Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

undecafluorohexanoic acid, PFHxA [1]; sodium undecafluorohexanoate, NaPFHx [2]; ammonium undecafluorohexanoate, APFHx [3]; other inorganic salts of undecafluorohexanoic acid [4]

EC Number: 206-196-6[1]; 220-881-7[2]; 244-479-6[3]; - [4]
CAS Number: 307-24-4[1]; 2923-26-4[2]; 21615-47-4[3]; - [4]

CLH-O-0000007429-65-01/F

Adopted

14 March 2024

14 March 2024

CLH-O-0000007429-65-01/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted on **14 March 2024** by **consensus** an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name:	**undecafluorohexanoic acid, PFHxA [1]; sodium undecafluorohexanoate, NaPFHx [2]; ammonium undecafluorohexanoate, APFHx [3]; other inorganic salts of undecafluorohexanoic acid [4]**
EC Number:	**206-196-6[1]; 220-881-7[2]; 244-479-6[3]; - [4]**
CAS Number:	**307-24-4[1]; 2923-26-4[2]; 21615-47-4[3]; - [4]**

Rapporteur, appointed by RAC: **Betty Hakkert**

Administrative information on the opinion

Germany on **11 April 2023** has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report.

The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **2 May 2023**.

Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **3 July 2023**.

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The following table provides a summary of the Current Annex VI entries, Dossier submitter proposals, RAC opinions and potential Annex VI entries if agreed by the Commission.

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	TBD	undecafluorohexanoic acid, PFHxA [1]; sodium undecafluorohexanoate, NaPFHx [2]; ammonium undecafluorohexanoate, APFHx [3]; other inorganic salts of undecafluorohexanoic acid [4]	206-196-6[1]; 220-881-7[2]; 244-479-6[3]; - [4]	307-24-4[1]; 2923-26-4[2]; 21615-47-4[3]; - [4]	Repr. 1B	H360D	GHS08 Dgr	H360D			
RAC opinion	TBD	undecafluorohexanoic acid, PFHxA [1]; sodium undecafluorohexanoate, NaPFHx [2]; ammonium undecafluorohexanoate, APFHx [3]; other inorganic salts of undecafluorohexanoic acid [4]	206-196-6[1]; 220-881-7[2]; 244-479-6[3]; - [4]	307-24-4[1]; 2923-26-4[2]; 21615-47-4[3]; - [4]	Repr. 1B	H360D	GHS08 Dgr	H360D			
Resulting Annex VI entry if agreed by COM	TBD	undecafluorohexanoic acid, PFHxA [1]; sodium undecafluorohexanoate, NaPFHx [2]; ammonium undecafluorohexanoate, APFHx [3]; other inorganic salts of undecafluorohexanoic acid [4]	206-196-6[1]; 220-881-7[2]; 244-479-6[3]; - [4]	307-24-4[1]; 2923-26-4[2]; 21615-47-4[3]; - [4]	Repr. 1B	H360D	GHS08 Dgr	H360D			

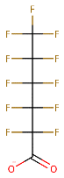
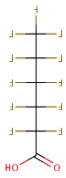
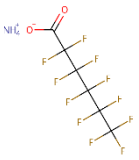
GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Undecafluorohexanoic acid (PFHxA), its sodium and ammonium salts, as well as other inorganic salts, are considered together in this opinion. At neutral pH, PFHxA will be completely transformed into its conjugate anion (PFHx). In aqueous solution, the sodium and ammonium perfluorohexanoate salts will be present as PFHx and the respective cations (Na^+ or NH_4^+). Read-across between the acid and the neutral salts is therefore considered appropriate by RAC.

Some mammalian toxicity studies are conducted with neutral PFHx salts, as the acid has shown to be more irritating. In interpreting the doses administered in these studies, caution should be paid to the relative mass attributed to the active substance (PFHx), which are 92.7% for sodium perfluorohexanoate and 94.2% for ammonium perfluorohexanoate respectively (Table 1).

***Table 1**: Substances considered in the opinion and the percentage of mass[1] attributed to the PFHx anion*

Chemical name	Perfluorohexanoate	Perfluorohexanoic acid	Sodium perfluorohexanoate	Ammonium perfluorohexanoate
CAS nr.	92612-52-7	307-24-4	2923-26-4	21615-47-4
Structural formula				
Average mass	313.047 g/mol	314.054 g/mol	336.036 g/mol	331.085 g/mol
Percentage of mass attributed to the PFHx anion	100.0%	99.3%	92.7%	94.2%

[1]Information retrieved from https://comptox.epa.gov/dashboard/ using CAS nr.

Toxicokinetic considerations regarding elimination

For PFHxA, studies show sex- and species differences in elimination that are important to understand the results from toxicity experiments using different experimental animal species.

In a 28-day oral gavage toxicity study, male rats showed a 1.6- to 3-fold higher PFHxA plasma concentration at the end of the experiment compared to female rats (NTP, 2019) which may be attributed, at least in part, to sex specific elimination kinetics. Hence, male rats are generally expected to have higher PFHxA plasma levels compared to female rats.

A study by Iwai (2011) showed that repeated oral dosing of 50 mg/kg bw APFHx for 13 consecutive days in male and female rats and mice resulted in mean (+/- SD) plasma values of 0.5 (0.0) and 0.3 (0.1) mg/mL in male and female rats at 24h post-dosing, compared to 1.0 (0.3) and 0.5 (0.1) mg/mL in male and female mice, respectively. These data suggested that in these two species, male mice are somewhat less efficient in eliminating PFHxA than female mice, and mice are somewhat less efficient in eliminating PFHxA compared to rats. In the toxicokinetic study, Gannon et al. (2011) illustrated findings that are in line with the latter, and report that mice had a slightly lower elimination of PFHxA compared to rats.

In humans, the elimination half-life has primarily been studied in the male sex. Estimated half-lives range from 5.1 days (Luz et al. 2019) to 14-49 days (Russell et al. 2013). In one study with employees at an airport in northern Sweden accidentally exposed to short-chain PFAS through drinking water, the half-life could not be reliably calculated because no decrease was noted in more than half of the seventeen participants during a 5-month follow-up period (Xu et al. 2020). This shows that humans are less effective in eliminating PFHxA compared to laboratory rodent species.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

There are five studies presented in the CLH dossier for assessment of specific target organ toxicity after repeated exposure (STOT RE):

In a 28-day study, similar to OECD TG 407, SD rats were dosed at 0, 62.6, 125, 250, 500 and 1000 PFHxA mg/kg bw/day via oral gavage to 10 animals/sex/group (NTP, 2019). In males (1000 mg/kg bw/day), terminal mean body weight was decreased by 13% compared to controls. Liver weights were increased in males from 500 mg/kg bw/day onward and in females from 250 mg/kg bw/day onward. Liver enzymes (AST, ALT and ALP) were also elevated in males and females both at 500 mg/kg bw/day. Hepatocyte hypertrophy (minimal to mild severity) was observed in males from 500 mg/kg bw/day onwards and in females at 1000 mg/kg bw/day. A dose-response reduction in haemoglobin (Hb), haematocrit (Ht) and red blood cells (RBC), and some clinical chemistry parameters (e.g. cholesterol) was observed in males and females. Hb and RBCs were decreased by >19% from 500 mg/kg bw/day onward in males and an increase in extramedullary haematopoiesis was observed. From the lowest dose onwards decreased thyroid hormone, T4 (total and free) and T3 (total), plasma levels were observed in males without effects on TSH or thyroid histopathology. Increased incidences in olfactory degeneration and hyperplasia (minimal to moderate severity for all lesions) were observed in males and females from 250 mg/kg bw/day onwards. Kidney weights in males (top dose) and females (500 mg/kg bw/day onwards) were decreased with minimal progressive nephropathy in top dose females.

A combined repeated-dose/reproduction toxicity screening study in SD rats (OECD TG 422) was performed, administering PFHxA at a dose of 0, 50, 150, or 450/300 mg/kg bw/day by oral gavage to 10 animals/sex/group (WIL Research Laboratories, 2005). Excessive mortality was reported at the top dose (450 mg/kg bw/day), which was subsequently reduced on study day four to 300 mg/kg bw/day. Increased liver weight compared to controls was observed in males at the mid and top dose and in females at the top dose. Higher liver weights correlated to hepatocellular hypertrophy (minimal to mild severity, occurring from the mid dose onwards in both males and females) which was reversible in males (in females not assessed due to mortality). Papillary necrosis in the kidney occurred in 5/15 females and 2/11 males at the top dose. In all dosed males, a reduction in Hb was observed. Thymus weight was reduced in top dose males, and top dose males and females had treatment-related changes in lymphoid organs.

A non-guideline, non GLP, 90-day study with a 28-day recovery period was performed in SD rats. PFHxA was administered via oral gavage at doses of 0, 10, 50 and 200 mg/kg bw/day via oral gavage to 10 animals/sex/group (Chengelis et al., 2009). Mean body weights were reduced throughout the study (males and females) and at the end of the study by -11% and -8% for mid and top dose males compared to controls. Liver weight and liver enzyme levels (ALT: +237%)

were increased in top dose males and minimal hepatocellular hypertrophy (7/10 animals) was observed which was reversible after recovery and necrosis was observed in one animal. RBCs, Hb and Ht were reduced by more than 10% in top dose males.

In a 90-day study with 30 and 90 day recovery periods (OECD TG 408), NaPFHx was administered to SD rats via oral gavage in 10 animals/sex/group at dose levels of 0, 20, 100 and 500 mg/kg bw/day (Loveless et al., 2009). Liver weights were reduced in top dose males and females with corresponding non-reversible hepatocellular hypertrophy . Hypertrophy also occurred in 4/10 mid dose males but was reversible after recovery. Liver enzymes (either AST, ALT or ALP) were elevated in males in all dose groups. In top dose males and females, reductions for RBCs, Hb and Ht (-13 to -18% in females, -31 to -36% in males) were observed. In the thyroid of top dose males and females, minimal follicular cell hypertrophy was observed, which persisted until 30 days after cessation of dosing. No data on thyroid hormones was presented. Several lesions were observed in nasal tissue, mainly in the top dose males and females which persisted during the full recovery period of 90 days. Atrophy of the olfactory epithelium was observed in males and females at the mid dose (but resolved after 30 days recovery), and respiratory metaplasia occurred in high dose males and females.

A chronic toxicity/carcinogenicity study in SD rats (OECD TG 453) was performed, administering PFHxA at a dose of 0, 2.5, 15, and 100 mg/kg bw/day (males) and 0, 5, 30, and 200 mg/kg bw/day (females) via oral gavage to 60-70 animals/sex/group (Klaunig et al. 2015). Treatment-related mortalities and clinical signs were observed in top dose females. Body weights were not affected. Renal tubular degeneration and papillary necrosis was observed in top dose females (17/56 versus 1/39 controls). Incidence of hepatocellular necrosis (minimal to severe) was increased in mid- and top dose females (mid dose: 9/60, top dose: 16/70 versus 2/60 controls). The DS notes that this occurred primarily in animals that died before scheduled necropsy. Airway lesions were attributed to reflux injury. Treatment-related effects on tumour incidence were not noted.

The DS concluded that the liver is the primary organ of toxicity, but the information available did not show severe liver effects of relevance to human health at or below the STOT RE guidance values. The DS further concluded that effects observed on the thyroid, kidney and red blood cell parameters did not shown significant and/or severe effects at or below the STOT RE guidance values. With respect to adverse effects on the nose, the DS concluded that hyperplasia of the olfactory epithelium due to degeneration of the tissue seemed plausible, as the olfactory epithelial lesions did not point to gavage-related reflux. While the observed effects occurred below the guidance value for STOT RE Cat 2, the DS noted no conclusions could be drawn about the underlying mechanism (i.e. local irritation or substance-specific systemic effect) and did not further consider these effects in terms of STOT RE classification.

Overall, a classification for STOT RE was considered not appropriate by the DS.

Comments received during consultation

One MSCA and two industry or trade organisations provided comments during the consultation.

The MSCA supported the read-across to other perfluoro carboxylic acids (PFCAs). Regarding STOT RE, the MSCA supported no classification, but noted that the repeated dose studies were not performed in mice, which are regarded as the most sensitive species for testing several PFCAs.

The two industry or trade organisations disagreed with the group approach for PFHxA and its inorganic salt forms and disagreed with the mouse as most appropriate test species.

Assessment and comparison with the classification criteria

Haematological effects

Effects indicative of anaemia were observed consistently across all repeated dose studies, but reductions in red blood cells or Hb that may be considered adverse (i.e. above 10 or 20% change (Muller et al., 2005)) were only seen at high doses of 500 mg/kg bw/day in one 28-day study (NTP, 2019) and a 90-day study (Loveless et al., 2009). This dose exceeds the STOT RE guidance values, and therefore RAC concludes classification based on these observations is not warranted.

Thyroid

In one 28-day study, decreased thyroid hormone (T3 and T4) levels were observed in males from a dose of 62.6 PFHxA mg/kg bw/day onwards in absence of an increase in TSH and histopathologic changes in the thyroid (NTP, 2019). In a 90-day study, minimal follicular hypertrophy was observed at 500 mg/kg bw/day, (Loveless et al., 2009). Thyroid hormone levels were not measured in this study. The thyroid histopathology effects occurred above the STOT RE guidance values, therefore RAC concludes no classification for STOT RE is needed.

Kidneys

Papillary necrosis in the kidney was observed after 90 days exposure in males and females to 450/300 mg PFHxA /kg bw/day (WIL Research, 2005) and after 104 weeks exposure in females to 200 mg PFHxA/kg bw/day (Klaunig et al., 2015). RAC conlcudes the effects on kidneys were observed above the STOT RE guidance value, and therefore no classification is warranted.

Lymphoid system

Histopathological lesions (necrosis and atrophy of thymus and spleen and depletion of lymph nodes) occurred in 450/300 mg/kg bw/day dosed males and females in a combined repeated dose toxicity/reproductive toxicity study (WIL Research Laboratories, 2005). It is unclear if changes occurred at lower dose levels because data were either not reported or not conducted. RAC concludes no classification is possible due to lack of information.

Liver

The liver is the primary organ of toxicity, with dose-dependent effects occurring consistently across all studies with different exposure durations. Several findings, such as liver hypertrophy and changes in enzyme activities, occurred at dose levels below the guidance values (Table 2).

***Table 2**: Liver effects compared to STOT RE guideline values*

Effect	Effect level	Equivalent guidance values	Comparison with STOT RE guidance values
Hepatocellular hypertrophy	150 mg PFHxA/kg bw/d (39 days) In 2/9 female rats Minimal severity	STOT RE 1: ≤ 23.1 mg/kg bw/d STOT RE 2: ≤ 231 mg/kg bw/d	Within STOT RE 2 guidance value
	150 mg PFHxA/kg bw/d (32 days) In 2/10 male rats Minimal severity	STOT RE 1: ≤ 28.1 mg/kg bw/d STOT RE 2: ≤ 281 mg/kg bw/d	Within STOT RE 2 guidance value
	100 mg NaPFHx/kg bw/d (90 days) In 4/10 male rats Minimal severity	STOT RE 1: ≤ 10 mg/kg bw/d STOT RE 2: ≤ 100 mg/kg bw/d	Within STOT RE 2 guidance value

Effect	Effect level	Equivalent guidance values	Comparison with STOT RE guidance values
ALT	20 mg NaPFHx/kg bw/d (90 days) Factor 2.3 higher compared to controls in male rats (P<0.05)	STOT RE 1: ≤ 10 mg/kg bw/d STOT RE 2: ≤ 100 mg/kg bw/d	Within STOT RE 2 guidance value

However, as shown in Table 2, RAC agrees with the DS that these findings were not severe enough to be considered adverse. At dose levels below the STOT RE 2 guidance values, incidences of hepatocellular hypertrophy were low and of minimal severance. The effect on ALT were not dose related in the 90-day study in rats (Loveless et al., 2009). RAC agrees with DS that no classification for STOT RE is needed on the basis of the available information in rats.

Nasal effects

Hyperplasia and degeneration of the olfactory epithelium (minimal to moderate severity) occurred from 250 PFHxA mg/kg bw/day onward in both males and females in a 28-day study (NTP, 2019). This effect can be considered adverse and is below the guidance value of 300 mg/kg bw/day which may warrant classification as STOT RE 2 (Table 5). Olfactory epithelium degeneration also occurred in the 90-day study at 100 mg NaPFHx/kg bw/day (Loveless et al., 2009). Details on the incidences of effects in both studies are provided in Table 3 and 4.

Table 3*: Nasal lesions in the 28 day study with PFHxA in rats (NTP, 2019)*

28-day study with PFHxA in rats	Males						Females					
Dose (mg/kg bw/d)	0	62.6	125	250	500	1000	0	62.6	125	250	500	1000
Number examined	10	10	10	10	10	10	10	10	10	10	10	10
Degeneration of olfactory epithelium (average severity score [a])	0	0	1 (1.0)	6** (1.0)	6** (1.7)	6** (2.7)	0	1 (1.0)	3 (1.0)	9** (1.1)	9** (1.7)	6** (2.5)
Hyperplasia of olfactory epithelium (average severity score [a])	0	0	0	6** (1.0)	5* (1.0)	6** (1.2)	0	0	3 (1.0)	4* (1.0)	7** (1.0)	3 (1.0)

[a] Severity scores: 1=minimal, 2=mild, 3=moderate, 4=severe
*P<0.05; **P<0.01

Table 4*: Nasal lesions in a 90-day study with NaPFHx in rats (Loveless, 2009)*

90-day study with NaPFHx in rats	Males				Females			
Dose (mg/kg bw/d)	0	20	100	500	0	20	100	500
Number examined	10	10	10	10	10	10	11 or 9 [a]	10
Degeneration of olfactory epithelium [b,c]	0	0	4	7	0	0	5	4

[a] 11 animals in the main study and 9 animals in the 90-day recovery group
[b] Severity of the lesions was minimal to mild and not further specified per dose group
[c] After a recovery period of 30 (control and high dose only) or 90 days, degeneration of the olfactory epithelium resolved

Table 5: *Effects on the nose compared to STOT RE guidance values*

Effect	Effect level	Equivalent guidance values	Classification justified
Degeneration of olfactory epithelium	250 mg PFHxA/kg bw/d (28 days) In 6/10 males and 9/10 females Minimal severity	STOT RE 1: ≤ 30 mg/kg bw/d STOT RE 2: ≤ 300 mg/kg bw/d	Within STOT RE 2 guidance value
	100 mg NaPFHx/kg bw/d (90 days) In 4/10 males and 5/11 females Minimal to mild severity	STOT RE 1: ≤ 10 mg/kg bw/d STOT RE 2: ≤ 100 mg/kg bw/d	Within STOT RE 2 guidance value
Hyperplasia of olfactory epithelium	250 mg/kg bw/d (28 days) In 6/10 males and 4/10 females Minimal severity	STOT RE 1: ≤ 30 mg/kg bw/d STOT RE 2: ≤ 300 mg/kg bw/d	Within STOT RE 2 guidance value

There is no information available which shows significant and/or severe target organ toxic effects of relevance to human health and produced at generally low exposure concentrations of PFHxA and its inorganic salts (see Table 5). Assignment to the classification Category 1 (STOT RE) is therefore not appropriate.

Evidence from two repeated dose toxicity studies showed significant adverse effects upon exposure to PFHxA or NaPFHx that are of relevance to human health. Hyperplasia of the olfactory epithelium was observed in a single 28-day study performed with PFHxA (NTP, 2019). There was no dose dependent increase in severity of hyperplasia. Degeneration of the olfactory epithelium was observed in both a 28-day and 90-day study (NTP, 2019 and Loveless et al., 2009, both Klimisch score 1). In a second 90-day study, nasal tissues were examined, but the authors did not report treatment-related nasal lesions (Chengelis et al., 2009, Klimisch score 2). There is no temporal concordance of the effects, especially hyperplasia did not become more apparent in studies with longer exposure duration, such as the 90-day studies (Loveless et al., 2009 and Chengelis et al., 2009) or the 2-year study (Klaunig et al., 2015).

Since no consistent pattern was noted in the available studies and the effects may, in part, be due to reflux, RAC agrees with the DS that no classification for STOT RE is warranted for nasal cavity.

Overall, **RAC concludes that no classification for STOT RE is warranted.**

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

There are six studies presented in the CLH-dossier for assessment of adverse effects on fertility and development by PFHxA or its salts:

- A one-generation reproduction toxicity study in Crl:CD(SD) rats according to OECD TG 415 protocol, administering NaPFHx (100% purity) at a dose of 0, 20, 100, or 500 mg/kg bw/day by oral gavage to 20 animals/sex/group (Loveless et al. 2009).

- A prenatal developmental toxicity study in Crl:CD(SD) rats according to OECD TG 414 protocol administering NaPFHx (100% purity) at a dose of of 0, 20, 100, or 500 mg/kg bw/day on gestational days (GD) 6-20 via oral gavage to 22 pregnant dams/group (Loveless et al. 2009).

- A combined repeated-dose toxicity study with reproduction/developmental toxicity screening test in SD rats according to OECD TG 422, administering PFHxA (85% purity) at a dose of 0, 50, 150, or 450/300 mg/kg bw/day by oral gavage to 10-15 animals/sex/group (WIL Research Laboratories, 2005).

- A 28-day repeated-dose toxicity study in SD rats, similar to OECD TG 407, administering PFHxA (purity >99%) at a dose of 0, 62.6, 125, 250, 500 and 1000 mg/kg bw/day via oral gavage to 10 animals/sex/group (NTP, 2019).

- A 'phase I' reproductive and developmental toxicity study in Crl:CD I (ICR) mice, designed to evaluate the ICH Harmonised Tripartite Guideline including effects on the reproductive process, gestations, parturition, development and lactation, GLP, administering APFHx (purity 93.4%) at a dose of 0, 100, 350, and 500 mg/kg bw/day on GD 6-18 via oral gavage to 20 dams/sex/group (Charles River Laboratories 2011a; Charles River Laboratories 2012; Iwai and Hoberman 2014).

- A 'phase II' reproductive and developmental study in Crl:CD I (ICR) mice (see Phase I), GLP, administering APFHx (purity 93.4%) at a dose of 0, 7, 35 and 175 mg/kg bw/day on GD 6-18 via oral gavage to 20 dams/sex/group (Charles River Laboratories 2011b; Iwai and Hoberman 2014).

In the one-generation reproduction study (OECD TG 415) by Loveless et al. (2009) with rats, no substance-related effects on sexual function and fertility parameters (i.e. mating, fertility, gestation length, number of implantation sites, oestrus cyclicity, sperm parameters, litter size) were recorded, apart from decreased relative F1 adult testes weight at 20 and 500 mg/kg bw/day. No statistically significant changes were seen in the mid dose. Histopathological examination of the testes was not performed in any of the groups. With regard to developmental toxicity, mean pup weights were decreased during lactation and postweaning, up to 17-18% at 500 mg/kg bw/day compared to controls. The effect on body weight persisted until PND 35 and PND 49 in males and females respectively, at the highest dose tested. No other substance-related developmental effects were seen, i.e. on litter size, sex ratio, pup survival at birth, pup clinical observations, sexual maturation, or F1 developmental parameters.

In the developmental toxicity study (OECD TG 414) by Loveless et al. (2009) with rats, no effects on pregnancy or developmental toxicity were observed, i.e. no treatment-related observations were noted on ovaries and uterine content (including gravid uterus weight, number of corpora lutea, implantations, early/late resorptions), or pregnancy duration. A decrease in mean body

weight was observed in F1 pups, up to -10% at 500 mg /kg bw/day (not reaching statistical significance). No other substance-related developmental effects were seen, i.e. on pup viability, sex ratio, external and skeletal alterations, or soft tissue and visceral head examinations. Litter size and litter weight were not reported in this study. In dams, a statistically significant decrease in body weight was observed during gestation (GD 19-21), as well as on net body weight (minus gravid uterus weight; GD 21) at 500 mg /kg bw/day.

In the OECD TG 422 by WIL Research Laboratories (2005) with rats, no effects on sexual function, fertility, or pregnancy were observed, i.e. there were no effects seen on reproductive performance indices, gestation length, and parturition. With regard to developmental toxicity, no effects on litter size, viability, postnatal survival, sex ratio, or pup body weight (no values reported) were seen. Dams showed an increase in mortality (6/15) at the highest dose tested. In this dose group, a decrease in mean body weight (of -5.8%) was seen on GD 20, but on all other days (GD 0-17) mean body weight was not different from controls. Other systemic effects, such as effects on liver weight, haematology, and clinical chemistry observations, were noted in P0 males and females.

In the 28-day study according to NTP protocol (NTP, 2019) with rats, a treatment-related decrease of -13% terminal body weight was reported at 1000 mg/kg bw/day. At this dose, a statistically significant reduction of -25% cauda epididymal sperm count and -18% total sperm count (normalised to grams of cauda epididymis) were seen, as well as a transient decrease in epididymal weight of -5% (not statistically significant from controls). From the lowest dose onwards, males showed reduced total T4, free T4, and total T3 plasma levels. No effect on TSH and testosterone was observed, nor effects on the histopathology of the thyroid gland or the testes and epididymis. In females, no effects on the thyroid hormone system were seen at any dose.

In the 'phase I' reproduction study as summarised by Iwai and Hobermann (2014) based on the Charles River Laboratories reports (2011a; 2012) performed with mice (ICH guideline, GLP), PFHxA treatment-related effects on development were observed. Specifically, an increase in the number of stillborn pups of 5/245 (2%) and 16/177 (9%) was observed at 350 and 500 mg/kg bw/day, respectively, compared to controls (4/221; 1.8%). The effect occurred in five different dams in these groups. Furthermore, the number of liveborn pups dying at PND 0 (21/150; 14.0%) and between PND 1-4 (20/129; 15.5%) was statistically significantly increased at 500 mg /kg bw/day. At the mid-dose, a (non-statistically significant) trend in the number of liveborn pups dying at PND 0 (3/232; 1.3%) and statistically significant increase in the number of liveborn pups dying between PND 1-4 (25/229; 10.9%) was also apparent. At the mid- and high dose, viability indices were significantly reduced at 350 mg/kg bw/day (PND 7) and at 500 mg/kg bw/day (PND 4 and PND 7). Pup body weights were decreased $\geqslant$100 mg/kg bw/day at PND 0, which persisted until PND 20 in the mid- and high dose groups. Lastly, the percentage of pups with delayed eye opening was statically significantly increased at PND 14 in the mid- and high dose groups. In dams, no increased mortality in treatment groups occurred compared to controls. At day 0 postpartum, the mean body weight of dams was slightly increased in the top dose. At other timepoints (including during lactation), no effects on mean body weight were observed at any dose.

Also in the 'phase II' study (on mice with administration of a top dose lower than the mid dose in the 'phase I' study), a statistically significant increase in the number of stillborn pups of 3/241 (1.2%) was seen at 175 mg mg/kg bw/day compared to controls (0/249; 0%), as well as an increase in the number of liveborn pups dying at PND 0 (4/238; 1.4%). At this dose, pup weight was statistically significantly decreased at PND 0 as well. The percentage of pups with delayed eye opening was unaffected in this study at PND 14 compared to controls. Some effects on the eyes (corneal opacity, microphthalmia, lenticular opacity) were seen in pups (from different litters) at the highest dose tested. In dams, no increased mortality in treatment groups occurred

compared to controls. During gestation and lactation, no effect on mean body weights were seen in the dams at any dose.

In a re-analysis, Iwai et al. (2019) changed their interpretation regarding a PFHxA treatment-related effect on the number of stillborn pups, and concluded there was no treatment-related effect of PFHxA on stillborn pups in the phase II study after applying a non-conventional statistical approach of pooling control data from two independent experiments. They provided historical control data for several effects (stillbirth, postpartum mortality, eye anomalies) and they provided weight-of-evidence-based re-analyses of pup viability during the first days of life and eye effects observed in pups.

Conclusions by the DS

Statistical analysis and use of historical control data

The DS mentioned that *"Iwai et al. (2019) suggested using the individual pup as statistical unit. However, litter dependency (intra-litter likeness) was not considered as recommended for analysis of developmental endpoints in offspring when the individual pup is used as the statistical unit according to European Food Safety Authority (EFSA) (2017); Golub and Sobin (2020); Orelien et al. (2002)."*

Furthermore, the DS noted that Iwai et al. (2019) conducted a pooled analysis of the control groups of phase I and phase II studies. The DS noted that this combination of the control is not in accordance with generally accepted procedures for the combination of historical control data (see e.g Guidance Document 116, ENV/JM/MONO(2011)47, OECD (2014)). According to accepted procedures for the use of historical control data (HCD), the control group with the stillborn pups in phase I should have been replaced by HCD. It is noted that such a replacement has to meet certain requirements including the proof that the concurrent control group of phase I is an outlier. For such a proof the exact number of stillborn and alive pups in each historical study would have been needed, but is not provided in Iwai et al. (2019). Only averages and percentages of historical control data were presented in Iwai et al. (2019). On this basis thereof, the DS concluded that the combination of the control groups as described in Iwai et al. (2019) is not acceptable. For these reasons the DS disagreed with the re-analysis and did not consider the conclusion of Iwai et al. (2019).

Sexual function and fertility

Based on the above observations, the DS proposed no classification for sexual function and fertility. There is no information available on PFHxA adverse effects on sexual function and fertility in humans. The effects of PFHxA on the male reproductive system in rats were only seen in a selected set of studies at doses that also evoked a marked decrease in body weight and other systemic effects. No effects on sexual functioning and fertility were observed. The DS therefore considered there is not sufficient evidence for PFHxA and its inorganic salts to warrant classification for sexual function and fertility.

Developmental toxicity

Regarding developmental toxicity, the DS proposed classification in Category 1B; H360D, based on an increase in peri- and postnatal pup mortality, considered to be adverse, treatment- and dose-related. The DS furthermore indicated that the same developmental toxicity pattern of reduced pup survival in mice is reported for perfluoroheptanoic acid (C7) and perfluorooctanoic acid, PFOA (C8), both classified as Repr. 1B; H360D as well as for perfluorononanoic acid (C9), classified as Repr. 1B; H360Df.

Lactation effects

The DS proposed no classification for lactation. The DS specified that no information is available to assess whether PFHxA and its inorganic salts interfere with lactation or is present in breast milk in amounts sufficient to cause concern for the health of a breastfed child.

Comments received during consultation

One MSCA, one national authority, and two industry or trade organisations provided comments during the public consultation.

The MSCA supported the read-across to other perfluoro carboxylic acids (PFCAs) and supported the proposal for classification for adverse effects on development, Repr. 1B, H360D. The MSCA supported no classification for adverse effects via lactation. Regarding the evaluation for adverse effects on fertility, the MSCA supported no classification, but noted that the fertility studies were not performed in mice, which are regarded as the most sensitive species for testing several PFCAs. Consequently, the MSCA reasoned it cannot be ruled out that adverse effects would have been seen in fertility studies when performed with mice.

The national authority welcomed a discussion of the perinatal pup mortality reported between PND 0-4 and whether they may be linked to effects on or via lactation.

The two industry or trade organisations did not agree with the proposal for classification for adverse effects on development, Repr. 1B, H360D. They disagreed with the group approach for PFHxA and its inorganic salt forms, disagreed with the mouse as an appropriate test species, disagreed that the statistical analysis as described in the paper by Iwai et al. (2019) was not robust, commented on the use of Klimisch grade 2 studies in the weight-of-evidence approach by the DS, and commented on the difficulty to distinguish between maternal toxicity and developmental toxicity as the cause for decreased pup weight.

Additional key elements

In the CLH-dossier, reference is made to the RAC opinion on the Annex XV dossier proposing restrictions on PFHxA, its salts and related substances (ECHA 2021). In this opinion is indicated that PFHxA was measured in human milk in the studies by Kang et al. (2016) and Nyberg et al. (2018).

The study by Kang et al. (2016) measured PFHxA in breastmilk of 264 Korean women sampled in 2013. The median and interquartile range was 47 (19-79) pg PFHxA/mL. The detection frequency in these samples was 70.8%. In comparison, PFOA levels in these samples were 72 (52-110) pg/mL (detection frequency 98.5%).

The study by Nyberg et al. (2018) showed that during the last decade, a steep increase in the concentration of PFHxA in mother milk samples from Stockholm was observed, with levels ranging from 34-132 pg/mL in 2016 (N = 10; 100% detection frequency). In comparison, levels of 3-83 pg/mL PFOA were seen in these samples, with an overall decreasing trend.

These two studies indicate that **PFHxA is transferred to breastmilk**.

Assessment and comparison with the classification criteria

Adverse effects on sexual function and fertility

The one-generation OECD TG 415 study with PFHxA provides some indication that the substance may target the testes. In the robust study summary is stated that in F1 adult males *"testes*

weight and relative testis weight (relative to bw) were decreased by 7% and 11% (statistically significant) in the 20 and 500 mg/kg bw/day group, respectively, compared with the control." However, no statistically significant changes in testes weight in the 100 mg/kg bw/day group were observed. Histopathological examination in F1 males was not performed. The data documentation is scarce and original data were not available to the DS. Apart from the above effect reported, there are no other studies indicating PFHxA may target the testes.

In the 28-day study performed by NTP (2019), cauda epididymal sperm counts in male rats were significantly lower (25%) in the highest dose group compared to controls and occurred in the presence of a slight decrease in epididymal weight (5%). These effects on cauda epididymal sperm counts in the rat were only apparent in one subacute (28 days) repeated dose toxicity study at fairly high dose levels (1000 mg/kg bw/day) causing marked (-13%) body weight reductions. Sperm motility and spermatid counts were not affected. In a reproduction/developmental toxicity screening study (OECD TG 422) in rats no effects on fertility were observed. Effects on sexual function and fertility were not seen in the studies ('Phase I/Phase II') conducted with mice.

Comparison with the criteria

Classification of a substance in Category 1A is largely based on evidence from humans. There is no information available which supports classification in this category. Assignment of PFHxA to classification category 1A is therefore not appropriate.

Classification of a substance in Category 1B is largely based on data from animal studies. Such studies shall provide clear evidence of an adverse effect on sexual functioning and fertility or on development in absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate.

Overall, RAC observes the following:

- In the one-generation study (OECD TG 415), the mean weight of the testes was statistically significantly decreased at 20 and 500 mg/kg bw/day, but not at 100 mg/kg bw/day. Histopathological examination of the testes was not performed in any of the groups. Other fertility parameters were not affected. Due to limited and insufficiently reported data, specific effects on fertility cannot be concluded here.

- In one study (28-day, NTP 2019), a statistically significant reduction of -25% cauda epididymal sperm count and -18% total sperm count (normalised to g of cauda epididymis) were seen at 1000 mg/kg bw/day, as well as a transient decrease in epididymal weight of -5% (not statistically significant from controls). Effects on the male reproductive system were only apparent in this one subacute repeated dose toxicity study at fairly high dose levels causing body weight reduction of 13%. Sperm motility and spermatid counts were not affected.

Based on the above, RAC concludes that **no classification for sexual function and fertility** is warranted for PFHxA and its inorganic salts (this is in agreement with the DS proposal).

Adverse effects on development

There are two reproductive and developmental toxicity studies in Crl:CD I (ICR) mice, designed to evaluate the ICH Harmonised Tripartite Guideline including the reproductive process. These studies were conducted under GLP and dosing was from GD 6-18. F1 generation pups were not directly administered the test substance and/or vehicle, but may have possibly been exposed during maternal gestation (*in utero* exposure) or via maternal milk during the lactation period.

According to the CLH-report of the DS as well as in the publications of Iwai and Hoberman (2014) and Iwai et al. (2019), the study with 100, 350 and 500 mg/kg bw/day was conducted first (therefore called 'phase I study'), after which a study with lower dose levels (7, 35, 175 mg/kg bw/day) was conducted (specified as 'phase II study').

However, according to the original study reports, the order appeared to be the other way around. The study with the low doses (7-175 mg/kg bw/day) was conducted first. The original study report specified that, due to no observed mortality and adverse clinical signs in the first study with mice (Charles River Laboratories 2011b, with a top dose of 175 mg/kg bw/day), the study was repeated with higher doses up to 500 mg/kg bw/day (Charles River Laboratories 2011a). Also some pup-data in the original report did not match with the ones of CLH-report, and corrected numbers as well as original numbers are therefore specified in the tables below as well as in the 'Supplemental Information – In depth analysis by RAC'. Some incidences (see tables) in the original study reports appeared higher than presented by the DS, making the conclusions on developmental toxicity even stronger.

Increased number of stillborn pups

In the studies in mice (Charles River 2011a; 2011b), a significantly increased number of stillborn pups at 500 mg/kg bw/day and increased number of stillborn pups at 350 mg/kg bw/day as well as significantly increased number of stillborn pups at 175 mg/kg bw/day were seen as compared to the respective controls (Table 6).

Table 6: *Number of stillborn pups and percentage of stillborn pups relative to the total number of pups delivered in the mouse studies.*

Dose (mg/kg bw/day)	**0**	**7**	**35**	**100**	**175**	**350**	**500**
CRL (2011b), N/N (%)	0/249	0/213	0/232	-	3/241 (1.2)**	-	-
CRL (2011a), N/N (%)	4/221 (1.8)	-	-	0/250	-	5/245 (2.0)	19/180 (11)**
CLH report#	*4/221 (1.8)*	-	-	*0/250*	-	*5/245 (2.0)*	*16/177 (9.0.**)*

**, statistically significant ($p \leqslant 0.01$)

data from CLH report are given if different from the original study report. The degree of pup mortality and number of nests affected was higher (19 instead of 16 pups respectively, in 7 instead of 6 nests).

According to Iwai et al 2019, the HCD for 2004-2015 was 0-1.8%.

Discussion on statistical analysis of stillborn pups

In Iwai et al (2019), the statistical analysis of the Charles River Laboraties (2011b) study was re-assessed. They pooled control data of both mice studies and stated the effects seen at 175 mg/kg bw/day in Charles River Laboratories (2011b) were not statically significant different from the controls. Moreover, it was noted that the incidence in the 175 mg/kg bw/day group was within the HCD of that lab (0-1.8%). The comments made by the DS regarding the combination of control data and the use of historical control data are shared by RAC (see previous section), and the re-assessment of the Charles River Laboratories (2011b) data on stillborn pups in Iwai et al. (2019) is not considered reliable.

The DS also commented it would be inappropriate to use the pup instead of the litter as statistical unit in the analysis, as this would not take sufficiently account of intra-litter likeness. RAC notes that intra-litter likeness is particularly important to take into consideration as there is the possibility that shared genetics and/or the maternal environment causes a strong similarity in the outcome (Golub and Sobin 2020). Here, effects on stillborn pups were not seen in a single

litter, but in five and seven different litters in the 350 and 500 mg/kg bw/day dose groups, respectively. Re-analysis of the individual data illustrated that there is a clear trend in the stillborn pup data, indicating both an increase in the number of stillborn pups with dose and an increase in the number of litters affected with dose (see Supplemental Information in the Appendix).

Increased number of liveborn pups that died during the first days of life

In the studies in mice (Charles River 2011a; 2011b), the total number of liveborn pups that died during the study between PND0-PND20 substantially increased with treatment (Table 7). Neonatal mortality was most prominent during the first days of life, indicating this is a sensitive timeframe of exposure to PFHxA.

***Table 7**: Number of liveborn pups found dead or presumed cannibalised relative to the total number of pups during PND0-20, and percentage of liveborn pups found dead during PND0-20*

Dose (mg/kg bw/day)		**0**	**7**	**35**	**100**	**175**	**350**	**500**
CRL (2011b), N/N	PND 0-20	4/249	8/213	2/232	-	11/241	-	-
CLH report#	*PND 0*	*0/249 (0.0)*	*0/211 (0.0)*	*0/232 (0.0)*	-	*4/238 (1.7)***	-	-
	PND 1-4	*3/249 (1.2)*	*6/211 (2.8)*	*2/232 (0.9)*	-	*3/234 (1.3)*	-	-
	PND 5-7	*1/246 (0.4)*	*0/205 (0.0)*	*0/230 (0.0)*	-	*3/231 (1.3)*	-	-
	PND 8-14	*0/245 (0.0)*	*0/205 (0.0)*	*0/230 (0.0)*	-	*0/228 (0.0)*	-	-
	PND 15-20	*0/245 (0.0)*	*0/205 (0.0)*	*0/230 (0.0)*	-	*1/228 (0.4)*	-	-
CRL (2011a), N/N	PND 0-20	3/221	-	-	7/250	-	42/245	52/180
CLH report#	*PND0*	*0/217 (0.0)*	-	-	*0/250 (0.0)*	-	*3/232 (1.3)*	*21/150 (14.0)***
	PND 1-4	*2/217 (0.9)*	-	-	*3/250 (1.2)*	-	*25/229 (10.9)***	*20/129 (15.5)***
	PND 5-7	*1/215 (0.5)*	-	-	*1/247 (0.4)*	-	*3/204 (1.5)*	*0/109 (0.0)*
	PND 8-14	*0/214 (0.0)*	-	-	*1/244[a] (0.4)*	-	*3/201 (1.5)*	*0/109 (0.0)*
	PND 15-20	*0/214 (0.0)*	-	-	*2/215[a] (0.9)*	-	*0/198 (0.0)*	*0/109 (0.0)*

**, statistically significant ($p \leqslant 0.01$)

[a] Excludes mortality of pups that remained on study after dam was found dead. See footnotes Table S1b.

data from CLH report are given if different from the original study report. The total number of liveborn pups that died during the study includes pups reported as 'missing (presumed cannibalized)', but not includes pups defined as 'unknown vital status (partly cannibalized)'. At 7, 350 and 500 mg/kg bw/day, 2, 8 and 11 pups with reported status 'uncertain vital status (partly cannibalized)' were not included in the total number of pups that died during the study as reflected in the CLH report.

Decreased pup weight

A decreasing trend in the body weights of pups was observed in four different studies:

- Charles River Laboratories (2011b) mouse: At 175 mg/kg bw/day, pup weight was statistically significantly decreased at PND 0 (-12.5%) (Table 8).
- Charles River Laboratories (2011a) mouse: pup body weights were decreased $\geqslant$100 mg/kg bw/day at PND 0, and persisted until PND 20 in the mid- and high dose groups (Table 8).
- OECD TG 415 rat: mean pup weights were decreased during lactation and postweaning, up to 17-18% at 500 mg /kg bw/day compared to controls. The effect on body weight persisted until PND 35 and PND 49 in males and females respectively, at the highest dose tested (Table 9).

- OECD TG 414 rat: a decrease in mean body weight was observed in mean fetal pup body weight, up to -10% at 500 mg /kg bw/day (not reaching statistical significance) (Table 9).

In the OECD TG 422 study with rats, the study summary specified that no effect on pup body weight was reported. No values were provided to support this statement.

Table 8: *Pup weight/litter (mean ± S.D. in grams) in the mouse studies (Charles River 2011a; 2011b; 2012)*

Dose (mg /kg bw/day)		**0**	**7**	**35**	**100**	**175**	**350**	**500**
CRL 2011b	PND 0	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	-	1.4 ± 0.2*	-	-
	PND 4	2.8 ± 0.3	2.8 ± 0.3	3.0 ± 0.3	-	2.7 ± 0.5	-	-
	PND 7	4.2 ± 0.6	4.2 ± 0.4	4.4 ± 0.4	-	4.2 ± 0.6	-	-
	PND 14	6.8 ± 1.2	6.7 ± 0.6	7.0 ± 0.7	-	6.8 ± 0.9	-	-
	PND 20	10.2 ± 1.8	10.0 ± 1.2	10.8 ± 1.3	-	10.4 ± 1.4	-	-
CRL 2011a	PND 0	1.6 ± 0.2	-	-	1.5 ± 0.1*	-	1.4 ± 0.2**	1.4 ± 0.2**
	PND 4	3.0 ± 0.4	-	-	2.8 ± 0.2	-	2.2 ± 0.6**	2.4 ± 0.5**
	PND 7	4.4 ± 0.8	-	-	4.1 ± 0.4	-	3.6 ± 1.0**	3.9 ± 0.8
	PND 14	7.4 ± 1.9	-	-	6.8 ± 0.8	-	6.4 ± 1.4	6.8 ± 1.1
	PND 20	11.0 ± 3.0	-	-	9.8 ± 1.5	-	8.8 ± 2.7	9.7 ± 2.0

* Significantly different from the control group value ($p \leqslant 0.05$).

** Significantly different from the control group value ($p \leqslant 0.01$)

Table 9: *Pup weight/litter (mean ± S.D. in grams) in the rat studies (Loveless et al. 2009).*

Dose (mg/kg bw/day)		**0**	**20**	**100**	**500**
OECD TG 415	PND 0	7.1 ± 0.9	6.8 ± 0.6	6.3 ± 0.4	5.8 ± 0.4[#]
	PND 7	18 ± 2.7	18 ± 2.2	17 ± 1.3	15 ± 1.4[#]
	PND 14	36 ± 3.4	37 ± 3.0	34 ± 2.6	30 ± 2.5[#]
	PND 21	60 ± 5.3	62 ± 5.0	57 ± 5.3	49 ± 4.1[#]
OECD TG 414	GD21	5.8 ± 0.3	5.7 ± 0.3	5.8 ± 0.3	5.3 ± 0.6

[#]Statistically significant difference from control at $p < 0.05$ by analysis of covariance and Dunnett–Hsu.

Delayed eye opening

In the Charles River Laboratories (2011a) study in mice, the percentage of pups with delayed eye opening was statically significantly increased at PND 14 in the mid- and high dose groups (Table 10). Clear effects on eye opening were noted in the mid and high dose groups.

Table 10 *: Percentage of pups meeting the criterion for eye opening (mean ± S.D. per litter) in the mouse studies (Iwai and Hoberman 2014)*

Dose (mg /kg bw/day)		**0**	**7**	**35**	**100**	**175**	**350**	**500**
CRL 2011b	PND 10	0.4 ± 1.7	2.1 ± 4.8	1.0 ± 2.9	-	1.3 ± 3.2	-	-
	PND 11	0.4 ± 1.7	2.2 ± 4.1	1.7 ± 3.5	-	1.2 ± 3.1	-	-
	PND 12	3.5 ± 6.5	7.0 ± 10.7	5.5 ± 6.6	-	2.8 ± 4.0	-	-
	PND 13	37.6 ± 34.1	35.3 ± 23.5	50.4 ± 35.5	-	29.7 ± 25.6	-	-
	PND 14	85.5 ± 22.7	87.6 ± 24.4	89.3 ± 22.7	-	78.9 ± 27.4	-	-
	PND 15	99.6 ± 1.6	99.2 ± 3.3	99.6 ± 1.9	-	94.2 ± 22.3	-	-
	PPD 16	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	-	100.0 ± 0.0	-	-
CRL 2011a	PND 10	0.4 ± 1.7	-	-	0.4 ± 1.5	-	0.5 ± 2.2	0.0 ± 0.0
	PND 11	0.4 ± 1.7	-	-	0.4 ± 1.5	-	0.5 ± 2.2	0.0 ± 0.0
	PND 12	6.8 ± 23.4	-	-	0.8 ± 2.4	-	1.1 ± 3.0	1.3 ± 4.3
	PND 13	31.7 ± 37.9	-	-	14.0 ± 19.2	-	13.2 ± 25.8	14.2 ± 29.4
	PND 14	82.5 ± 24.4	-	-	68.6 ± 34.9	-	42.0 ± 39.5**	50.2 ± 38.0*
	PND 15	98.4 ± 3.7	-	-	88.2 ± 25.6	-	76.1 ± 37.8	73.4 ± 42.4
	PND 16	100.0 ± 0.0	-	-	99.2 ± 3.3	-	91.1 ± 22.7	99.2 ± 2.5
	PND 17	100.0 ± 0.0	-	-	100.0 ± 0.0	-	100.0 ± 0.0	100.0 ± 0.0

* Significantly different from the control group value ($p \leqslant 0.05$).

** Significantly different from the control group value ($p \leqslant 0.01$)

Eye deficits

Two litters in the 175 mg/kg/day dosage group of the Charles River Laboratories study (2011b) had one pup each with corneal opacity (2/238 pups, 0.8%) and one pup each with microphthalmia (undersized eye, 2/238 pups, 0.8%). One litter in this dosage group also had a pup with lenticular opacity (1/238 pups, 0.4%). In the other dose groups of this study, no such effects were seen. Due to the high incidence of pup mortality in the other Charles River Laboratories study (2011a) mid and high dose groups, evaluation of dose-dependency of the ocular effects using this study is difficult.

In Iwai et al. (2019), historical control data for CD-1 mice is reported ranging from eye anomalies in general to specific cornea lesions, ranging from 4- to 104-week studies. The CLP Guidance specifies that "*Use of historical control data should be on a case by case basis with due consideration of the appropriateness and relevance of the historical control data for the study under evaluation. In a general sense, the historical control data set should be matched as closely as possible to the study being evaluated. The historical data must be from the same animal strain/species, and ideally, be from the same laboratory to minimise any potential confounding due to variations in laboratory conditions, study conditions, animal suppliers, husbandry etc..*"

From the reporting in Iwai et al. (2019), it remains unclear if the historical data reported match the same laboratory facility. Furthermore, it remains unclear how specific observations such as lenticular opacity or microphthalmia relate to reported incidences of spontaneous lesions of the eye in general.

In view of the reporting and the lack of measurements in all the pups that died, it is not possible to draw conclusions on eye defects.

Post-implantation loss

In Charles River Laboratories (2011a; 2011b), the number of implants and the number of pups delivered were provided, but the post-implantation loss was not quantified. Based on the numbers provided, the post-implantation loss was calculated as the number of implants minus the pups delivered (sum of stillborn and liveborn pups). At 500 mg/kg bw/day, the implantation loss was higher compared to the incidences in the lower groups in the same study (Table 11).

Table 11*: Incidence of post-implantation loss*

Dose (mg /kg bw/day)		**0**	**7**	**35**	**100**	**175**	**350**	**500**
CRL (2011b)	Implants (N)	261	220	239	-	252	-	-
	Pups delivered (N)	249	213	232	-	241	-	-
	Post-implantation loss[a] (N)	12	7	7	-	11	-	-
CRL (2011a)	Implants (N)	245	-	-	276	-	266	239
	Pups delivered (N)	221	-	-	250	-	245	180
	Post-implantation loss[a] (N)	24	-	-	26	-	21	59

[a]ECHA (2023) specifies that "If there is extensive cannibalism that seem to affect the parameter, it is proposed that instead of "post-implantation loss" the term "post-implantation and postnatal loss immediately after birth" is used". Since cannibalism was observed in CRL (2011a), it cannot be excluded that this value also reflects any malformed or dead pups that were cannibalised immediately after birth. Such pups are not recorded, and are consequently not reflected in the number of pups delivered.

General toxicity of the dams

Mouse

In the studies with mice, no increased incidence in mortality nor a change in mean body weight was observed in dams compared to controls. The mortality incidences in Charles River Laboratories (2011a) were 3/20, 6/20, 1/20, and 3/20 for the control to high dose groups respectively. Iwai and Hoberman (2014) specified that the deaths of the animals in the control group (3) and low-dose group (6) were within the historical range for the testing facility. Additionally, two out of three of the deaths in the highest dose group were suggested to be due

to stress of nursing, but this was not substantiated by data. It is noted that the number of dead animals in the mid (1) and high (3) dose are within the historical control data and are equal or lower than the number of deaths in the concurrent control and low dose group. No mortality occurred in Charles River Laboratories (2011b) in any of the dose groups.

At day 0 postpartum, the mean body weight of dams was slightly increased in the top dose in Charles River Laboratories (2011a). At other timepoints (including during lactation), no effects on mean body weight were observed at any dose in Charles River Laboratories (2011a; 2011b). Individual data of dams and their nest revealed no correlation between dams with lower body weigths and affected nests (Supplemental Information – In depth analysis by RAC).

Rat

In the OECD TG 415 study, some mortalities at 500 mg/kg bw/day were observed, reported as non-treatment related. Information on mortality of controls is lacking. The body weights are also not provided in Loveless et al. (2009). Overall body weight gain between gestation day 0-21 did not differ among control and treated groups. In the OECD TG 414 study, no mortality was reported. Dams had a statistically significant decrease in body weight during GD 19-21 as well as on net body weight (minus gravid uterus weight; GD 21) at 500 mg /kg bw/day. Mean body weight of dams was varying between -5% to -7% compared to the control group.

Relative species sensitivity

The effects observed in mice are consistent in Charles River Laboratories studies (2011a; 2011b). In rats the effects were observed at higher doses and were mainly related to pup body weight. There are no reasons to consider mice less relevant for assessment of PFHxA than rats. In contrast, kinetics show that rats are somewhat more efficient in eliminating PFHxA than mice. Moreover, the elimination kinetics in humans is longer than in rodents.

Comparison with the criteria

Overall, RAC observes that exposure to PFHxA results in:

a) increased stillbirths in mice pups in two different studies, showing a clear trend and reaching statistical significance in both studies. Effects were seen in five and seven different litters in the two highest treatment groups, respectively;
b) increased neonatal mortality in mice pups in two different studies, showing a clear trend and reaching statistical significance in both studies;
c) delayed development in mouse and rat offspring in four different studies, e.g. indicated by substance-induced pup weight decrements and delayed eye opening;

The effects in (a), (b), and (c) are severe and are not resulting from marked maternal toxicity. Furthermore, exposure to PFHxA results in increased post-implantation loss in mice pups in one study (Charles River Laboratories, 2011a).

There are no reasons to consider rodents are more sensitive to PFHxA-induced effects compared to humans. In contrast, in view of the slower elimination kinetics in humans (e.g. Xu et al., 2020), the effects observed in rodents may underestimate effects in humans.

Classification of a substance in Category 1B is largely based on data from animal studies. Such studies shall provide clear evidence of an adverse effect on development in absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate.

Based on the observations listed above, RAC concludes that the adverse effects of PFHxA and its inorganic salts on development **warrant classification as Repr. 1B; H360D**.

Adverse effects on or via lactation

In the Charles River Laboratories mouse studies (2011a; 2011b), an increased number of pups dying between PND 0-4 was observed at 175, 350 and 500 mg PFHx/kg bw/day. In Charles River Laboratories (2011a), viability indices were furthermore statistically significantly reduced at PND 7 (350 mg/kg bw/day) and PND 4 and PND 7 (500 mg/kg bw/day) respectively. It might be these effects are substantiated via lactational exposure.

Classification for lactation effects is appropriate based on either one of three criteria:

a) Human evidence indicating a hazard to babies during the lactation period; and/or
b) Results of one or two generation studies in animals which provide clear evidence of adverse effect in the offspring due to transfer in the milk or adverse effect on the quality of the milk; and/or
c) Absorption, metabolism, distribution and excretion studies that indicate the likelihood that the substance is present in potentially toxic levels in breast milk.

For PFHxA, there is no information available to evaluate whether criterium (a) applies. With regard to criteria (b) and (c), the CLP Guidance specifies that "*In general, positive data should usually be available to show that a substance leads to adverse effect in offspring due to effects on lactation to support classification. However, in exceptional circumstances, if there are substantiated grounds for concern that the substance may have an adverse effect via lactation then it may be classified as such in the absence of direct evidence. This should be based on a quantitative comparison of the estimated transfer via the milk and the threshold for toxicity in the pups.*"

In the mouse studies, PFHxA was not quantified in the milk of the dams, but it was quantified in the blood and the liver in F0 females and F1 males and females at study termination (analytical methods nor limit of detection (LOD) specified in the study). In dams, PFHxA was detected in livers of the highest dose groups. In the F1 generation, PFHxA was not found in the blood or livers at any dose group. Based on the presence of PFHxA in human milk in the human biomonitoring studies by Kang et al. (2016) and Nyberg et al. (2018), presence of PFHxA in the milk of dams might be plausible.

However, since no quantified PFHxA blood levels are reported in Iwai and Hoberman (2014), nor a LOD is provided, estimating the transfer from blood to milk is not feasible. Therefore, RAC concludes on **no classification for adverse effects on or via lactation <u>due to lack of data</u>** to evaluate whether PFHxA reaches toxic levels in the milk.

Therefore, considering all the above elements , RAC concludes that **PFHxA and its inorganic salts warrant classification as Repr. 1B; H360D.**

Additional references

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ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).

Appendix

Supplemental information – In depth analyses by RAC

Discussion on statistical analysis of stillborn pups

Tables S1a and S1b illustrate the details on litters with stillborn, dying, missing, or uncertain vital status pups as provided in the CLH-dossier (Table S1a) and the reports in Charles River Laboratories (CRL, (2011a; 2011b) (Table S1b). Note that the number of nests with affected pups is higher than the number of nests with stillborn pups.

Table S1a: Details on litters with stillborn, dying, missing, or uncertain vital status pups

Dose (mg/kg bw/day)	0	7	35	175
CRL 2011b, N per litter (N total in litter)	Litter 402: 1d (15 Litter 405: 1d (9) Litter 407: 1m (14) Litter 414L 1m (13)	Litter 427: 1m (15) Litter 429: 1m (13) Litter 430: 1d, 2u (3) Litter 434: 1d (14) Litter 435: 1m (13) Litter 438: 1d (16)	Litter 445: 1d (15) Litter 460: 1d (11)	Litter 467: 1d (11) Litter 470: 3s, 2d, 1m (8) Litter 473: 1d, 2m (15) Litter 476: 1m (9) Litter 479: 1d (11)
CLH report	*0*	*0*	*0*	*Litter 470: 3s, 2d, 1m (8)*

s = stillborn; d = died; u = uncertain vital status (partly cannibalized) ; m = missing (presumed cannibalized); - = not applicable

Table S1b: Details on litters with stillborn, dying, missing, or uncertain vital status pups

Dose (mg/kg bw/day)	0	100	350	500
CRL 2011a, N per litter (N total in litter)	Litter 8312: 1d (15) Litter 8317: 1s (12) Litter 8321: 3s (7) Litter 8329: 1d (1)	Litter 8331: 1d (15) Litter 8332: 1d (13) Litter 8334: 1m (12) Litter 8336: 1m (12) Litter 8339: 1m (14) Litter 8341: 1d (13) Litter 8343: 1d[a] (15) Litter 8346: 0d[b] (13) Litter 8347: 0d[c] (17) Litter 8348: 0d[d] (15)	Litter 8353: 1s, 1d, 1m (16) Litter 8354: 2d, 6u, 11m (19) Litter 8355: 2m (14) Litter 8356: 1u (12) Litter 8357: 1s, 1d (10) Litter 8358: 1s, 10d (11) Litter 8359: 1m (16) Litter 8360: 1s, 1d (14) Litter 8362: 1u (15) Litter 8364: 2d (14) Litter 8367: 1d (12) Litter 8369: 1m (12) Litter 8370: 1s (15)	Litter 8371: 2d (12) Litter 8373: 1d (11) Litter 8375: 3s (3) Litter 8378: 9s, 1d (10) Litter 8379: 1d (14) Litter 8381: 1s, 11d (12) Litter 8383: 2s, 6d (8) Litter 8385: 1s, 6d, 7u (14) Litter 8387: 5d, 1m (14) Litter 8388: 1s, 3d (11) Litter 8389: 4u, 2m (6) Litter 8390: 2s, 2m (13)
CLH report#	*Litter 8317: 1s (12)* *Litter 8321: 3s (7)*	*0*	*Litter 8353: 1s (16)* *Litter 8357: 1s (10)* *Litter 8358: 1s (11)* *Litter 8360: 1s (14)* *Litter 8370: 1s (15)*	*Litter 8375: 3s (3)* *Litter 8378: 9s, 1d (10)* *Litter 8383: 2s, 6d (8)* *Litter 8385: 1s, 6d, 7u (14)* *Litter 8388: 1s, 1d (11)* *Litter 8389: 4u (6)*

s = stillborn; d = died; u = uncertain vital status (partly cannibalized) ; m = missing (presumed cannibalized); - = not applicable

[a] Mouse dam (8343) was found dead on day 14 of lactation. Any mortality of pups after day 14 postpartum were excluded from summarization and statistical analyses. 3d pups were excluded from the dataset and further calculations.
[b] Mouse dam (8346) was found dead on day 13 of lactation. Any mortality of pups after day 13 postpartum were excluded from summarization and statistical analyses. 7d pups were excluded from the dataset and further calculations.
[c] Mouse dam (8347) was found dead on day 13 of lactation. Any mortality of pups after day 13 postpartum were excluded from summarization and statistical analyses. 13d pups were excluded from the dataset and further calculations.
[d] Mouse dam (8348) was found dead on day 13 of lactation. Any mortality of pups after day 13 postpartum were excluded from summarization and statistical analyses. 7d pups were excluded from the dataset and further calculations.

data from CLH report are given if different from the original study report. 1: Any mortality of pups after the death of four dams at 100 mg/kg bw/day was excluded from summarization and statistical analyses. These were 3+7+13+7 = 30 pups respectively. 2: The number of stillborn pups was 19 in total at 500 mg/kg bw/day.

Golub and Sobin (2020) express their preference for using data from all littermates in all litters and including litter as a random effect in a mixed effect model to test for intra-litter likeness. Such an analysis was not provided by the DS.

To review the the comments of the DS on analysis of the stillborn pup data in Iwai et al. (2019), a dose-response analysis was performed based on the raw data as provided in the full study reports (CRL 2011a; 2011b) using the individual litter data (See PROAST analyses as provided in the RCOM). This analysis confirmed the litter data could best be described by incorporating an additional parameter (parameter alpha) in the models to take into account litter effects. Furthermore, the dose-response analysis showed the data were best described using an exponential model and not the null model (horizontal line), confirming a clear substance related dose-response in these data. Hence, re-analysis of the individual data illustrates that there is a clear trend in the data, indicating both an increase in the number of stillborn pups with dose and an increase in the number of litters affected with dose.

Individual maternal body weights and litter effects in the 0, 350 and 500 mg/kg bw/day groups (CRL 2011a).

Table S2. Individual maternal body weights and litter effects in the 0 mg/kg bw/day group (CRL 2011a)

Animal ID	**BW Lactation Day 1 (gr)**	**BW Lactation Day 20 (gr)**	**Litter effects**
8311	35.8	48.8	
8312	33.1	44.3	
8313	Not pregnant		
8314	34.4	Found dead LD16	
8315	34.2	42.7	1d (15)
8316	33.8	Found dead LD16	
8317	35.4	43.5	1s (12)
8318	31.6	37.2	
8319	35.3	45.0	
8320	30.4	34.0	
8321	36.5	43.3	3s (7)
8322	34.4	47.6	
8323	32.5	40.9	
8324	36.0	40.3	
8325	34.6	42.9	
8326	35.5	51.3	
8327	31.4	42.7	
8328	32.9	Found dead LD14	
8329	31.4	Sacrificed LD3 (no pups)	1d (1)
8330	35.9	49.6	
Mean	35.3 +/- 2.4	42.8 +/- 3.7	

Table S3. Individual maternal body weights and litter effects in the 350 mg/kg bw/day group (CRL 2011a)

Animal ID	**BW Lactation Day 1 (gr)**	**BW Lactation Day 20 (gr)**	**Litter effects**
8351	36.3	53.3	
8352	34.4	46.1	
8353	**39.2**	**45.7**	**1s, 1d, 1m (16)**
8354	**37.8**	**Sacrificed LD1 (no pups)**	**2d, 6u, 11m (19)**
8355	33.0	42.9	2m (14)
8356	32.4	46.4	1u (12)
8357	31.7	35.1	1s, 1d (10)
8358	**36.3**	**Sacrificed LD1 (no pups)**	**1s, 10d (11)**
8359	32.6	44.3	1m (16)
8360	31.6	44.3	1s, 1d (14)
8361	Found dead GD13		
8362	32.9	43.3	1u (15)
8363	31.1	35.7	
8364	**34.4**	**47.6**	**2d (14)**
8365	36.4	39.9	
8366	34.8	40.7	
8367	27.9	47.4	1d (12)
8368	34.4	43.5	
8369	**39.4**	**47.9**	**1m (12)**
8370	**38.5**	**49.9**	**1s (15)**
Mean	35.0 +/- 2.4	43.8 +/- 4.5	
Control mean	35.3 +/- 2.4	42.8 +/- 3.7	

Table S4. Individual maternal body weights and litter effects in the 500 mg/kg bw/day group (CRL 2011a)

Animal ID	BW Lactation Day 1 (gr)	BW Lactation Day 20 (gr)	Litter effects
8371	**34.5**	**42.6**	**2d (12)**
8372	Not pregnant		
8373	**39.4**	**41.2**	**1d (11)**
8374	33.2	43.0	
8375	28.9	Sacrificed LD0 (no pups)	3s (3)
8376	33.5	44.2	
8377	32.5	38.9	
8378	30.0	Sacrificed LD0 (no pups)	9s, 1d (10)
8379	**37.5**	**44.5**	**1d (14)**
8380	35.6	38.6	
8381	**34.2**	**Sacrificed LD1 (no pups)**	**1s, 11d (12)**
8382	36.9	50.7	
8383	**38.9**	**Sacrificed LD1 (no pups)**	**2s, 6d (8)**
8384	Not pregnant		
8385	**39.9**	**Sacrificed LD0 (no pups)**	**1s, 6d, 7u (14)**
8386	Found dead GD8		
8387	**36.7**	**Found dead LD13**	**5d, 1m (14)**
8388	**35.1**	**Found dead LD13**	**1s, 3d (11)**
8389	**37.4**	**Sacrificed LD1 (no pups)**	**4u, 2m (6)**
8390	**36.7**	**41.5**	**2s, 2m (13)**
Mean	35.8 +/- 2.5	41.1 +/- 3.8	
Control mean	35.3 +/- 2.4	42.8 +/- 3.7	