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Statement concerning the proposed classification and labeling of PFHxA and its inorganic salts

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Summary

A harmonized classification and labeling (CLH) of undecafluorohexanoic acid (perfluorohexanoic acid, PFHxA) and its salts as reproductive toxicants of category 1B has been proposed by a panel of the European Chemicals Agency (ECHA). The experts of EuDiCo GmbH were asked to prepare a statement on the CLH dossier of ECHA and to assess the proposed classification as reproductive toxicant category 1B.

The dossier submitters (DS, rapporteur), the Federal Institute for Occupational Safety and Health (BAuA) from Germany, submitted its CLH report or proposal for harmonised classification and labelling in April 2023. It is now in consultations since May 2nd, 2023 and open for comments until July 3rd, 2023.

So far none of the substances is listed in the CLP regulation (EC 1272/2008), but there exist self-classifications in the C&L inventory for two of them (Table 1).

Table 1: Overview over existing self-classifications for PFHxA, NaPFHx and APFHx according to the ECHA C&L inventory.

PFHxA	NaPFHx	APFHx
<ul style="list-style-type: none"> Acute Tox. 3, H301 Acute Tox. 3, H311 Acute Tox. 2, H330 Skin Corr. 1B, H314 Skin Corr. 1B, H314, H335 Eye Dam. 1, H318 	<p>No self-classification available</p>	<ul style="list-style-type: none"> Acute Tox. 4, H302 Skin Corr. 1B, H314 Skin Sens. 1, H317 Eye Dam. 1, H318

A justification for the harmonised classification as an action at community level is provided by the DS. It is stated, that data on PFHxA, APFHx and NaPFHx are available which identify reproductive toxicity properties. According to the authors, since PFHxA and its salts are expected to dissociate into the PFHx-anion in biological media, they can be evaluated as a group.

The authors of the opinion disagree with this group approach, based on scientific data. In the overview of physico-chemical properties, differences in water solubility, partition coefficients and pKa values are shown for PFHxA and APFHx. No reliable data were available for NaPFHx. All of the aforementioned parameters influence resorption and distribution through the body. Also the half live in the body is influenced since renal filtration is influenced by solubility. Therefor a group approach for PFHxA and its inorganic salts is not appropriate.

During evaluation of adverse effects on the development the DS conclude, that the presented data would provide clear evidence of major manifestations of developmental toxicity including death of the developing organism. Thus, a classification in reproductive toxicity category 1B would be appropriate according to the authors.

The major criticism regarding the conclusion is that maternal toxicity was recognized in several studies. Therefor it cannot be distinguished whether maternal toxicity or developmental toxicity is the reason for the decreased weights or postnatal survival.

In their “Comparison with the CLP criteria” for category 1B the DS explain their conclusion. According to the text, the only reasoning that a classification in the category 1B is appropriate is the increased peri- and postnatal pup mortality from a study using AFPHx as test substance. It is also stated that this is the same developmental toxicity pattern as with perfluoroheptanoic, perfluorooctanoic and perfluorononanoic acid.

In Table 20 of the CLH report, the authors name not only the decreased postnatal survival from the Iwai, Hoberman, 2014, study but also an increased relative liver weight in pups from the same study. Additionally, from Loveless et al., 2009, decreased maternal as well as decreased pup weights are listed as main reproductive effects on development.

Several questions arose for the authors of this opinion regarding the conclusion and its rationale.

1. From the Loveless at al., 2009, study the DS mention as reproductive effects a decreased pup weight as well as a decreased maternal weight. When the mother’s weight is reduced it is most likely that the dam will birth lighter pups since she is not able to fully provide for them. Therefor the authors of this opinion consider it justified that the decreased pup weight is attributable to maternal toxicity instead of reproductive toxicity.

2. The DS mention an increased relative liver weight in pups according to the Iwai, Hoberman, 2014, study. According to the study itself the relative liver weight was not increased but actually decreased in the highest dose group of phase I. The absolute liver weights as well as terminal body weights were not significantly reduced in this dose group. Additionally, the DS only state this “fact” in the summary table 20 of the CLH report. Thus the authors of this opinion find it quite confounding to mention this false fact in the summary table which is what most people will look at if they want to gain a first insight on the topic.

3. The only discussed reason for a classification in reproductive toxicity category 1B in the CLH report is an increased peri- and postnatal pup mortality from Iwai, Hoberman, 2014. In contrast to this, only the decreased postnatal survival, i.e. increased postnatal pup mortality, is mentioned in table 20 as a main reproductive effect of PFHxA.

While this could be attributed to maternal toxicity in the two highest dose groups of phase I (p.42, CLH report), in the highest dose group of phase II it can be argued with intra-litter likeness since all three stillbirths occur in one litter. The latter has also been hinted by the DS though not clearly stated (p. 38).

Apart from this, the reliability of the Iwai, Hoberman, 2014 study itself is questionable. It was scored by the DS with a Klimisch score of 1, which is “reliable without restrictions”. The authors of this opinion disagree with this rating. Reasons to downgrade the studies by Charles River Laboratories include not working according to an OECD guideline, the use of a sub-optimal test organism according to the selected testing guideline, not-robust statistical analyses leading to several re-evaluations leading to different result interpretations due to differing result significances.

Additionally, there exist several studies regarding reproductive toxicity of PFHxA and its salts that were performed according to OECD guidelines. Some of these are mentioned and discussed in the CLH report. All of them come to the conclusion, that there exist no developmental effects without maternal toxicity. Thus, no developmental toxicity could be derived from any of them.

In conclusion, the before mentioned points left the authors of this opinion with the question why the authors scored the study performed by CLR with a Klimisch score of 1. Since they already had some criticism with the study of their own it is questionable why they used it in their conclusion of a classification as reproductive toxicant when there also exist several reliable studies indicating otherwise.

Due to the before-mentioned reasons, the authors of this opinion do not support the classification of PFHxA and its inorganic salts as reproductive toxicant category 1B.

Leverkusen, 30.06.2023

██████████

Toxicologist

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Biochemist

Description of the problem/task

The association textil + mode is the overall association of the German textile and fashion industry and thus represents the interests of over 1.000 companies in its member associations.

TEGEWA e.V. is the association of manufacturers of textile, paper, leather and fur auxiliaries and colorants, surfactants, complexing agents, antimicrobials, polymer flocculants, cosmetic raw materials and pharmaceutical excipients or related products and thus represents a broad spectrum of companies from Germany, Switzerland and the Netherlands.

A harmonized classification and labeling (CLH) of undecafluorohexanoic acid (perfluorohexanoic acid, PFHxA) and its salts as reproductive toxicants of category 1B has been proposed by a panel of the European Chemicals Agency (ECHA). The experts of EuDiCo GmbH were asked to prepare a statement on the CLH dossier of ECHA and to assess the proposed classification as reproductive toxicant category 1B.

Background information

Harmonised classification and labelling (CLH) process

Manufacturers, importers or downstream users are obliged to classify and label hazardous substances and mixtures to ensure a high level of protection of human health and the environment. If no classifications have been made previously, self-classification is also allowed.

For hazards of highest concern (carcinogenicity, mutagenicity, reproductive toxicity (CMR) and respiratory sensitizers), classification and labelling is harmonised (CLH) throughout the EU to ensure an adequate risk management. Harmonised classifications are listed in CLP Regulation (EC No. 1272/2008) Annex VI and must be applied by all manufacturers, importers or downstream users of such substances and of mixtures containing such substances.

-generic approach and criticism

A proposal for CLH can be made either for substances without a current entry or for substances with an existing classification, if new relevant information is available. These proposals can be submitted by a Member State competent authority (MSCA), e.g. for

Germany, the Federal Institute for Occupational Safety and Health (BAuA), or by a manufacturer, importer and downstream user of a substance. This could happen in three situations:

- If there is new evidence, that a substance is either CMR or a respiratory sensitiser.
- When it is justified that a classification for a substance at EU level is needed for other hazard classes than CMR or respiratory sensitizer.
- To add one or more new hazard classes to an existing entry (under the conditions mentioned before).

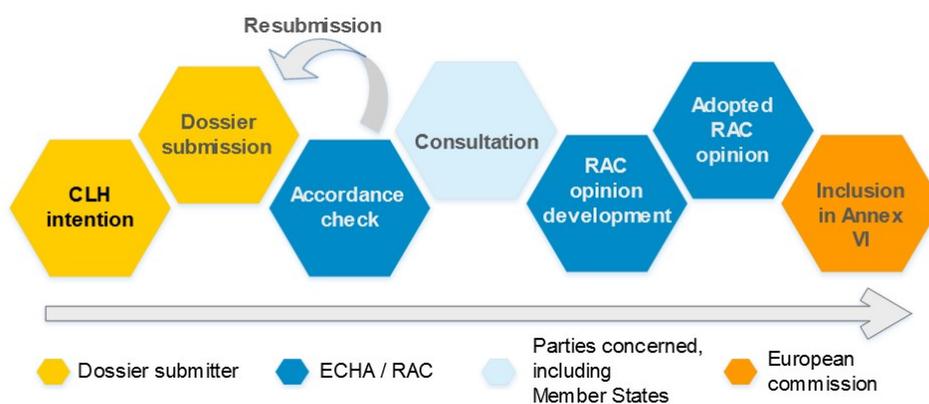


Figure 1: Steps of the CLH process.¹

PFHxA and its inorganic salts

Undecafluorohexanoic Acid (Perfluorohexanoic Acid, PFHxA)

PFHxA belongs to the class of per- and polyfluoroalkyl substances (PFAS). PFAS are mostly synthetic and used in a wide range of consumer products. PFAS all have in common a carbon chain surrounded by fluorine atoms and they are differing in varying terminal groups. PFHxA is not registered under REACH (EC No. 1907/2006) as well as it is not listed in CLP regulation (EC No. 1272/2008) Annex VI Table 3.

There exist several self-classifications noted in the C&L inventory of ECHA, which are listed in the following:

¹ <https://echa.europa.eu/regulations/clp/harmonised-classification-and-labelling>, last visited on 12.06.2023.

- Acute Tox. 3, H301
- Acute Tox. 3, H311
- Acute Tox. 2, H330
- Skin Corr. 1B, H314
- Skin Corr. 1B, H314, H335
- Eye Dam. 1, H318

Sodium Undecafluorohexanoate (NaPFHx)

NaPFHx is not registered under REACH (EC No. 1907/2006) as well as it is not listed in Annex VI of the CLP Regulation (EC No. 1272/2008). Furthermore, no self-classifications are available in the C&L inventory.

Ammonium Undecafluorohexanoate (APFHx)

APFHx is registered under REACH (EC No. 1907/2006). In contrast, APFHx is not listed in CLP Regulation (EC No. 1272/2008) Annex VI Table 3 of the C&L inventory the following self-classifications are listed:

- Acute Tox. 4, H302
- Skin Corr. 1B, H314
- Skin Sens. 1, H317
- Eye Dam. 1, H318

The dossier submitters state that they expect PFHxA as well as its inorganic salts, APFHx and NaPFHx, to dissociate in biological media. Hence, the PFHx-anion would be formed in the bloodstream, regardless whether the acid or the salts were administered. This is the dossier submitters reasoning to jointly consider PFHxA and its inorganic salts.

Statement / Opinion

The dossier submitters (DS, rapporteur), the Federal Institute for Occupational Safety and Health (BAuA) from Germany, submitted its CLH report or proposal for harmonised classification and labelling in April 2023. It is now in consultations since May 2nd, 2023 and open for comments until July 3rd, 2023.

The authors of the dossier start by giving details about the substance identities and their molecular formulas. Besides PFHxA, APFHx, NaPFHx and other inorganic salts of PFHxA were named. Lists of current self-classifications are provided (see last chapter), as well as uses, physicochemical properties and details on toxicokinetics.

A justification for the harmonised classification as an action at community level is provided by the DS. It is stated, that data on PFHxA, APFHx and NaPFHx are available which identify reproductive toxicity properties. According to the authors, since PFHxA and its salts are expected to dissociate into the PFHx-anion in biological media, they can be evaluated as a group.

The authors of the opinion disagree with this group approach. In the overview of physicochemical properties, differences in water solubility, partition coefficients and pKa values are shown for PFHxA and APFHx. No reliable data were available for NaPFHx. All of this influences resorption and distribution through the body. Also the dwell time in the body is influenced since renal filtration is influenced by solubility. Therefore a group approach for PFHxA and its inorganic salts is not appropriate.

In the main part of the report, the DS focus on the evaluation of health, environmental and additional hazards whereby only reproductive toxicity and specific target organ toxicity after repeated exposure were actually evaluated.

Reproductive toxicity

A summary table of all animal studies used in the evaluation on adverse effects on sexual function and fertility and development is provided (Table 2).

Table 2: Overview of the cited studies in the evaluation of the reproductive toxicity of PFHxA and its inorganic salts. * = Addendum available: Iwai et al., 2019². # = Addendum available: CLR, 2012³.

Study details	Test substance	Test species	Reference
One-generation reproduction toxicity study	NaPFHx	Crl:CD(SD)-Rat	Loveless et al., 2009 ⁴
Prenatal developmental toxicity study	NaPFHx	Crl:CD(SD)-Rat	Loveless et al., 2009 ⁴
Combined RDT study with reproduction/developmental toxicity screening test	PFHxA	SD-Rat	WIL Research Laboratories, 2005 ⁵
Reproductive and developmental toxicity Study phase I	APFHxA	Crl:CDI(ICR)-mouse	Charles River Laboratories (CLR), 2011a ^{6, #} , published also by Iwai, Hoberman, 2014 ^{7, *}
Reproductive and developmental toxicity Study phase II	APFHxA	Crl:CDI(ICR)-mouse	CLR, 2011b ^{8, #} , published also by Iwai, Hoberman, 2014 ^{7, *}
RDT 28-day study	PFHxA	SD-rat	NTP, 2019 ⁹

² Iwai H., Hoberman A.M., Goodrum P.E., Mendelsohn E., and Anderson J.K. (2019): Addendum to Iwai and Hoberman (2014) – Reassessment of developmental toxicity of PFHxA in mice. *International Journal of Toxicology* 38 (3), 183-191. DOI: 10.1177/1091581819837904.

³ Charles River Laboratories (2012): Final report amendment: Oral (gavage) combined developmental and perinatal/postnatal reproduction toxicity study of PFH ammonium salt (ammonium salt of perfluorinated hexanoic acid) in mice, date: 2012-09-28.

⁴ Loveless S.E., Slezak B., Serex T., Lewis J., Mukerji P., O'Connor J.C., Donner E.M., Frame S.R., Korzeniowski S.H., and Buck R.C. (2009): Toxicological evaluation of sodium perfluorohexanoate. *Toxicology* 264 (1-2), 32-44. DOI: 10.1016/j.tox.2009.07.011.

⁵ WIL Research Laboratories (2005): A combined 28-day repeated dose oral toxicity study with the reproduction/developmental toxicity screening test of perfluorhexanoic acid and 1H,1H,2H,2H-tridecafluoro-1-octanol in rats, with recovery.

⁶ Charles River Laboratories (2011a): Oral (gavage) combined developmental and perinatal/postnatal reproduction toxicity study of PFH ammonium salt (ammonium salt of perfluorinated hexanoic acid) in mice, date: 2011-07-26.

⁷ Iwai H. and Hoberman A.M. (2014): Oral (gavage) combined developmental and perinatal/postnatal reproduction toxicity study of ammonium salt of perfluorinated hexanoic acid in mice. *International Journal of Toxicology* 33 (3), 219-237. DOI: 10.1177/1091581814529449.

⁸ Charles River Laboratories (2011b): Oral (gavage) combined developmental and perinatal/postnatal reproduction toxicity study of PFH ammonium salt (ammonium salt of perfluorinated hexanoic acid) in mice., date: 2011-08-25.

Adverse effects on sexual function and fertility

Using the first three studies as well as the last study the DS conclude, that the data from these animal studies did not provide clear evidence of an adverse effect on sexual function and fertility. Thus, an assignment to category 1, 1A, 1B or 2 based on adverse effects on sexual function and fertility would not be appropriate.

Adverse effects on development

Using all studies but the last the DS conclude, that the presented data would provide clear evidence of major manifestations of developmental toxicity including death of the developing organism. Thus, a classification in reproductive toxicity category 1B would be appropriate according to the authors.

The major criticism regarding the conclusion is that maternal toxicity was recognized in several studies. Therefore it cannot be distinguished whether maternal toxicity or developmental toxicity is the reason for the decreased weights or postnatal survival.

The conclusion for the proposed classification is largely based on the phase I and II study performed at Charles River Laboratories, 2011, which was later published as one paper by Iwai and Hoberman⁷ in 2014.

In their “Comparison with the CLP criteria” for category 1B the DS explain their conclusion. According to the text, the only reasoning that a classification in the category 1B is appropriate is the increased peri- and postnatal pup mortality from a study using AFPHx as test substance. It is also stated that this is the same developmental toxicity pattern as with perfluoroheptanoic, perfluorooctanoic and perfluorononanoic acid.

In Table 20 of the CLH report, the authors name not only the decreased postnatal survival from the Iwai, Hoberman, 2014, study but also an increased relative liver weight in pups from the same study. Additionally, from Loveless et al., 2009, decreased maternal as well as decreased pup weights are listed as main reproductive effects on development (Figure 2).

⁹ NTP (2019): NTP technical report on the toxicity studies of perfluoroalkyl carboxylates (perfluorohexanoic acid, perfluorooctanoic acid, perfluorononanoic acid, and perfluorodecanoic acid) administered by gavage to Sprague Dawley (Hsd:Sprague Dawley SD) rats. Toxicity report 97, date: 2019-08. National Toxicology Program, Public Health Service, U.S. Department of Health and Human Services, Research Triangle Park, North Carolina, USA.

Substance	Harmonised classification for Repr.	Reproductive effects	
		Main reproductive effects of PFHxA	
PFHxA C6 (available studies on sodium and ammonium salts)	Proposed: Repr. 1B, H360D (classification proposed for acid, sodium, ammonium and other salts)	↓ maternal weight (Loveless et al., 2009) ↓ pup weight (Loveless et al., 2009) ↑ pup rel. liver weight (Iwai and Hoberman, 2014) ↓ postnatal survival (Iwai and Hoberman, 2014)	

Figure 2: Table 20 from the CLH report on PFHxA and its inorganic salts.

Several questions arose for the authors of this opinion regarding the conclusion and its rationale.

1. From the Loveless et al., 2009, study the DS mention as reproductive effects a decreased pup weight as well as a decreased maternal weight. When the mother's weight is reduced it is most likely that the dam will birth lighter pups since she is not able to fully provide for them. Therefore the authors of this opinion consider it justified that the decreased pup weight is attributable to maternal toxicity instead of reproductive toxicity.

From the citation in table 20, CLH report, it is unclear whether this is deduced from the one-generation reproduction toxicity study (OECD TG 415) or the prenatal developmental toxicity study (OECD TG 414). Regarding the TG 415 study it is stated on p. 35 and p. 41 that treatment-related effects on mean pup body weights were observed at ≥ 100 mg/kg bw/d. This could not be confirmed by the authors of this opinion. In table 16 of the CLH report (depicted in Figure 3 for convenience) significant changes in body weights of the F1 generation pups are only observed at 500 mg/kg bw/d. In the parental generation P0 there are no data shown for absolute body weights but only for body weight gains which is also significantly reduced in the 500 mg/kg bw/d dose group for males and for females during the first week of gestation. This is not only supporting the opinion of these authors, but is showing clear evidence that the decreased pup weight is due to maternal toxicity.

	Day	P0 Dosage (mg/kg bw/d)				
		Control	20	100	500	
Body weight gain P0 ♂	Test	0 - 105	340 ± 44	329 ± 47	299 ± 43*	241 ± 40*
Body weight gain P0 ♀	Gestation	0 - 7	36 ± 10	38 ± 8	37 ± 10	25 ± 8*
		0 - 21	140 ± 25	145 ± 16	147 ± 23	134 ± 19
	Lactation	0 - 21	5.1 ± 2.6	7.4 ± 2.0	20 ± 15	25 ± 12*
<i>F1, not dosed</i>						
Body weight F1 pups	Postnatal	0	7.1 ± 0.9	6.8 ± 0.6	6.3 ± 0.4	5.8 ± 0.4#
		7	18 ± 2.7	18 ± 2.2	17 ± 1.3	15 ± 1.4#
		14	36 ± 3.4	37 ± 3.0	34 ± 2.6	30.0 ± 2.5#
		21	59.6 ± 5.3	62 ± 5.0	57 ± 5.3	49 ± 4.1#
Body weight gain F1 ♂	Postweaning ^a	0 - 39	320 ± 25	327 ± 42	320 ± 27	321 ± 25
Body weight gain F1 ♀	Postweaning ^a	0 - 39	183 ± 21	178 ± 18	173 ± 21	183 ± 24

^aAge of animals at postweaning day 0 = 21 days old.

*Statistically significant difference from control at $p < 0.05$ by Dunnett/Tamhane–Dunnett.

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

Figure 3: Table 16 from the CLH report on PFHxA and its inorganic salts: Mean ± SD body weight gains (g) in NaPFHx-treated P0 rats and non-treated F1 rats and body weights (g) of non-treated F1 pups (Loveless et al., 2009).

Regarding the TG 414 study it is stated by the DS on p. 36 and p. 42 that developmental toxicity occurred due to lower foetal body weights. Even though the body weight is slightly decreased in the 500 mg/kg bw/d dose group according to table 17 of the CLH report (depicted in Figure 4 for convenience) it is not statistically significant. Thus it should not be taken into account for developmental toxicity. In the same dose group maternal body weights are significantly reduced. This strongly supports the conclusion of the authors and provides even more evidence of maternal toxicity effects rather than developmental toxicity.

Group	Day	P0 Dosage (mg/kg bw/d)			
		Control	20	100	500
Maternal bw (g)					
	GD 19	361.1 ± 22.2	365.8 ± 18.3	353.7 ± 25.9	343.9 ± 25.9*
	GD 20	377.4 ± 24.5	383.0 ± 19.0	371.5 ± 25.5	354.6 ± 28.5*
	GD 21	400.0 ± 27.6	405.6 ± 19.2	392.7 ± 27.3	371.5 ± 32.9*
	GD 21 (net bw ^a)	304.1 ± 16.3	308.2 ± 18.2	294.1 ± 21.1	288.1 ± 22.7*
Maternal bw gain (g)	GD 6-21	165 ± 18	167 ± 13	161 ± 17	134 ± 27@
	GD 6-21 ^b	69 ± 10	69 ± 10	62 ± 11	51 ± 20@
Foetal bw (g)		5.8 ± 0.3	5.7 ± 0.3	5.8 ± 0.3	5.3 ± 0.6

^aNet body weight on gestation day 21 = terminal body weight minus the gravid uterus weight.

^bTotal body weight gain (gestation days 6–21) minus products of conception on day 21.

* Parametric comparison to control (Dunnett/Tamhane–Dunnett) significant (p-value not reported).

@ Statistically significant from control at $p < 0.005$ by Dunn's test (as stated in the registration data, while reported in the publication as being < 0.05).

Figure 4: Table 17 from the CLH report on PFHxA and its inorganic salts: Mean ± SD maternal body weight, body weight gain in NaPFHx-treated P0 rats and foetal body weights (Loveless et al., 2009).

2. The DS mention an increased relative liver weight in pups according to the Iwai, Hoberman, 2014, study. According to the study itself the relative liver weight was not increased but actually decreased in the highest dose group of phase I. The absolute liver weights as well as terminal body weights were not significantly reduced in this dose group. Additionally, the DS only state this “fact” in the summary table 20 of the CLH report (see Figure 2). It is neither mentioned in the summary table of animal studies (pp. 22-27) nor in the text describing the study (pp. 37-41). In the “Comparison with the CLP criteria”, where the authors explain their conclusion, relative pup weights are not mentioned either. Thus the authors of this opinion find it quite confounding to mention this false fact in the summary table which is what most people will look at if they want to gain a first insight on the topic.

3. The only discussed reason for a classification in reproductive toxicity category 1B in the CLH report is an increased peri- and postnatal pup mortality from Iwai, Hoberman, 2014. In contrast to this, only the decreased postnatal survival, i.e. increased postnatal pup mortality, is mentioned in table 20 as a main reproductive effect of PFHxA.

While this could be attributed to maternal toxicity in the two highest dose groups of phase I (p.42, CLH report), in the highest dose group of phase II it can be argued with intra-litter likeness since all three stillbirths occur in one litter. The latter has also been hinted by the DS though not clearly stated (p. 38).

Apart from this, the reliability of the Iwai, Hoberman, 2014 study itself is questionable. It was scored by the DS with a Klimisch score of 1, which is “reliable without restrictions”. “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.”¹⁰

The study by Iwai and Hoberman was performed according to International Conference on Harmonisation (ICH) Harmonised Tripartite Guideline stages C through F. Since the study is from 2014, the most recent version of the guideline was from 2005, which is used here for further evaluation. It has since been reviewed and adopted in 2020.

¹⁰ Klimisch, H.J.; Andrae, M.; Tillmann, U. (1997). "A Systematic Approach for Evaluating the Quality of Experimental Toxicological and Ecotoxicological Data". *Regulatory Toxicology and Pharmacology*. 25 (1): 1–5. doi:10.1006/rtph.1996.1076. PMID 9056496.

OECD Guidelines for the testing of chemicals are state-of-the-art and the most relevant internationally agreed testing methods used by governments, industry and academia to assess the safety of chemicals. Studies performed according to OECD guidelines and under GLP conditions are covered by the OECD Mutual Acceptance of Data (MAD) system. This means that these study results are accepted in all OECD countries and adherent countries for the purpose of safety assessment and other uses relating to the protection of human health and the environment.¹¹ Thus, OECD guidelines under GLP conditions usually are rated with a Klimisch score of 1.

Studies performed under different conditions than the OECD guidelines even though performed according to other guidelines will always be compared to this gold standard.

There were several inconsistencies noticed in the CLR, 2011 / Iwai, Hoberman, 2014, studies.

Phase 1 and 2 are inverted from the CLR to Iwai, Hoberman. According to the final report by the study director from CLR the study part using lower dosages (7, 35, 175 mg/kg bw/d) was performed first. Since no NOAEL could be derived, a second study phase was initiated using higher concentrations (100, 350, 500 mg/kg bw/d). It is reported the vice versa by Iwai, Hoberman and also in the CLH report.

Furthermore, it is necessary to criticize the inaccurate handling of the two toxicological endpoints NOAEL (no observed adverse effect level) and NOEL (no observed effect level). By close evaluation of the respective studies it could be determined that the authors always meant to give a NOAEL.

Within the determined NOAELs (maternal and developmental) already in the two CLR study phases exist inconsistencies regarding the NOAELs, especially for maternal toxicity, from one study phase to the next to the addendum. Iwai, Hoberman, 2014 adopt the conclusion from the CLR addendum. Nevertheless, it was necessary to reanalyze the data on several toxicological endpoints in yet another addendum (Iwai, et al., 2019). The statistical reanalysis leads to the determination of yet again different NOAELs.

¹¹ <https://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.html>, last visited on June 26th, 2023.

<https://www.oecd.org/chemicalsafety/testing/oecd-guidelines-testing-chemicals-related-documents.html>, last visited on June 26th, 2023.

Table 3: Overview of NOAELs derived from CLR, 2011, study data in different publications.

Publication	NOAEL (maternal tox.)	NOAEL (developmental tox.)
CLR, 2011, lower dosages ("phase 2" – performed first)	175	35
CLR, 2011, higher dosages ("phase 1" – performed second)	100	< 100
CLR, 2012, addendum	100	100
Iwai, Hoberman, 2014	100	100
Iwai, et al., 2019	175	175

Moreover, the authors of the study decided to use mice as a test organism even though rats are the preferred organism, not only in the reproductive toxicity OECD guideline studies, but also in the ICH Harmonised Tripartite Guideline for reproductive toxicity testing. The latter is the guideline which CLR, by their own account, worked in accordance to. Mice as test organism have several disadvantages including fast metabolic rate, stress sensitivity, malformation clusters (which occur in all species) particularly evident, small fetus.¹² Other disadvantages are surface-body weight-ratio, their small gastrointestinal organs (important for gavage feeding) and low body weights in general. Also, the authors did not report viability parameters like motion or apathy of the dams or monitor food consumption as recommended by the ICH Guideline¹².

Another question the authors of this opinion asked themselves about the study was about the solubility of APFHxA in water. It was reported by the DS to be 57 g/L at 20 °C. According to the title of the given reference, this is an experimental value. Solubility was determined as critical micelle concentration since APFHx has an amphiphilic property. This seems to be the same source as used during REACH registration as it is the same value which can be viewed on the ECHA Homepage. During the study CLR reported administering a

¹² ICH Harmonised Tripartite Guideline: Detection of Toxicity to Reproduction for Medicinal Products & Toxicity to Male Fertility, version incorporated in November 2005.

concentration of 100 mg/mL and 70 mg/mL APFHx in deionised water in the two highest dose groups. It was stated that all solutions were clear and for all solutions HPLC analyses were performed which were within a range of $\pm 10\%$. 100 mg/mL and 70 mg/mL are equal to 100 g/L and 70 g/L. Thus, concentrations of APFHx were used which are above water solubility and formation of a turbid emulsion containing micellar APFHx rather than a solution would be expected. This would have implications on ADME parameters of APFHx.

In addition to this, there exist several studies regarding reproductive toxicity of PFHxA and its salts that were performed according to OECD guidelines. Some of these are mentioned and discussed in the CLH report. All of them come to the conclusion, that there exist no developmental effects without maternal toxicity. An additional study¹³ by DuPont de Nemours, Inc. came to the same conclusions. It was performed according to OECD Guideline 414 in 2007. Though fetal weight was reduced in the highest dose group (500 mg/kg bw/d) maternal toxicity, in the form of decreased body weight parameters and food consumption, was observed in the same dosage group. No reduction in live fetuses was observed during this study. Thus, no developmental toxicity could be derived from any of the studies.

In conclusion, the before mentioned points left the authors of this opinion with the question why the authors scored the study performed by CLR with a Klimisch score of 1. Since they already had some criticism with the study of their own it is questionable why they used it in their conclusion of a classification as reproductive toxicant when there also exist several reliable studies indicating otherwise. Even though Klimisch 2 studies may be used in a “weight-of-evidence” approach during the evaluation of a toxicological endpoint, studies with a Klimisch score of 1 should always be preferred. Since the Klimisch 1 studies use the recommended test species and did not see adverse effects on peri- or postnatal mortality, the authors of strongly suspect that the DS are picking the study supporting their argument and purpose.

Due to the before-mentioned reasons, the authors of this opinion do not support the classification of PFHxA and its inorganic salts as reproductive toxicant category 1B.

¹³ E.I. du Pont de Nemours and Company (2007): H-27579: Developmental Toxicity Study in Rats. Date: 26-04-2007.

Comparison with Klimisch- and Guideline Criteria

In the report several studies were consulted by the DS in order to collect information valuable for a possible classification.

Six studies in total were evaluated, all of them using GLP documentation standards. Four of them were performed according or similar to OECD guidelines:

- TG 407: Repeated Dose 28-Day Oral Toxicity Study in Rodents
- TG 414: Prenatal Developmental Toxicity Study
- TG 415: One-Generation Reproduction Toxicity Study (effectively deleted in 2019)
- TG 422: Combined Repeated Dose Toxicity Study with the Reproduction/ Developmental Toxicity Screening Test

Klimisch et al. developed a well-accepted scoring system to assess the reliability of data, particularly from toxicological and ecotoxicological studies. It assigns studies to four categories depending on data reliability: reliable without restrictions, reliable with restrictions, not reliable and not assignable. Data reliability depends on many factors including characterisation of test substances, reporting of information and quality assurance.¹⁰

Table 4 shows an overview of the studies used in the evaluation on adverse effects on sexual function and fertility and development and its scoring by the DS of the CLH report as well as a revised scoring by the authors. Reasons to downgrade the studies by Charles River Laboratories are extensively discussed throughout the opinion and include not working according to an OECD guideline, the use of a sub-optimal test organism according to the selected testing guideline, not-robust statistical analyses leading to several re-evaluations leading to different result interpretations due to differing result significances.

Table 4: Overview over OECD guideline as well as GLP accordance and Klimisch scores of the studies used in the evaluation of the reproductive toxicity of PFHxA and its inorganic salts.

Study details	Reference	OECD Guideline	GLP	Klimisch Score (DS)	Klimisch Score (authors of this opinion)
One-generation reproduction toxicity study	Loveless et al., 2009 ⁴	TG 415	yes	1	1
Prenatal developmental toxicity study	Loveless et al., 2009 ⁴	TG 414	yes	1	1
Combined RDT study with reproduction/developmental toxicity screening test	WIL Research Laboratories, 2005 ⁵	TG 422	yes	1	1
Reproductive and developmental toxicity study phase I	CLR, 2011a ^{6, #} , published also by Iwai, Hoberman, 2014 ^{7, *}	/	yes	1	2
Reproductive and developmental toxicity study phase II	CLR, 2011b ^{6, #} , published also by Iwai, Hoberman, 2014 ^{7, *}	/	yes	1	2
RDT 28-day study	NTP, 2019 ⁹	Similar to TG 407	yes	1	1