability scores were:
1. reliable without restriction
2. reliable with restrictions
3. insufficient reliability
4. reliability cannot be evaluated
5. reliability not evaluated since the study is not relevant and/or not required for the risk assessment.

These reliability scores were defined as follows:
1. Reliable without restriction
"This includes studies or data from the literature or reports which were carried out or generated according to generally valid and/or internationally accepted testing guidelines (preferably performed according to GLP) or in which the test parameters documented are based on a specific (national) testing guideline (preferably performed according to GLP) or in which all parameters described are closely related/comparable to a guideline method."
2. Reliable with restrictions
"This includes studies or data from the literature, reports (mostly not performed according to GLP), in which the test parameters documented do not totally comply with the specific testing guideline but are sufficient to accept the data or in which investigations are described which cannot be subsumed under a testing guideline, but which are nevertheless well documented and scientifically acceptable."
3. *Insufficient reliability*
"This includes studies or data from the literature/reports in which there are interferences between the measuring system and the test substance or in which organisms/test systems were used which are not relevant in relation to the exposure (…) or which were carried out or generated according to a method which is not acceptable, the documentation of which is not sufficient for an assessment and which is not convincing for an expert judgment."
4. *Reliability cannot be evaluated*
"This includes studies or data from the literature, which do not give sufficient experimental details and which are only listed in short abstracts or secondary literature (books, reviews, etc.)."
5. *Reliability not evaluated*
The study is not relevant and/or not useful for the risk assessment.

Generally, the assignment of a reliability score is expert judgement based on defined criteria. Each reliability box in the summary tables started with the reliability score, followed by comments justifying the score. This is equally applicable *for in vitro* and *in vivo* studies.

The relevance of the test results is mainly, but not exclusively, based on:

- Genetic endpoint (high relevance for gene mutations, structural and numerical chromosomal alterations as well as results obtained in an in vitro comet assay; lower relevance for other genotoxic effects). Other test systems although potentially considered of limited or low relevance may provide useful supporting information.
- Cell lines (*e.g.* human vs other mammalians) in case of *in vitro* studies.
- Route of administration (*e.g.* oral vs intravenous, subcutaneous or intraperitoneal injection) in case of *in vivo* studies.
- Status of validation (*e.g.* for which an OECD Test Guideline (TG) exists or is in the course of development, internationally recommended protocol, validation at national level only, no validation).

Tables were used in order to structure the outcome of the evaluations in a transparent way and to provide a possibility to consider the relevance of study results in a weight-of-evidence approach. Remarks were inserted in the columns "Reliability" and assigned relevance to the test results in order to justify the judgments. Minor and/or major deviations from OECD TGs were reported in column "Reliability" (*e.g.* lack of positive control, inappropriate exposure conditions, limited reporting etc.).

The studies were grouped in these tables based on genetic endpoints or test systems and chronologically within these groups. The results were evaluated by the SCCS and presented as positive, negative, equivocal or inconclusive:

1. The result should be considered **clearly positive** if all three of the following criteria are fulfilled (WHO, 2020):
   a. At least one of the test concentrations (or doses) results in a statistically significant increase compared with the concurrent negative control.
   b. The increase is dose related when evaluated with an appropriate trend test.
   c. Any of the results are outside the distribution of the historical negative control data (*e.g.* statistically based control limits).
2. In contrast, results are considered **clearly negative** if none of the three criteria is fulfilled, given a lack of major methodological deficiencies.
3. The term **„equivocal result"** usually refers to a situation where not all the requirements for a clear positive result have been met (EFSA, 2011). An example could be where a positive trend was observed, but the dose-response relationship is not statistically significant. Equivocal can, therefore, be interpreted as possibly relating to the true state of nature as the true result is on the borderline of the decision criteria for positive or negative. In the context of testing, it could imply a weak positive result as opposed to a clear positive or negative. Repeated testing would then result in results falling just one side or the other of the decision criteria. Equivocal results are generally less relevant than clearly positive results, however, they may be considered as an indication for a possible genotoxic potential which should be clarified by further testing. A modification of the experimental conditions may be taken into consideration.
4. An **"inconclusive result"** could be considered one where no clear result was achieved but this may have been a consequence of some limitation of the test or procedure (EFSA, 2011). In this case, repeating the test under the correct conditions should produce a clear result.

Evaluation of reliability and relevance of the test system/test design was always performed irrespectively whether a study has been conducted in compliance with Good Laboratory Practice (GLP) or not. The type of a document (*i.e.*, publication or unpublished study report) and the question if the study has been performed according to GLP or not, do not necessarily have an impact on the reliability score. The details reported are key for judgment of the reliability and relevance of the information irrespective of whether or not published in a peer-reviewed journal.

I.2. Criteria for inclusion and exclusion applied to screening of publications retrieved in the literature search

The screening process of the papers retrieved by the literature search (see list of references) was performed using an online tool (PubReMiner, https://hgserver2.amc.nl/cgi-bin/miner/miner2.cgi). The screening was performed by one reviewer in two steps: title and abstract (TiAb) and initial screening for relevance (full text).
At the TiAb screening, the following criteria for **exclusion** were applied:
• Non-biological, toxicological or genotoxicity studies (*e.g.* synthesis, photocatalytic performance)
• Studies on non-mammal species (*e.g.* fish, *Drosophila*) or plants
• *In vivo* studies that have used a non-relevant route of administration (*e.g.* inhalation).
• Reviews, editorials, letters to the editors, etc.
As a general principle, in case of doubt or insufficient information in the abstract to draw a conclusion on possible exclusion, the approach taken has been to bring the publication to the following step, *i.e.* full-text screening.

As a first step, full text of the publications were screened by 2 reviewers to confirm relevance of the test material: butyl paraben. At this step, publications with test material(s) not relevant for the assessment of butyl paraben were excluded.
At the same time, detailed information on the test material was extracted, including:

1. Source, manufacturer
2. CAS number
3. Purity of the test material

In a second step, the full-text publications were screened for relevance along with a classification of the studies according to the following areas of assessment:

- *In vitro/in vivo*
- Genotoxicity endpoint

In addition, information on the study design was extracted from the publications (*e.g.* type of cells/animal species, concentrations/doses tested, duration of the studies, etc).
Final conclusion was made as consensus risen from discussion between 2 genotoxicity experts.

I.3 References

1. ECHA Guidance IR/CSA R.4. ECHA Guidance Documents and Practical Guides: http://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment. Guidance on collection of available information (Chapter R.3), evaluation of information (Chapter R.4)
2. EFSA Scientific Committee; Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA Journal 2011;9(9):2379. [69 pp.] doi:10.2903/j.efsa.2011.2379.
3. Klimish H-J, Andreae M, Tillmann U. 1997. A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. Reg Toxicol Pharmacol 25(1): 1-5. https://doi.org/10.1006/rtph.1996.1076
4. OECD (2019), *Guiding Principles and Key Elements for Establishing a Weight of Evidence for Chemical Assessment*, Series on Testing and Assessment No. 311, Environment, Health and Safety Division, Environment Directorate.
5. WHO EHC 240: Principles for Risk Assessment of Chemicals in Food (2009) - the updated section 4.5 on genotoxicity published in November 2020.

II. RESULTS OF SEARCH ON BUTYL PARABEN GENOTOXICITY (access date: 2020-05-12)

The types of documents include:

- peer reviewed articles
- journal entries
- book chapters
- government and non-government funded publications.

Results from PubMed search with PubReMiner
https://hgserver2.amc.nl/cgi-bin/miner/miner2.cgi

Key words including MeSH terms	No of entries
Butyl paraben AND genotoxicity	5
Butyl paraben AND gene mutations	0
Butyl paraben AND micronucleus test	1

Results from Find-eR search
https://ec-europa-finder.primo.exlibrisgroup.com/discovery/search?vid=32EUC_INST:VU1

Key words including MeSH terms	No of entries
Butyl paraben AND genotoxicity	40
Butyl paraben AND gene mutations	21
Butyl paraben AND micronucleus test	3

Results from https://www.lens.org/ (Scholarly works) search

Key words including MeSH terms	No of entries
Butyl paraben AND genotoxicity	28
Butyl paraben AND gene mutations	10
Butyl paraben AND micronucleus test	5

Results from https://scholar.google.com/ **search**

Key words including MeSH terms	No of entries
Butyl paraben AND genotoxicity	774
Butyl paraben AND gene mutations	668
Butyl paraben AND micronucleus test	144

III. DETAILED RESULTS FOR THE DIFFERENT *IN VITRO* AND *IN VIVO* TESTS

1
2 **Table 2.1: Bacterial gene mutation assays (Ames test)**
3

	Test system/ Test object	Exposure conditions (concentration/duration/metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments by SCCS	Relevance of the result as evaluated by SCCS	Authors_year
1	*S. typhimurium* TA92, TA94, TA98, TA100, TA1535, TA1537	Maximum dose: 1 mg/plate (highest non-cytotoxic dose); solvent DMSO. Pre-incubation with both the test sample and the S-9 mix for 20 min at 37°C before plating. Incubation at 37°C for 2 days. The result was considered positive if the number of colonies found was twice the number in the control (exposed to the appropriate solvent or untreated).	BPB supplied from the Japan Food Additives Association, Tokyo, at the request of the Ministry of Health and Welfare of Japan, where the purity and quality of each sample were checked. Purity 99%; no CAS number was provided for butyl p-hydroxybenzoate.	**Negative (-/+ S9-mix)**	1	High	Ishidate M Jr, Sofuni T, Yoshikawa K, Hayashi M, Nohmi T, Sawada M, Matsuoka A. Primary mutagenicity screening of food additives currently used in Japan. Food Chem Toxicol. **1984** Aug;22(8):623-36. doi: 10.1016/0278-6915(84)90271-0.
2	*S. typhimurium* TA97, TA98, TA100, TA1535	1, 3, 10, 33, 100, 166, 333, 1000, 3333 µg BP/plate; without and with 10%, 30 rat S9, 10%, 30% hamster S9. 1: Vehicle Control: DMSO, positive controls: 2-Aminoanthracene (0.5 ug/plate); 2-Aminoanthracene (1 ug/plate); 2-Aminoanthracene (2.5 ug/plate); sodium azide (5 ug/plate); 2-Aminoanthracene (5 ug/plate); 9-	Purity not stated; CAS number: 94-26-8	**Negative (-/+ S9-mix)**	1 4 out of 5 OECD TG 471 recommended strains were used. The 5th recommended strain should be selected from E. coli	Limited 1 recommended strain was not used.	NTP G06: Ames Summary Data; Study Number: 926250, 2018

	Test system/ Test object	Exposure conditions (concentration/duration/metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments by SCCS	Relevance of the result as evaluated by SCCS	Authors_year
		Aminoacridine (50 ug/plate); 4-Nitro-O-Phenylenediamine (2.5 ug/plate)			WP2 uvrA, or E. coli WP2 uvrA (pKM101), or S. typhimurium TA102.		
3	*S. typhimurium* TA98 and TA100	≤1000 mg/plate (5.148 mmol/plate) -S9 fraction No important details are available to the SCCS to assess the study.	Not provided	- According to NTP (2005) the result was negative.	4 Important details are not available to the SCCS to assess the study.	Low	Haresaku M, Nabeshima J, Ishigaki K, Hashimoto N and Tovoda Y, 1985. Mutagenicity study (Ames test) of toothpaste ingredients. Journal of the Society of Cosmetic Chemists 19(2), 100-104. (In Japanese)

SCCS comment on bacterial gene mutation studies based on Table 2.1:

Butylparaben was tested on *S. typhimurium* TA92, TA94, TA97, TA98, TA100, TA1535, TA1537 strains in studies of high or limited reliability and relevance with negative results.

However, the SCCS noted that 1 strain combination recommended by the OECD TG 471 that is sensitive for a variety of oxidative agents and crosslinking agents has not been represented (E. coli WP2 uvrA, or E. coli WP2 uvrA (pKM101), or S. typhimurium TA102). As it is known that these 4 *S. typhimurium* strains may not detect these types of mutagens, the SCCS is of the opinion that unless a documented negative result is available to the SCCS, a valid Ames test with lacking bacterial strain combination should be provided.

There are other reports existing in the open literature in which theoretically BPB was tested, but to which the SCCS had no access:

1. Fujita, H., and Hiraga, K. 1980. Mutagenicity of paired fungicide mixtures in the Salmonella/microsome test. Tokyo Toritsu Eisei Kenkyusho Kenkyu Nenpo, 0(31-32):29-32. Abstract from TOXCENTER 1981:107663.
   S. typhimurium TA98, TA100 TA1538 strains were used.
2. Fujita, H., Kojima, A., Sasaki, M., and Hiraga, K. 1985. Mutagenicity test of antioxidants and fungicides with Salmonella typhimurium TA97a, TA102. Tokyo Toritsu Eisei Kenkyusho Kenkyu Nenpo, 36:413-417. Abstract from TOXCENTER 1986:120075.
   Salmonella typhimurium TA97a, TA102 were used.
3. Kojima, A., and Hiraga, K. 1978. Mutagenicity of citrus fungicides in the microbial system. Tokyo Toritsu Eisei Kenkyusho Kenkyo Nempo, 29:83-85. Search result from EMIC (secondary source ID: EMICBACK/39451).
   Bacillus subtilis strains H17A and M45T were used.
4. Morita, K., Ishigaki, M., and Abe, T. 1981. Mutagenicity of materials related with cosmetics. J SCCJ, 15:243-53. Abstract from TOXCENTER 1982:96081.
   Escherichia coli strain WP2 was used.

Table 2.2: *In vitro* mammalian cell chromosomal aberrations/ micronucleus test

	Test system/ Test object	Exposure conditions (concentration/duration/ metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability / Comments	Relevance of the result as evaluated by SCCS	Authors_year
1	Chinese hamster cells Chromosomal aberrations	No information if S9 fraction was used. Important details are not available to the SCCS to assess the study.	Not provided	**-** According to NTP (2005) the result was **positive:** 1-3% increase in polypoid cell production was observed. Aberrations included chromatid breaks, chromatid gaps, chromosomal exchanges, and ring formations.	4 Important details are not available to the SCCS to assess the study.	Low	Ishidate, M., Hayashi, M., Sawada, M., Matsuoka, A., *et al.* **1978.** Cytotoxicity test on medical drugs. Chromosome aberration tests with Chinese hamster cells in vitro. Eisei Shikensho Hokoku 96:55-61. **Cited by CIR (1984)**.

2	Chinese hamster fibroblast cell line (CHL) Chromosomal aberrations	The cells were exposed to BPB at 3 different concentrations for 24 and 48 hr without S9. **Maximum tested concentration of 60 µg/mL.** Results were treated as positive if between 10.0 and 19.9% (+), 20.0 and 49.9% (++) or more than 50.0% (+++) cells had aberrations.	BPB supplied from the Japan Food Additives Association, Tokyo, at the request of the Ministry of Health and Welfare of Japan, where the purity and quality of each sample were checked. Purity 99%; CAS number was not provided for butyl p-hyroxybenzoate.	**Negative – S9**	2 Relatively low concentrations tested. S9-mix was not used. 100 well-spread metaphases were scored per concentration.	Limited	Ishidate M Jr, Sofuni T, Yoshikawa K, Hayashi M, Nohmi T, Sawada M, Matsuoka A. Primary mutagenicity screening of food additives currently used in Japan. Food Chem Toxicol. **1984** Aug;22(8):623-36. doi: 10.1016/0278-6915(84)90271-0.
3	Human peripheral lymphocytes for 1 female volunteer Chromosomal aberrations	Cells were exposed to BPB with or without S9 fraction. 48 hours after the start of the culture, the cells were treated for **4 hours ±S9-mix** with BPB (5, 10, 25, 50 µg/mL) or for **26 h –S9-mix**. Positive controls: Thio-TEPA without S9 and cyclophosphamide with S9. Cells stained with 5% Giemsa. At least 200 well-spaced metaphases were analysed.	BPB from (TCI) TOKYO Chemical Industry CO., LTD CAS 94-26-8	**Inconclusive** Only 2 lowest concentrations were analysable; 2 highest concentrations were considered too toxic. Lack of data on historical controls significantly hampers drawing conclusions.	3	Low	Chrz J, Hošíková B, Svobodova L, Očadlíková, D, Kolářová H, Dvořaková M, & Mannerström M. **2020**. Comparison of methods used for evaluation of mutagenicity/genotoxicity of model chemicals-Parabens. Physiological Research, 69(Suppl 4), S661. doi.org/10.33549/physiolres.934615

				Range of cells with CA in the study negative controls is 2-5.5% vs. 9% in the cells exposed for 26 h –S9. 200 metaphases were analysed which is not in line with OECD TG 473 (recommending scoring of 300 metaphases). THIOTEPA is not among the positive controls recommended by OECD TG 473.			
4	Chinese hamster CHO-K1 ovary cells	BPB was added (0.1, 0.25, 0.5, 0.75 mM), and the culture was incubated for 3 h. After washing 5-bromo-2-deoxyuridine was added to	Butyl p-hydroxybenzoate (purity > 99%) from Kanto Chemical Co., Inc. (Tokyo, Japan).	**Equivocal** According to the authors the result was positive at	3 Apparently MMC (0.12 or 0.24 µM) did not induce	Low	Tayama S, Nakagawa Y, Tayama K. Genotoxic effects of environmental estrogen-like compounds in CHO-K1 cells. Mutat Res. **2008** Jan 8;649(1-2):114-25. doi:

	Chromosomal aberrations	each culture, and the cultures were incubated in the dark for 27 h (two rounds of replication), after which they were harvested. Two hours before harvesting, colcemid was added. 100 metaphases were scored. MMC and H2O2 were used as positive controls.		the highest concentration. SCCS: significant increase observed only at the highest concentration at which some cytotoxicity was observed.	any significant increase in CA. S9 fraction was not used. BPB induced CAs (cells with CAs/100 metaphases) only at the highest concentration (0.75 µM=146 µg/mL). For cytotoxicity the percent of metaphases without differently staining sister-chromatids was used. No historical data		10.1016/j.mrgentox.2007.08.006. Epub 2007 Aug 19.
5	Human lymphocytes from blood of healthy	Cells treated with BPB at 0.1, 0.25 or 0.5 **mg/L** for 24 h. Staining with 10% Giemsa solution.	Butylparaben (CAS Number: 94-26-8) (Sigma-Aldrich, St. Louis, MO, USA)	**Equivocal** The authors suggest an	3 Although the	Low	Todorovac E, Durmisevic I, Cajo S, Haverić A & Mesic A. **2020**. Evaluation of DNA and cellular damage caused by methyl-, ethyl- and butylparaben in vitro. Toxicological & Environmental

	female donors Chromosomal aberrations	For each treatment, four replicates were made. The analysis included the frequency of cytotoxic and genotoxic markers as well as assessment of the Mitotic Index. The frequencies of apoptotic and necrotic cells (cytotoxicity endpoints) and MI were analyzed in a total of 4000 cells per each tested concentration and controls. CAs were evaluated in a total of 400 well-spread metaphases per each treatment and controls.		increased number of polyploidies for BPB at the highest concentration tested.	authors suggest an increased number of polyploidies for BPB at the highest concentration tested (0.75% at 0.25 mg/L) the result is not clear considering the 0.5% polyploidy observed in DMSO (0.1%) control. Any firm conclusions cannot be drawn without reliable data on historical negative control data. No standard positive control substance was used to validate		Chemistry, DOI: 10.1080/02772248.2020.1851690

					the system. Very low concentrations of BPB were used (the highest was 0.5 µg/mL).		
6	Human peripheral blood leukocytes from two healthy donors as a male and a female Chromosomal aberrations	The cells treated with BPB (100, 50, 25 and 10 µg/mL), 18.5 µL/mL of DMSO as the solvent control, and 0.3 µg/mL of mitomycin C as a positive control for **24 and 48 h**. 100 metaphase cells per subject were examined for structural and numerical changes (total 200 metaphases per concentration).	Butyl paraben (butyl 4-hydroxybenzoate, CAS No: 94-26-8, from Sigma-Aldrich (St. Louis, MO, USA)	**Equivocal** Although BPB induced the CAs for both treatment periods, it significantly increased the CA values only at the highest concentration after 24 h where MI was decreased by more 3x.	2 BPB significantly decreased the MI at all concentrations for both treatment periods, and especially at the highest concentration after 24 h.	Limited	Bayülken GD & Tüylü, AB. **2019**. In vitro genotoxic and cytotoxic effects of some paraben esters on human peripheral lymphocytes. Drug and Chemical Toxicology, 42(4), 386-393. https://doi.org/10.1080/01480545.2018.1457049
7	MCF-10A (ATCC, #CRL-10317) human breast	The cells were treated with **BPB (100 µM=19.4 µg/mL) in combination with silver nanoparticles** for 24-h for MCF-10A, and 48-h for MCF-7 and MDA-MB-231 cells.	Butylparaben (BPB, #54680) from Sigma- Aldrich.	**Inconclusive** The cells were treated with BPB in combination	2 The cells were treated with BPB in combination with	Low The cells used are not recommended for regulatory	Roszak J, Domeradzka-Gajda K, Smok-Pieniążek A, Kozajda A, Spryszyńska S, Grobelny J, Tomaszewska E, Ranoszek-Soliwoda K, Cieślak M, Puchowicz D, Stępnik M. **2017.** Genotoxic effects in transformed and non-transformed human breast cell lines after exposure

	epithelial cells MCF-7 (ATCC, #HTB-22) and MDA-MB-231 (ATCC, #HTB-26) human breast cancer cells Cytokinesis-block in vitro micronucleus assay	Staining with propidium iodide with RNase. Micronuclei analyzed in 2000 binucleated cells per concentration (1000 cells per well in duplicate wells). Cytokinesis-Block Proliferation index (CBPI), Replication Index (RI) and %cytostasis were calculated in 500 mono-, bi- and multinuclears.		with silver nanoparticles. No data were provided for MN frequency induced by BPB alone.	silver nanoparticles. No data were provided for MN frequency induced by BPB alone.	purposes testing.	to silver nanoparticles in combination with aluminium chloride, butylparaben or di-n-butylphthalate. Toxicology in Vitro, 45, 181-193.
8	Human peripheral blood leukocytes from two healthy donors as a male and a female Micronucleus test	The cells treated with BPB (100, 50, 25 and 10 µg/mL), 18.5 µL/mL of DMSO as the solvent control, and 0.3 µg/mL of mitomycin C as a positive control for **24 and 48 h**. Staining with 5% Giemsa; micronuclei scored in 1000 binucleated cells per donor (total 2000 binucleated cells per concentration). The cell proliferation determined by the CBPI.	Butyl paraben (butyl 4-hydroxybenzoate, CAS No: 94-26-8, from Sigma-Aldrich (St. Louis, MO, USA)	**Positive** Significant and dose dependent increase in MN at 100 and 50 µg/mL after 24 and 48 h.	1 At the 2 highest concentrations a concentration-dependent decrease in CBPI was observed up to 40%	High	Bayülken GD & Tüylü, AB. **2019**. In vitro genotoxic and cytotoxic effects of some paraben esters on human peripheral lymphocytes. Drug and Chemical Toxicology, 42(4), 386-393. https://doi.org/10.1080/01480545.2018.1457049 Also in: Sinan GH, Bayülken DG & Tüylü BA. Assessment of the genotoxicity of butylparaben in human lymphocytes using the comet assay and cytokinesis-block micronucleus test. **2017**. The Turkish Journal of Occupational/Environmental Medicine and Safety, 2(1 (1)), 229-229.

					S9 fraction was not used. The MN frequency was comparable to MMC values.		

The SCCS comments on *in vitro* mammalian cell chromosomal aberrations/ micronucleus studies based on Table 2.2:

Butylparaben was tested in *in vitro* MN/CA tests:
- in 1 study of high relevance with a positive result on human peripheral blood leukocytes,
- in 2 study of limited relevance with a negative result (Chinese hamster fibroblast cell) or an equivocal result (human blood leukocytes),
- in 5 studies of low relevance which could not be assessed because of insufficient information (1), or with an inconclusive result (2), or with an equivocal result (2).

None of the studies were fully compatible with current OECD TG or were conducted according to GLP status.

There are other reports existing in the open literature in which theoretically BPB was tested, but to which the SCCS had no access:

1. Yoshida, S., Masubuchi, M., and Hiraga, K. 1978. Cytogenetic studies of antimicrobials on cultured cells. Tokyo Toritsu Eisei Kenkyusho Kenkyo Nempo, 29:86-88. Abstract from TOXCENTER 2002:329175.
2. Kawachi, T., Yahagi, T., Kada, T., Tazima, Y., Ishidate, M., Sasaki, M., and Sugiyama, T. 1980. Cooperative program on short-term assays for carcinogenicity in Japan. In: Molecular and Cellular Aspects of Carcinogen Screening Tests. IARC Sci Publ, No. 27. Lyon, France: IARC, pp. 323-330.
3. Odashima, S. 1980. Cooperative programme on long-term assays for carcinogenicity in Japan, vol 27. Lyon, France:IARC, pp. 315-322.

The available study results in the open literature on in chromosomal aberrations/*in vitro* micronucleus endpoint with butylparaben (Table 2.2) do not allow drawing firm conclusions. Hence, a valid study on chromosomal aberration endpoint with butylparaben is requested.

Table 2.3: *In vitro* mammalian cell gene mutation assays – NO DATA

Test system/ Test object	Exposure conditions (concentration/duration/metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year

The SCCS comment on *in vitro* mammalian cell gene mutation study results (based on Table 2.3)

No data on mammalian gene mutations with butylparaben have been found in the open literature.

Table 2.4: In vitro DNA damage (*e.g.* Comet assay)

	Test system/ Test object	Exposure conditions (concentration/duration/metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability / Comments	Relevance of the result as evaluated by SCCS	Authors_year
1	Chinese hamster CHO-K1 ovary cells Comet assay	BPB was added (0.2, 0.4, 0.6, 0.8, 1 mM), and the culture was incubated for **1 h**. Comet-assay kit (Trevigen Inc., Gaithersburg, MD) and silver-staining kit (Trevigen Inc.), and the cell-membrane integrity determined by trypan blue dye inclusion. The comets were classified into five patterns based on the area and intensity of staining of the tail, and over 200 cells were scored. H2O2 was used as a positive-control chemical.	Butyl p-hydroxybenzoate (purity > 99%) from Kanto Chemical Co., Inc. (Tokyo, Japan).	**Positive** Increase in mean comet points/cell significant from 0.4 mM (776 µg/mL). H2O2: significant increase in DNA damage at 0.9 mM.	2 SD values were not provided. Only one relatively short time of incubation was used. No historical control values provided.	Limited	Tayama S, Nakagawa Y, Tayama K. Genotoxic effects of environmental estrogen-like compounds in CHO-K1 cells. Mutat Res. **2008** Jan 8;649(1-2):114-25. doi: 10.1016/j.mrgentox.2007.08.006. Epub 2007 Aug 19.
2	Human peripheral blood leukocytes Comet assay	The cells treated with BPB (100, 50, 25 and 10 µg/mL), for 24 h.	Not provided	**-** According to the authors BPB increased the DNA migration in a dose-dependent manner.	4 Abstract and important details have not been provided enabling assessing the data.	Low	Sinan GH, Bayülken DG & Tüylü BA. Assessment of the genotoxicity of butylparaben in human lymphocytes using the comet assay and cytokinesis-block micronucleus test. **2017**. The Turkish Journal of Occupational/Environmental Medicine and Safety, 2(1 (1)), 229-229.

3	MCF-10A (ATCC, #CRL-10317) human breast epithelial cells MCF-7 (ATCC, #HTB-22) and MDA-MB-231 (ATCC, #HTB-26) human breast cancer cells Comet assay ±FpG	The cells were treated with **BP** for 6 and 24 h in preliminary experiments (100 and 200 µM) or for 24 h in the main experiment (100 µM=19.4 µg/mL). For each concentration, four slides (50 cells each) were prepared simultaneously: 2 for assessment without Fpg and the other 2, for FPG. % DNA in tail was used as the index of DNA damage. Image analysis system (Comet IV, Perceptive Instruments, UK) was used. Hydrogen peroxide used as a positive control.	Butylparaben (BP, #54680) from Sigma- Aldrich.	**Negative** No increase in comparison to control cells was observed.	2 In the main experiment only one concentration of BPB was used.	Limited The cells used are not recommended for regulatory purposes testing.	Roszak J, Domeradzka-Gajda K, Smok-Pieniążek A, Kozajda A, Spryszyńska S, Grobelny J, Tomaszewska E, Ranoszek-Soliwoda K, Cieślak M, Puchowicz D, Stępnik M. **2017.** Genotoxic effects in transformed and non-transformed human breast cell lines after exposure to silver nanoparticles in combination with aluminium chloride, butylparaben or di-n-butylphthalate. Toxicology in Vitro, 45, 181-193.
4	Human keratinocytes: HaCaT and SVK14 cell lines (both from ATCC, USA) Comet assay	Cells were exposed to BPB (10, 100, 250 µg/mL) for **24 h**. As a positive control, 1% H2O2 in PBS was applied for 15 min at 4°C. The experiment was repeated 3x in triplicates. From each sample, 100 cells were scored using the CometScore 1.5 software. The median values from each measurement were used for the amount of DNA in the head, the	BPB from (TCI) TOKYO Chemical Industry CO., LTD CAS 94-26-8	**Negative**	1 One time of exposure was used.	High	Chrz J, Hošíková B, Svobodova L, Očadlíková, D, Kolářová H, Dvořaková M, & Mannerström M. **2020**. Comparison of methods used for evaluation of mutagenicity/genotoxicity of model chemicals-Parabens. Physiological Research, 69(Suppl 4), S661. doi.org/10.33549/physiolres.934615

		Olive moment and DNA in the tail.					
5	Human lymphocytes from blood of healthy female donors Comet assay	Cells treated with BPB at 0.1, 0.25 or 0.5 **mg/L** for 24 h. DNA damage evaluated using Comet Assay IV software (Instem LSS Ltd., Staffordshire, UK), by measuring tail intensity. For each concentration, as well as for solvent (positive) and negative controls, 200 comets were analyzed.	Butylparaben (CAS Number: 94-26-8) (Sigma-Aldrich, St. Louis, MO, USA)	**Weakly positive** Slight increase (1.8x vs. untreated control, but significant and concentration dependent) was observed for the 2 highest BPB concentrations.	2 No standard positive control substance was used to validate the system. Very low concentrations of BPB were used (the highest was 0.5 µg/mL).	Limited	Todorovac E, Durmisevic I, Cajo S, Haverić A & Mesic A. **2020**. Evaluation of DNA and cellular damage caused by methyl-, ethyl- and butylparaben in vitro. Toxicological & Environmental Chemistry, DOI: 10.1080/02772248.2020.1851690

The SCCS comment on *in vitro* comet assay results, based on Table 2.4:

Butylparaben was tested using ***in vitro*** **comet assay**:
- in 1 study of high relevance with a negative result (HaCaT and SVK14 human keratinocytes),
- in 3 studies of limited relevance with a positive result (CHO-K1 cells), weakly positive (human lymphocytes), or a negative result (MCF-10A, MCF-7 and MDA-MB-231 cells),
- in 1 study of low relevance which could not be assessed because of insufficient information.

None of the studies were conducted according to GLP status. The results can only be considered as supportive in the overall WoE, however they may suggest a DNA damaging potential of butylparaben.

Table 2.5: Other *in vitro* assays

	Test system/Test object	Exposure conditions (concentration/duration/ metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
1	Chinese hamster CHO-K1 ovary cells Sister chromatid exchanges	BPB was added (0.1, 0.25, 0.5, 0.75 mM), and the culture was incubated for 3 h. After washing 5-bromo-2-deoxyuridine was added to each culture, and the cultures were incubated in the dark for 27 h (two rounds of replication), after which they were harvested. Two hours before harvesting, colcemid was added. 50 metaphases were scored. MMC and H2O2 were used as positive controls.	Butyl p-hydroxybenzoate (purity > 99%) from Kanto Chemical Co., Inc. (Tokyo, Japan).	**Equivocal** Significant increase in SCE observed only at the highest concentration at which some cytotoxicity was observed.	2 MMC (0.12 µM) induced almost 3-fold increase in SCE. BPB induced slight increase in SCE (by 42%) only at the highest concentration (0.75 µM). S9 fraction was not used. For cytotoxicity the percent of metaphases without differently staining sister-chromatids was used. No historical control values provided.	Limited The test is not recommended for regulatory testing purposes.	Tayama S, Nakagawa Y, Tayama K. Genotoxic effects of environmental estrogen-like compounds in CHO-K1 cells. Mutat Res. **2008** Jan 8;649(1-2):114-25. doi: 10.1016/j.mrgentox.2007.08.006. Epub 2007 Aug 19.

2	8-hydroxy-deoxyguanosine (8OHdG) Human spermatozoa	Cells exposed to BPB at 2.5 mM at 0 and 5 h. DNA damage assessed with fluorescent probe Oxy-DNA kit (Calbiochem, Merck, Whitehouse Station, NJ, USA) conjugated to fluorescein isothiocyanate (FITC) following cell fixation and permeabilization. Flow cytometric analysis.		Positive BPB induced loss of motility and vitality at the 0 h and 5 h incubation time points, while 8OHdG formation was highly significantly elevated.	2 Specificity of the assay is not determined.	Low Validity of the assay cannot be assessed. The test is not recommended for regulatory purposes.	Samarasinghe SVAC, Krishnan K, Naidu R, Megharaj M, Miller K, Fraser B & Aitken R J. **2018**. Parabens generate reactive oxygen species in human spermatozoa. Andrology, 6(4), 532-541.
3	Human peripheral blood leukocytes from two healthy donors as a male and a female Sister chromatid exchanges	The cells treated with BPB (100, 50, 25 and 10 µg/mL), 18.5 µL/mL of DMSO as the solvent control, and 0.3 µg/mL of mitomycin C as a positive control for **24 and 48 h**. SCE were analyzed in 50 metaphase cells (25 cells per donor) per concentration. Totally 200 cells were scored for PI. The results were expressed as the mean number of SCE/cell.	Butyl paraben (butyl 4-hydroxybenzoate, CAS No: 94-26-8, from Sigma-Aldrich (St. Louis, MO, USA)	**Positive** BPB significantly increased the SCE frequency at all concentrations for both treatment time. These increases were of concentration-dependent manner.	1 BPB significantly decreased the PI at the highest concentrations after 48 h.	Limited The test is not recommended for regulatory purposes.	Bayülken GD & Tüylü, AB. **2019**. In vitro genotoxic and cytotoxic effects of some paraben esters on human peripheral lymphocytes. Drug and Chemical Toxicology, 42(4), 386-393. https://doi.org/10.1080/01480545.2018.1457049

The SCCS comment on *in vitro* DNA damage results (based on Table 2.5):

Butylparaben was tested using *in vitro* sister chromatid exchanges test in 1 study of high reliability on human leukocytes with a positive result and in 1 study of limited reliability on CHO-K1 cells with an equivocal result.
Butylparaben was tested in human sperm cells with an Oxy-DNA kit designed to detect 8-hydroxy-deoxyguanosine levels. However, because validity of the test cannot be assessed the results have not been taken into consideration during WoE analysis of genotoxicity.
None of the studies were conducted according to GLP status. The results can only be considered as supportive in the overall WoE, however they may suggest a DNA damaging potential of butylparaben.

In vivo chromosome aberrations/ micronucleus test and in vivo mammalian gene mutation studies were not available in the literature (no Tables added)

Table 2.6: In vivo Comet assay

	Test system/Test object	Exposure conditions (concentration/duration)	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
1	Male ddY mice Comet assay on glandular stomach, colon, liver, kidney, urinary bladder, lung, brain, and bone marrow	Groups of 4 mice were treated once orally with BPB at 2000 mg/kg. After 3 and 24 h, slides were prepared for each analysed organ. To obtain nuclei, the homogenates were centrifuged and the precipitate was re-suspended in chilled homogenizing buffer at 1 g organ weight/mL. The slides were photographed at 200× and 50 nuclei per slide were analysed. The length of the whole comet ("length") and the diameter of the head ("diameter") were measured for 50 nuclei per organ per animal.	p-Hydroxybenzoic acid n-butyl ester CAS 94-26-8, purity >98.0 from Kanto Chemical Co. Inc., Tokyo, Japan	**Inconclusive** According to the authors BPB did not yield a statistically significant increase in DNA damage in any of the organs studied.	3 No data on cell cytotoxicity after isolation have been provided. No positive control substance has been used. The method of comet scoring is not clear (manual or automatic?), it seems to be developed by the laboratory, however no validation of the method	Low	Sasaki YF, Kawaguchi S, Kamaya A, Ohshita M, Kabasawa K, Iwama K, Taniguchi K, Tsuda S. The comet assay with 8 mouse organs: results with 39 currently used food additives. Mutat Res. **2002** Aug 26;519(1-2):103-19. doi: 10.1016/s1383-5718(02)00128-6.

		Migration was calculated as the difference between length and diameter for each of 50 nuclei. Mean migration of 50 nuclei from each organ was calculated for each individual animal.			has been described even in the previous papers by the authors. The result after 24 h in colon indicates an increased DNA damage (12.3±2 vs. 6.87±1 in control), however the number of animals per group (N=4) is too low according to OECD TG 489 (a minimum of 5 analysable animals of one sex).		
2	Human sperm cells Comet assay	Semen samples were immediately analysed (N=132). Comet extent, tail distributed moment (TDM), and percent DNA located in the tail (Tail%) for 100 sperm in each semen sample using VisComet software	/	**Positive** A statistically significant positive association between BPB concentration in urine and Tail% (p for trend=0.03).	1	Limited The relevance of the results is not clear at the moment without further research.	Meeker JD, Yang T, Ye X, Calafat AM & Hauser R. **2011**. Urinary concentrations of parabens and serum hormone levels, semen quality parameters, and sperm DNA damage. Environmental health perspectives, 119(2), 252-257.

		(Impuls Computergestutzte Bildanalyse GmbH, Gilching, Germany).					
3	Wistar albino rats Comet assay	5 groups with 6 rats. 200, 400 and 800 mg/kg bw/day BPB daily by oral gavage to male rats for **14 days**. At the end of the experiment blood samples were taken from heart. Genotoxic effect was measured in blood and liver samples with Comet assay.	Not provided	**-** According to the authors DNA damage level was statistically different from treatment groups compared to the oil control groups.	4 It is a abstract and important details have not been provided enabling assessing the data.	Low	Öztascı B, Barlas N. P10-064. Investigation of genotoxic effects of butylparaben (butyl 4-hydroxybenzoate) on pubertal male rats. Abstracts / Toxicology Letters 258S (2016) S62–S324.
4	Rats Comet assay on blood leukocytes and hepatocytes	8 groups of 6 rats: orally at 200, 400, or 800 mg/kg/day for 14 days and orally at 100, 200, or 400 mg/kg/day for the 28 days. Animals receiving only corn oil or a 60 mg/kg methyl methanesulfonate (MMS) intraperitoneal injection 24 hours before dissection served as control groups. 100 cells were analysed using the Comet Assay IV image analysis system (Perceptive Instruments/Instem, Suffolk, UK): tail moment, the intensity of the comet	Not provided	**Positive** DNA damage parameters were statistically significantly increased in leukocytes after 14 and 28 days with higher values after 14 days. In hepatocytes generally higher values of DNA damage parameters were observed	2 Hepatocyte isolation procedure lacks important details, *e.g.* cytotoxicity assessment.	Limited	Çömezoğlu B, Barlas N. Potential Genotoxic Effects of Butylparaben (Butyl 4-Hydroxybenzoate) in Lymphocytes and Liver Samples of Pubertal Male Rats. Erciyes Med J. 2022; 44(3): 279-285 | DOI: 10.14744/etd.2021.70750.

		tail (% of migrated DNA) and tail length (µm).		after 28 days of exposure.			

The SCCS comment on *in vivo* Comet assay study results (based on Table 2.6):

Butylparaben was tested using *in vivo* Comet assay after oral administration:
- in 2 studies of limited relevance with positive results (human sperm cells and rat blood leukocytes and hepatocytes),
- in 2 studies of low relevance which could not be assessed because of insufficient information or with an inconclusive result (cells from glandular stomach, colon, liver, kidney, urinary bladder, lung, brain, and bone marrow).

Based on the available study results on *in vivo* comet assay with butylparaben (Table 2.6) a DNA damaging effect cannot be excluded.

No other *in vivo* assays were available in the open literature (no Tabe added)

Table 2. 7. Summary of available data on carcinogenicity of butylparaben

	Results	REFERENCE	Reliability/Relevance/comments
1980	Negative results were reported in study on **mice** using the same doses (0.15, 0.3, or 0.6%? with diet??) but for a 106-week treatment time (Odashima, 1980). In the **rat**, butylparaben (0.6 or 1.2%) in the diet for up to 104 weeks did not produce any carcinogenic effects (Odashima, 1980).	Odashima, 1980 – publication not available	Unknown due to lack of availability
1985	… Table 2. Incidence of tumours in mice given n- or i-BHB in the diet for 102 wk Concn of hydroxybenzoate (% in diet) / Sex (no. of mice) / No. of 'effective' mice (% of total)* / No. of tumour-bearing mice (% of 'effective' mice) / No. of tumours 0 (control) M (50) 12 (24) 8 (67) 9† F (50) 22 (44) 12 (55) 12 **n-BHB** 0.15 M (50) 11 (22) 7 (64) 7 F (50) 31 (62) 14 (45) 14 0.3 M (50) 13 (26) 11 (85) 11 F (50) 22 (44) 8 (36) 8 0.6 M (50) 16 (32) 11 (69) 11 F (50) 34 (68) 21 (62) 22† **i-BHB** 0.15 M (50) 17 (34) 12 (71) 16‡ F (50) 27 (54) 10 (37) 10 0.3 M (50) 14 (28) 9 (64) 10† F (50) 30 (60) 12 (40) 12 0.6 M (50) 14 (28) 12 (86) 14§ F (50) 29 (58) 13 (45) 13 n-BHB = Butyl-*p*-hydroxybenzoate i-BHB = Isobutyl-*p*-hydroxybenzoate *'Effective' mice are those that survived for >78 wk and those that died with tumours during the experimental period (terminated at 106 wk). †One mouse had two tumours. ‡Four mice each had two tumours. §Two mice each had two tumours.	Inai, 1985	Limited reliability **1. Inai, 1985: rather poor survival** after 2 years, especially of controls and especially of males; at 0.6% survival ratio even higher than in controls (*e.g.* M: 16/50 vs 12/50 and F: 34/50 vs 22/50). - For comparison, please see combined survival data from NTP studies on B6C3F1 (Rao&Crockett, 2003) – mean survival in control dosed-feed groups was >74% (Table 3). - All tumor incidences of B6C3F1 mice (Rao&Crockett, 2003) was rather high

	Table 3. Incidence and time to development of neoplasms at different sites in mice given n- or i-BHI Incidence and time to development of neo Concn of hydroxybenzoate (% in diet) / Sex / Parameter* / All sites / Thymus and lymph node / Bone marrow / Lung / Soft tissue / Liver 0 (control) M N 9 3 (25) 1 (8) 3 (25) 1 (8) 0 T 53 ± 20 51 ± 20 68 70 ± 2 32 — F N 12 9 (41) 1 (5) 2 (9) 0 0 T 52 ± 20 44 ± 12 62 87 ± 12 — — n-BHB 0.15 M N 7 4 (36) 0 2 (18) 0 1 (9) T 54 ± 27 35 ± 9 — 93 ± 11 — 54 F N 14 8 (26) 0 4 (13) 1 (3) 0 T 79 ± 22 71 ± 21 — 92 ± 10 77 — 0.3 M N 11 5 (38) 2 (15) 2 (15) 2 (15) 0 T 55 ± 25 51 ± 9 38 ± 9 76 ± 21 64 ± 43 — F N 8 4 (18) 1 (5) 2 (9) 0 0 T 66 ± 20 57 ± 20 50 84 ± 12 — — 0.6 M N 11 2 (13) 3 (19) 3 (19) 2 (13) 1 (6) T 63 ± 18 63 ± 2 57 ± 5 75 ± 24 46 ± 12 81 F N 22 12 (35) 1 (3) 8 (26) 0 0 T 69 ± 25 61 ± 25 71 81 ± 22 — — • The estimated daily maximum ingested dose of n-BHB was about 40 mg/mouse (c. 940 mg/kg body weight). Such a dose is equivalent to a daily intake of about 65.8 g for man.		(depending on caging and diet type) >56% (Table 4). **2. Inai, 1985: 2 fold increase in tumor number at 0.6%** (especially in the lung) in females – hence, if survival was satisfactory, then tumor incidence could have been higher. **3. Inai, 1985: only one species tested** – guidelines require testing on two species, mice and rats.
1986	In a study on short term effects of various phenols and acids on the histological changes and the thymidine labelling index in the rat forestomach, finely ground substances of the p-hydroxybenzoic acid ester series were fed to groups of 5 weanling male Fischer 344 rats at 4% in the diet for 9 days. No proliferating activity was found with the free acid and the methyl ester. With the ethyl, propyl, and butyl ester, the activity in the prefundic region of the forestomach increased with alkyl chain length, 4% butyl paraben in the diet being nearly as effective as 2% BHA.	Rodrigues, 1986	Results showing changes in non-glandular stomach (forestomach) are of limited relevance for humans.

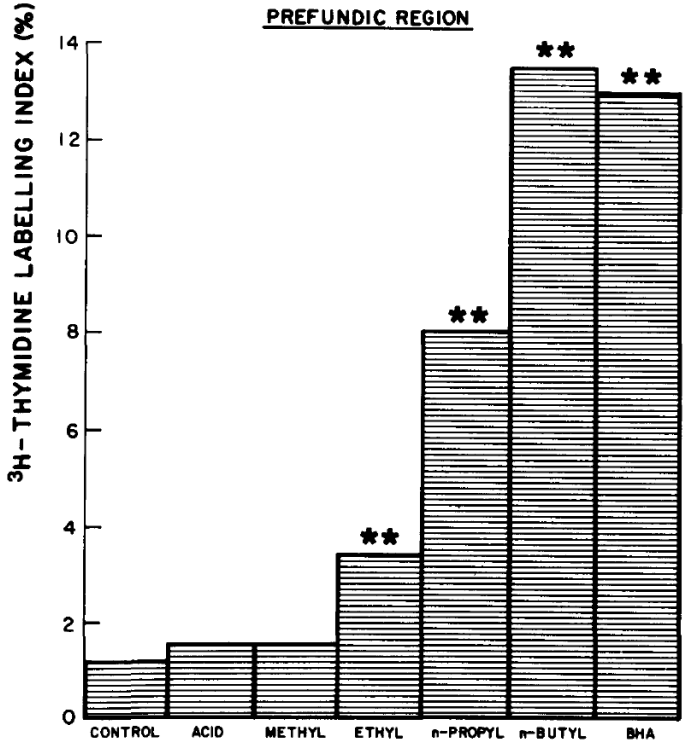


Fig. 4. The effect of 9 days exposure to 4% 4-hydroxybenzoic acid, 4% methyl 4-hydroxybenzoic acid ester, 4% ethyl 4-hydroxybenzoic acid ester, 4% *n*-propyl-4-hydroxybenzoic acid ester, 4% *n*-butyl-4-hydroxybenzoic acid ester or 2% BHA in the diet on the [*methyl*-^{3}H]thymidine labelling index in the prefundic and midregion of the male Fischer 344 rat forestomach (5 rats/group). $^{**}P < 0.01$.

- Among animals that received 4% n-butyl- and 4% n-propyl-4-hydroxybenzoic acid esters thickening of the mucosa along the lesser curvature was observed.
- Marked thickening of the mucosae with acanthosis, hyperkeratosis, prominent rete pegs and papillae were present along the lesser curvature in animals treated with **4% n-butyl-** and 4% n-propyl-4-hydroxybenzoic acid esters.

	• Ethyl, n-propyl- and **n-butyl-4-hydroxybenzoic acid esters** act entirely on the prefundic region of the forestomach epithelium proximate to the fundic mucosa • Authors: "Combined with our previous studies showing that the effects of BHA are dose dependent and show an apparent no observable effect level at a dietary level of 0.25%, the present results tend to emphasize the probable in vivo specificity of the effect of BHA on specific forestomach cells".		
2004	• Butyl paraben is not used as a food additive. • Limited in vitro data on the butyl ester (Bu-PB) suggest it may follow a different metabolic pathway. • The only long-term study specifically designed to address carcinogenicity was conducted on **Bu-PB** in mice, given up to 0.6% in the diet for two years. It reported no significant difference in tumour rates between treated and control animals but was **inadequate for assessment due to early deaths in treated and control groups and relatively high incidence of some tumours in the control group**. • A number of special studies on cell proliferation in the forestomach and glandular stomach of rats have been carried out using finely ground powdered parabens, fed for 9 days at up to 4% in the diet. Me-PB was without activity, Et-PB showed minimal activity, whilst Pr-PB and **Bu-PB** induced cell proliferation in the pre-fundic region of the forestomach. The potency depended on the alkyl chain length; 4% Pr-PB and **Bu-PB** had activities equivalent to 0.5% and **2% dietary BHA** respectively (Rodriguez *et al.*, 1986).	The EFSA Journal (2004) 83, 1-26	Summary opinion
2005	**9.3. Carcinogenicity** In eight-week-old female and male ICR/Jcl mice, oral administration of butylparaben (0.15, 0.3, or 0.6%) in the diet for up to 102 weeks produced neoplasms in the hematopoietic system, including thymic lymphoma, non-thymic lymphoid leukemia, and myeloid leukemia. Additionally, a moderately high incidence of lung adenomas and adenocarcinomas and of soft tissue myosarcomas and osteosarcomas were found. Tumor incidences, however, were not significantly different from those of the control group (Inai *et al.*, 1985). AFC (2004) judged this study to be inadequate due to excessive mortality in control and treated groups and high tumor incidences in the control group. Negative results were also reported in another study in mice using the same doses but for a 106 week	Butylparaben [CAS No. 94-26-8]. Review of Toxicological Literature Prepared for National Toxicology Program (NTP)	Summary opinion

	treatment time (Odashima, 1980). In the rat, butylparaben (0.6 or 1.2%) in the diet for up to 104 weeks did not produce any carcinogenic effects (Odashima, 1980).		
2018	**3.6 Genotoxicity / Carcinogenicity** Butylparaben was not genotoxic in an Ames assay (tested up to 1,000 mg/plate) and in Chinese hamster CHO-KI ovary cells. A 1-3% increase in polypoid cell production was found in Chinese hamster cells at 0.06 mg/mL (only dose tested), however no indications for chromosomal aberrations were found in Chinese hamster fibroblasts when butylparaben was tested at 60 mg/ml. An *in vivo* comet assay, in which animals were dosed with 2,000 mg/kg butylparaben, did not indicate treatment-related DNA damage. Taking all these data into account, butylparaben is not considered genotoxic [NTP, 2005; NICNAS, 2018]. Carcinogenic effects were investigated in mice (0.15, 0.3, or 0.6% in diet) after oral administration for up to 102 weeks. There were no statistically relevant findings that could be related to the treatment. However, as tumour incidences and mortality was high in both control and treatment groups, the reliability of the study was put into doubt by the EFSA Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) in 2004 [Cited in NTP, 2005, EFSA 2004]. In a similar study in which mice were treated with the same doses for 106 weeks, no carcinogenic effects were identified. In rats, oral administration of butylparaben (0.6 or 1.2% in the diet) did not reveal carcinogenic potential [NTP, 2005].	RIVM. Review on butylparaben: exposure, toxicity and risk assessment With a focus on endocrine disrupting properties and cumulative risk assessment RIVM Report 2018-0161	Summary opinion
2018	In mice fed **butylparaben** at 0, 0.15, 0.3 or 0.6 % in the diet for 106 weeks, tumour incidence was increased and time to tumour development was decreased in animals treated with the test chemical. However, the findings were not statistically significant (Inai *et al.*, 1985).	NICNAS	Summary opinion

References:

1. Butylparaben [CAS No. 94-26-8]. Review of Toxicological Literature Prepared for National Toxicology Program (NTP).
2. EFSA. Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a Request from the Commission related to para hydroxybenzoates (E 214-219). The EFSA Journal (2004) 83, 1-26.
3. Ghanta N Rao, Patrick W Crockett. Effect of diet and housing on growth, body weight, survival and tumor incidences of B6C3F1 mice in chronic studies. Toxicol Pathol. Mar-Apr 2003;31(2):243-50. doi: 10.1080/01926230390183742.

4. NICNAS, N.I.C.N.a.A.S. Parabens: Human Health Tier II Assessment, Accessed August 2018. 2018; Available from: https://www.nicnas.gov.au/chemical-information/imap-assessments/imap-group-assessment-report?assessment_id=1714.
5. Odashima S. Cooperative programme on long-term assays for carcinogenicity in Japan. IARC Sci Publ. 1980;(27):315-22. PMID: 7439964 No abstract available.
6. Rodrigues, C., Lok, E. Nera, E.A., Iverson, F., Page, D., Karpinski, K. and Clayson, D.B. (1986). Short-term effects of various phenols and acids in Fisher 344 male rat forestomach epithelium. Toxicology, 38, 103–117.

Appendix 3 on BMD modelling

Benchmark Dose Modeling: Report

European Food Safety Authority (EFSA)

4. Table of Contents

5. A. Data Description

Brief general description of the data. This section should include a table summarizing the data. In case that raw data is available, resulting in a too large table, summary statistics may be given instead. For quantal endpoints both the number of responding animals and the total number of animals should be given for each dose level; for continuous endpoints either the individual responses or the mean responses and the associated SDs (or SEMs) and sample sizes should be given for each dose level.

The endpoint to be analyzed is: mean.

Subset of the data is taken for dose, retaining value(s) 0, 10, 100, 500.

Data used for analysis:

dose	mean	sdt	n
0	2.13	0.1	16
10	2.14	0.1	13
100	2.01	0.1	15
500	2.00	0.1	17

6. B. Software Used

Results are obtained using the EFSA web-tool for Bayesion BMD analysis, which uses the R-package [BMABMDR] version 0.0.0.9057 for the underlying calculations.

7. C. Justification of any deviation from the procedure and assumptions

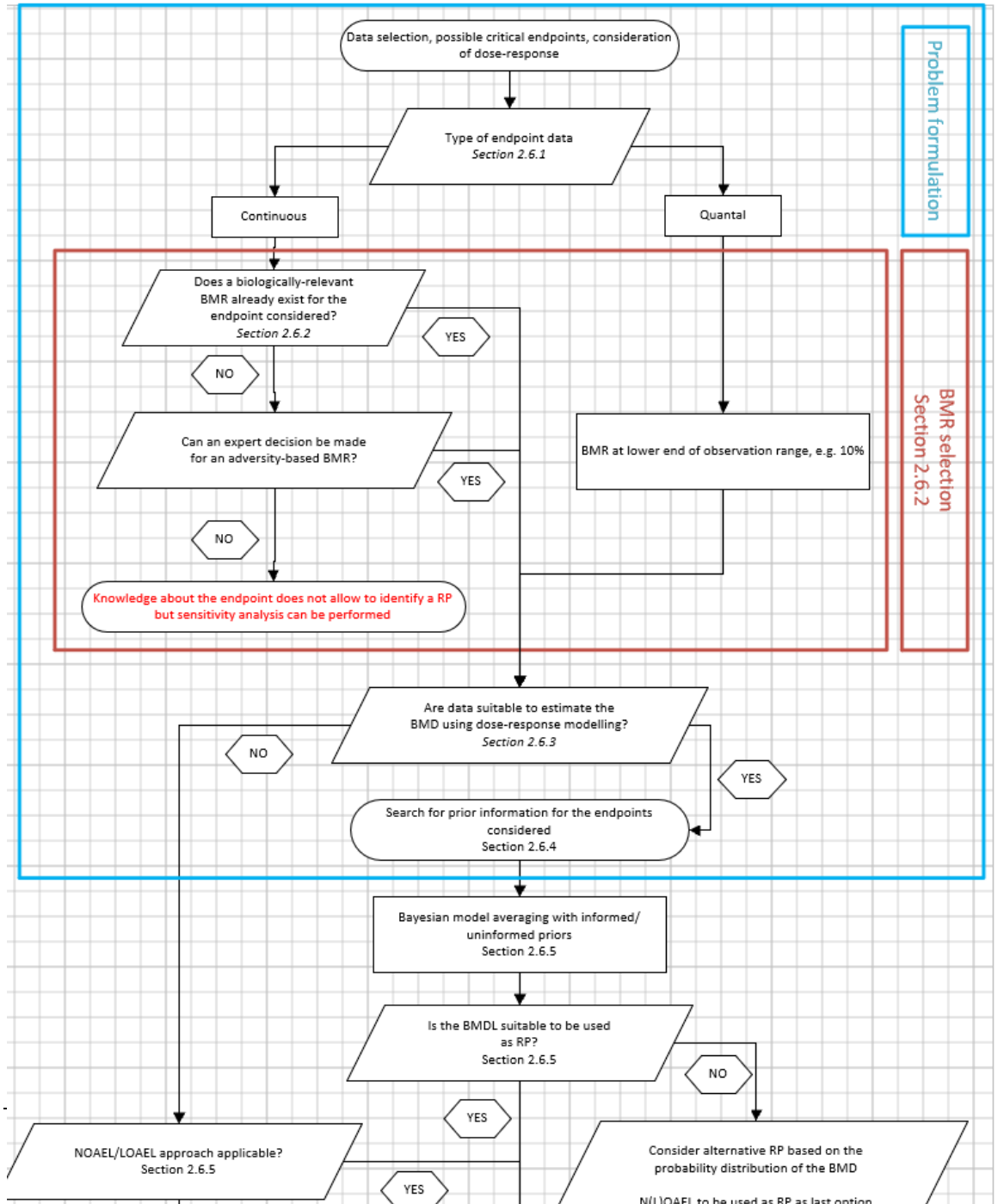
- *In case another approach than Bayesian model averaging was used, the rationale and details for deviating from the recommended approach should be provided.*

- *Assumptions made when deviating from the recommended defaults in this guidance document (e.g. gamma distributional assumption instead of normal and log-normal, heteroscedasticity instead of homoscedasticity).*

- *Other models than the recommended ones listed in Tables 2 and 3 of this guidance document that were fitted should be listed, with the reasons to include them.*

- *Description of any deviation from the procedure described in the flow chart to obtain the final BMD credible interval.*

The ‘Sampling Method’ is set to Bridge Sampling which differs from the default value. Please justify this deviation.


Problem formulation
BMR selection
Section 2.6.2
Data selection, possible critical endpoints, consideration of dose-response
Type of endpoint data
Section 2.6.1
Continuous
Quantal
Does a biologically-relevant BMR already exist for the endpoint considered?
Section 2.6.2
YES
NO
Can an expert decision be made for an adversity-based BMR?
YES
NO
BMR at lower end of observation range, e.g. 10%
Knowledge about the endpoint does not allow to identify a RP but sensitivity analysis can be performed
Are data suitable to estimate the BMD using dose-response modelling?
Section 2.6.3
NO
YES
Search for prior information for the endpoints considered
Section 2.6.4
Bayesian model averaging with informed/ uninformed priors
Section 2.6.5
Is the BMDL suitable to be used as RP?
Section 2.6.5
NO
YES
NOAEL/LOAEL approach applicable?
Section 2.6.5
YES
Consider alternative RP based on the probability distribution of the BMD

Flowchart to derive a Reference Point (RP) from a dose-response dataset of a specified endpoint, using BMD analysis

8. D. Results

Information pertaining to this endpoint.

8.1.1 Check for constant variance coefficient of variation

Distributional assumption of constant variance are met, Bartlett test p-value is 1

Distributional assumption of constant variance (on log-scale) are met, Bartlett test p-value is 0.9908

8.1.2 Goodness of Fit

Best fitting model fits sufficiently well (Bayes factor is 1.48e+00).

8.1.3 Model Averaged BMD

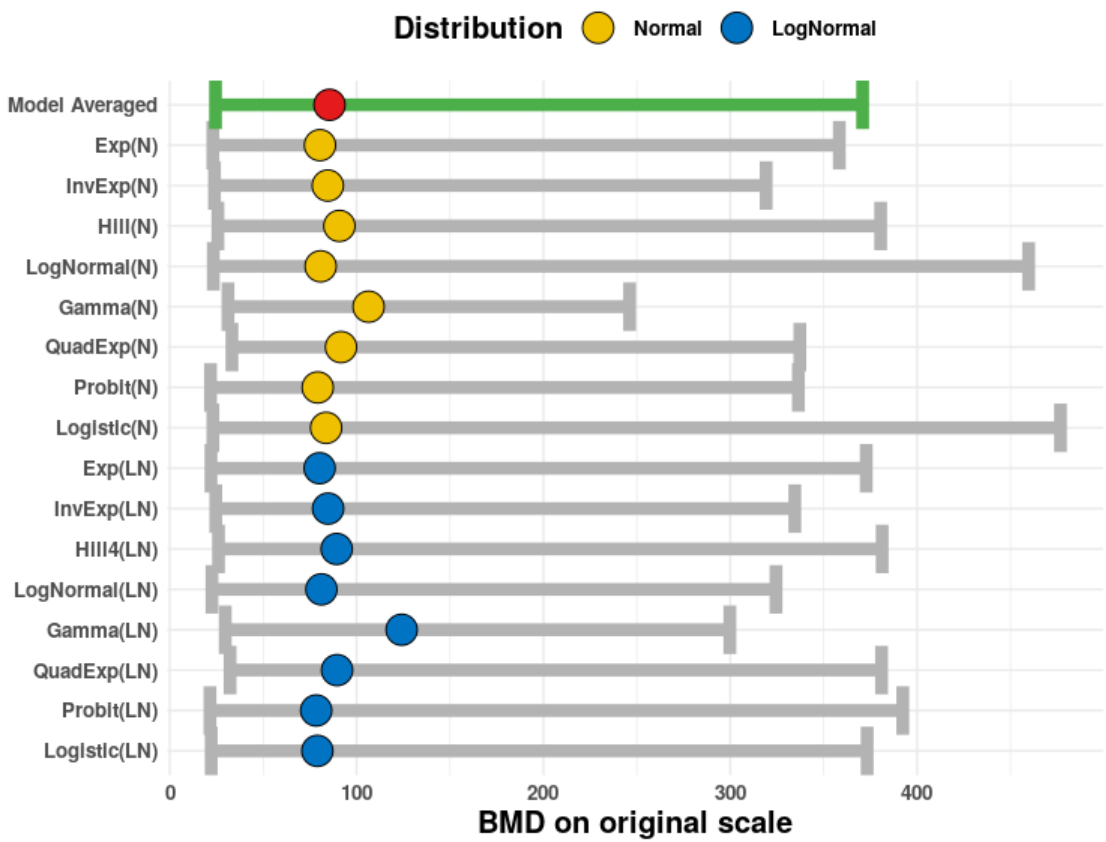
Model	Type	BMDL	BMD	BMDU
Model Averaged	BS	24.503	85.512	370.808

8.1.4 Estimated BMDs per model

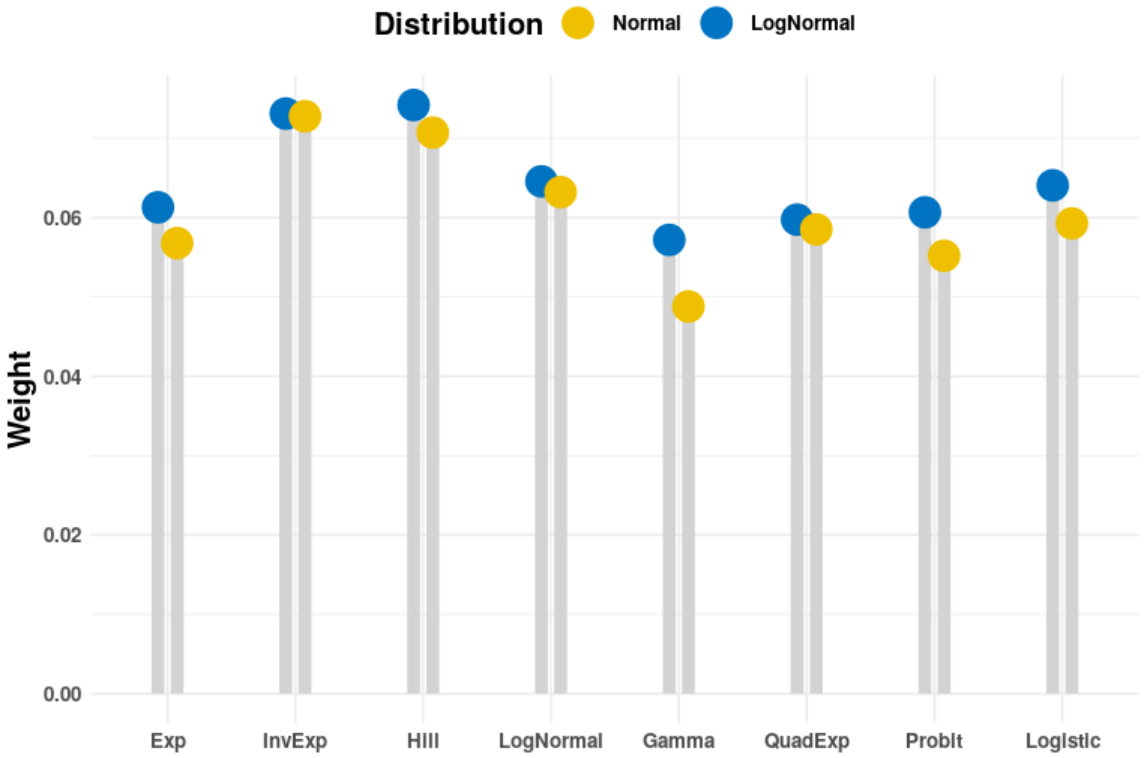
Model	BMDL	BMD	BMDU	Model Weights	Converged
E4_N	23.062	80.405	358.327	0.057	1
IE4_N	23.947	84.601	319.183	0.073	1
H4_N	25.766	90.792	380.459	0.071	1
LN4_N	23.315	80.759	459.673	0.063	0
G4_N	31.174	106.425	246.108	0.049	0
QE4_N	33.284	91.659	337.226	0.059	1
P4_N	21.818	79.200	336.461	0.055	1

L4_N	23.080	83.685	476.712	0.059	0
E4_LN	22.074	80.200	372.762	0.061	1
IE4_LN	24.642	84.758	334.535	0.073	1
H4_LN	26.100	89.341	381.289	0.074	1
LN4_LN	22.585	81.176	324.464	0.065	1
G4_LN	29.701	124.123	299.755	0.057	0
QE4_LN	32.278	89.545	380.946	0.060	1
P4_LN	21.540	78.389	392.325	0.061	1
L4_LN	22.202	78.993	373.235	0.064	1

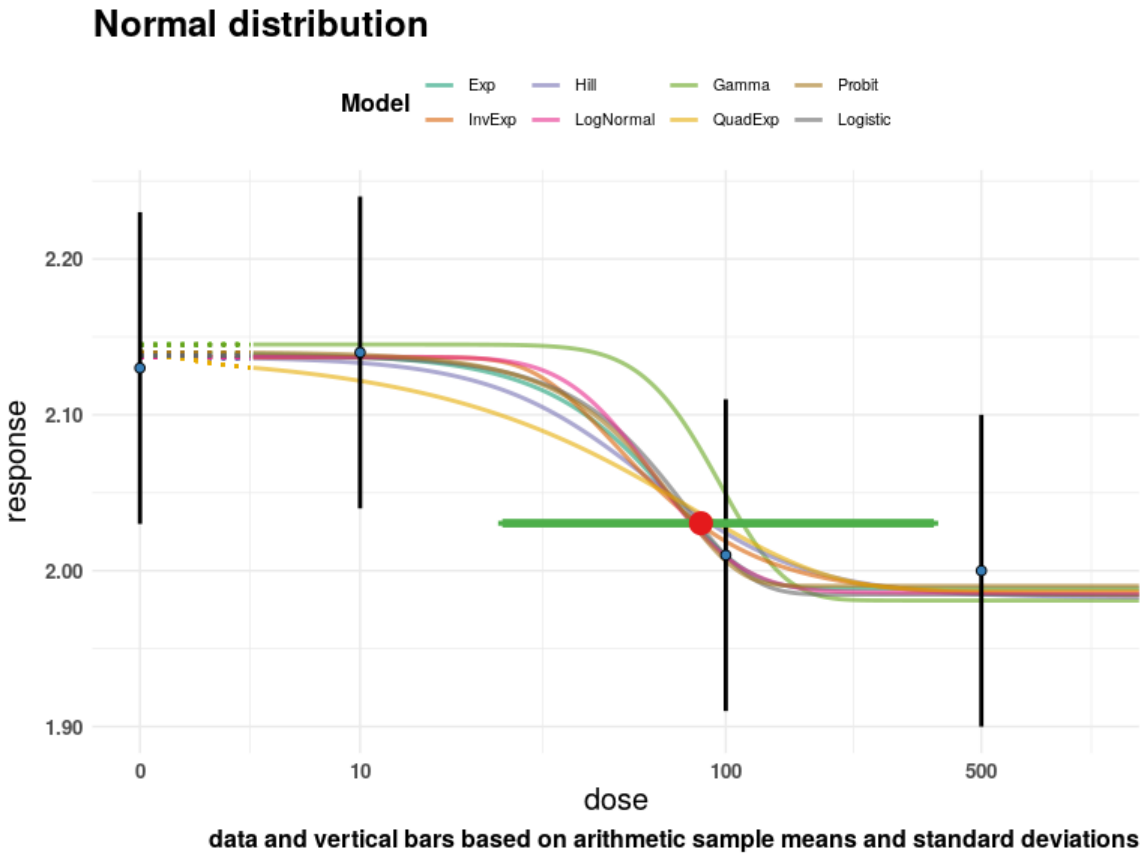
8.1.5 Plots of Fitted Models


Distribution
Normal
LogNormal
Model Averaged
Exp(N)
InvExp(N)
Hill(N)
LogNormal(N)
Gamma(N)
QuadExp(N)
Probit(N)
Logistic(N)
Exp(LN)
InvExp(LN)
Hill4(LN)
LogNormal(LN)
Gamma(LN)
QuadExp(LN)
Probit(LN)
Logistic(LN)
0
100
200
300
400
BMD on original scale

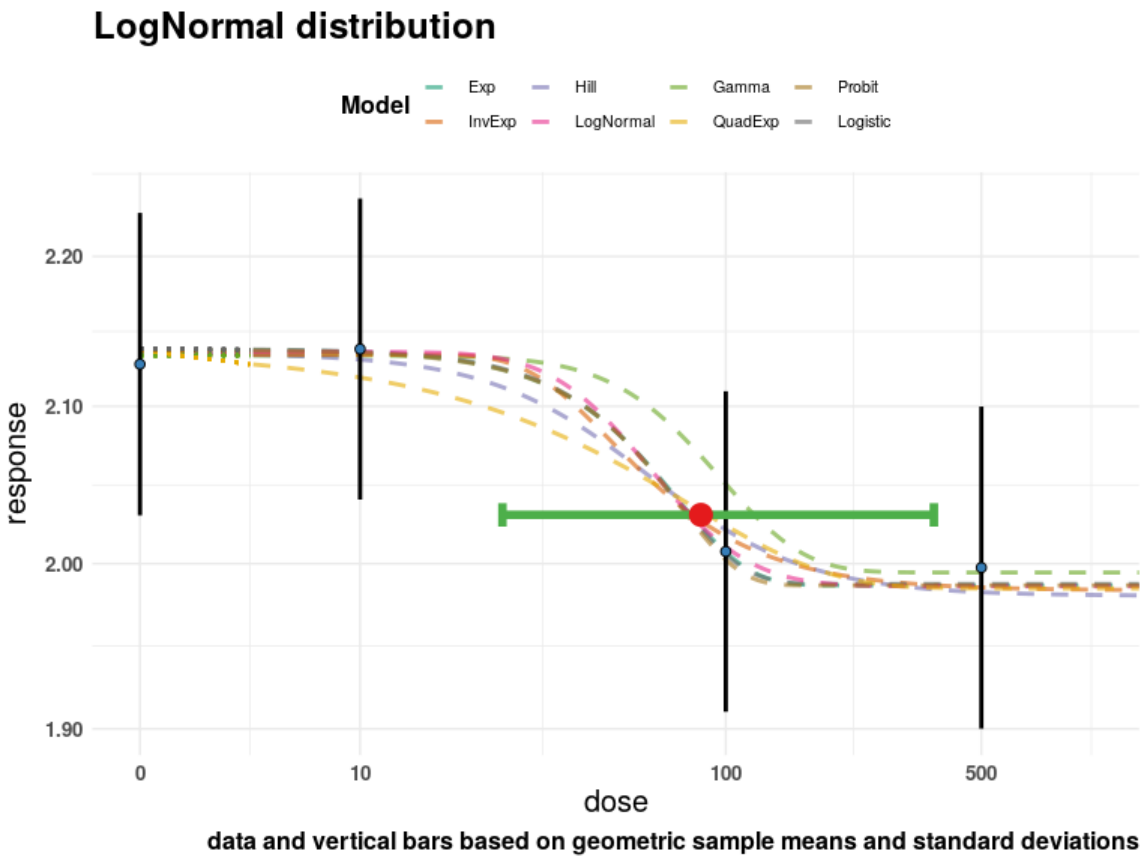
1


Distribution
Normal
LogNormal
Weight
0.06
0.04
0.02
0.00
Exp
InvExp
HIII
LogNormal
Gamma
QuadExp
Probit
Logistic

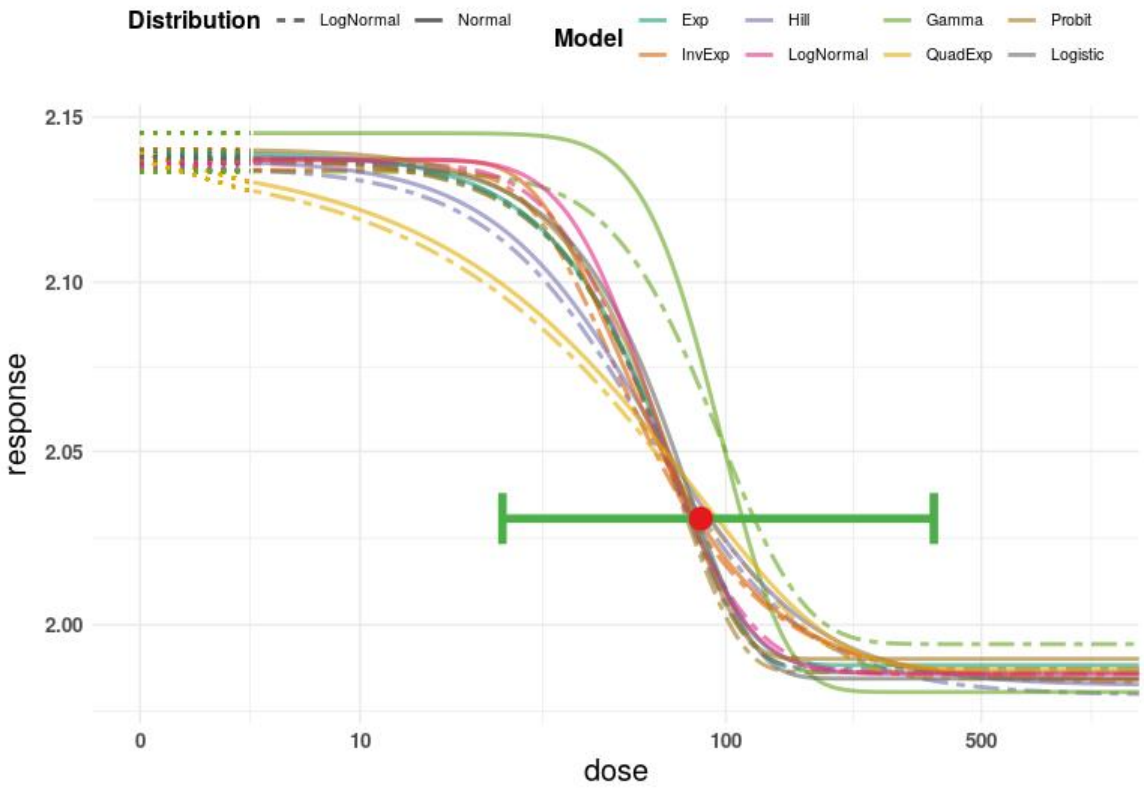
1


Normal distribution
Model
Exp
Hill
Gamma
Probit
InvExp
LogNormal
QuadExp
Logistic
response
2.20
2.10
2.00
1.90
0
10
100
500
dose
data and vertical bars based on arithmetic sample means and standard deviations

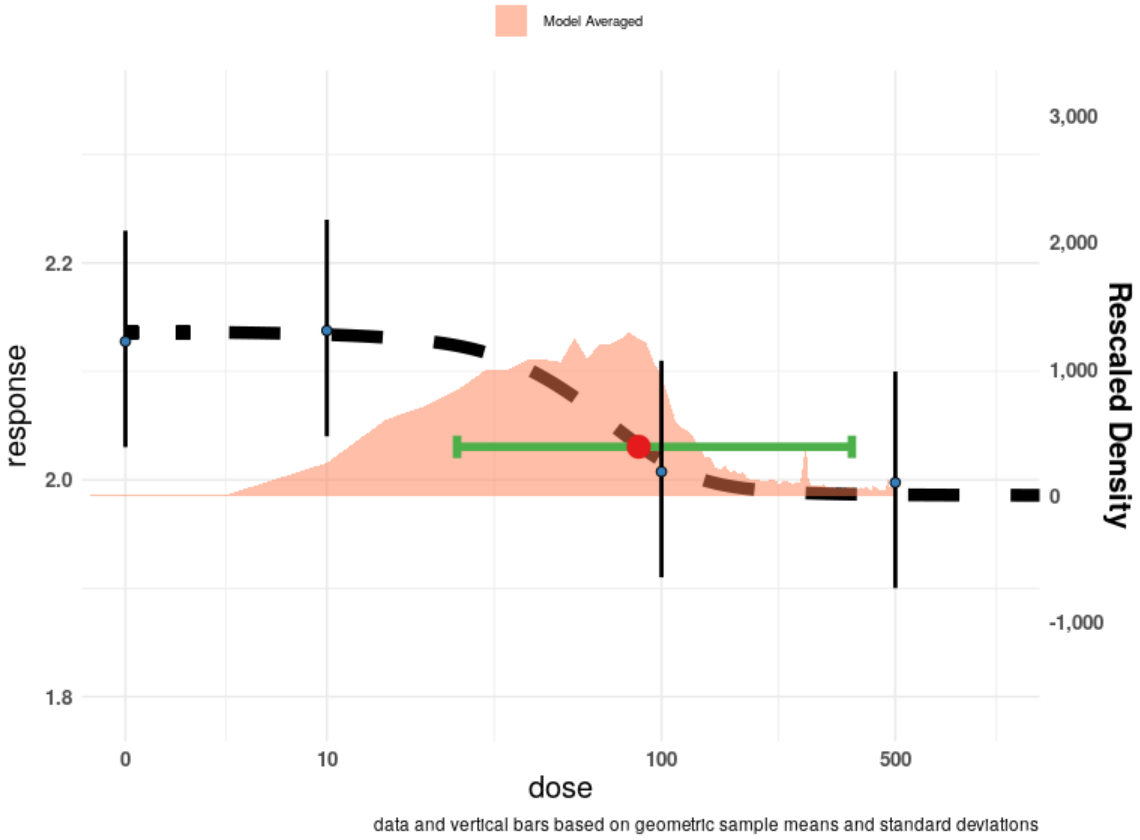
1


LogNormal distribution
Model
Exp
Hill
Gamma
Probit
InvExp
LogNormal
QuadExp
Logistic
response
2.20
2.10
2.00
1.90
0
10
100
500
dose
data and vertical bars based on geometric sample means and standard deviations

1


Distribution
LogNormal
Normal
Model
Exp
Hill
Gamma
Probit
InvExp
LogNormal
QuadExp
Logistic
2.15
2.10
2.05
2.00
response
0
10
100
500
dose

1



9. E. Conclusions

This section should summarize the results for each endpoint (dataset) that was analysed and provide a discussion of the rationale behind selecting the critical endpoint. The BMD confidence interval of the critical endpoint (and the BMDL selected as RP) should be reported and discussed.

10.Appendix

11.References