TASK ORDER 68HERH20F0407 UNDER
CONTRACT EP-C-17-017

EXTERNAL PANEL PEER REVIEW OF EPA'S DRAFT "IRIS TOXICOLOGICAL REVIEW OF PERFLUOROHEXANOIC ACID (PFHXA) AND RELATED SALTS"

FINAL PEER REVIEW REPORT

August 11, 2022

Submitted to:
U.S. Environmental Protection Agency
Office of Research and Development
Center for Public Health and Environmental Assessment
Attn: Vicki Soto
Soto.Vicki@EPA.gov

Submitted by:
ERG
110 Hartwell Avenue
Lexington, MA

ERG
www.erg.com

CONTENTS

1.0 INTRODUCTION

This report documents the results of an external independent peer review of the U.S. Environmental Protection Agency's (EPA's) draft "IRIS Toxicological Review of Perfluorohexanoic Acid (PFHxA) and Related Salts." ERG, a contractor to EPA, organized this review and developed this report. The peer review included a virtual meeting that was open to the public as observers and included an opportunity for oral public comment (in addition to opportunity for the public to submit written comments to EPA via the PFHxA docket).

Section 1.0 provides background about the review. Section 2.0 provides a high-level summary of key reviewer final comments. Section 3.0 presents reviewer final individual post-meeting comments. In Section 3.0, reviewer final comments are organized by charge question and presented exactly as submitted, without editing or correction of typographical errors (if any). Appendices A, B, and C, respectively, provide the list of reviewers, EPA's charge to reviewers, and the peer review meeting agenda.

1.1 Background

During the first half of 2022, ERG organized and managed an external peer review of EPA's draft "IRIS Toxicological Review of Perfluorohexanoic Acid (PFHxA) and Related Salts," developed in support of the Agency's online database, the Integrated Risk Information System (IRIS). IRIS assessments contain information about chemicals that encompasses hazard identification and dose-response assessment, two of the four steps in the human health risk assessment process. When used by risk managers in combination with information on human exposure and other considerations, IRIS assessments support the Agency's regulatory activities and decisions to protect public health. There is no existing IRIS assessment for PFHxA. EPA's draft Toxicological Review of PFHxA is based on a comprehensive review of the available scientific literature on the noncancer and cancer health effects in humans and experimental animals exposed to PFHxA or related salts.

1.2 Peer Review Process

During the first stage of the peer review process, ERG assembled a set of experts interested in serving as reviewers, who collectively spanned three key areas of expertise required by EPA for this and four other related peer reviews: environmental epidemiology, experimental toxicology, and the use of quantitative methods (e.g., dose-response modeling, PBPK model development) important for the derivation of toxicity values in human health assessments of environmental chemicals. To identify candidates, ERG used standard search processes and considered experts nominated by the public in response to a Federal Register Notice (FRN) requesting nominations. After considering comments on these candidates submitted by members of the public in response to a second FRN, ERG assembled a final pool of 20 experts from which to select reviewers for this and the four other related peer reviews. For this PFHxA review, ERG selected the following seven experts after confirming they had no conflict of interest for this review:

- Elaine M. Faustman, Ph.D., DABT (Panel Chair)
- Joseph T. Haney, Jr., M.S.
- Panagiotis G. Georgopoulos, Ph.D.
- Angela M. Leung, M.D.

- Carla A. Ng, Ph.D.
- David A. Savitz, Ph.D.
- R. Thomas Zoeller, Ph.D.

See Appendix A for a more detailed list of reviewers.

ERG provided reviewers with the draft PFHxA toxicological review document and with EPA's charge to reviewers (Appendix B), which asked reviewers to address each of the 10 questions and multiple sub questions and to categorize their advice to EPA into three tiers:

- Tier 1: *Necessary Revisions* – Use this category for any revisions you believe are necessary to adequately support and substantiate the analyses or scientific basis for the assessment conclusions, or to improve the clarity of the presentation in the PFHxA Toxicological Review.
- Tier 2: *Suggested Revisions* – Use this category for any revisions you encourage EPA to implement to strengthen the analyses or scientific basis for the assessment conclusions, or to improve the clarity of the presentation in the PFHxA Toxicological Review.
- Tier 3: *Future Considerations* – Use this category for any advice you have for scientific exploration that might inform future work. While these recommendations are generally outside the immediate scope or needs of the PFHxA Toxicological Review, they could inform future reviews or research efforts.

For their consideration, ERG also provided reviewers with written public comments submitted to EPA's docket for this review and with a table developed by EPA that listed published literature identified (1) during EPA literature search updates after release of the draft PFHxA toxicological review for public comment or (2) in public comments received through the EPA docket.

Working individually, each reviewer prepared written pre-meeting comments in response to the charge questions, and ERG compiled and distributed these preliminary comments to all reviewers a few days prior to the peer review meeting to help them prepare for discussions at the meeting.

ERG organized and facilitated a virtual peer review meeting, which took place via Zoom.gov on May 16 and 17, 2022. The meeting was open to members of the public to attend as observers and provided an opportunity for members of the public to make an oral comment. During this meeting, reviewers discussed and commented on EPA's draft PFHxA Toxicological Review, with discussion structured by EPA's charge questions. Appendix C provides the meeting agenda. After the meeting, reviewers prepared their individual final post-meeting comments (see Section 3.0) and ERG prepared a high-level summary (Section 2.0) of the key comments that reviewers had categorized into the three tiers described in EPA's charge (Appendix B).

2.0 SUMMARY OF KEY REVIEWER COMMENTS BY CHARGE QUESTION

This section summarizes the key comments that reviewers categorized into the three tiers described in EPA's charge (Appendix B). Comments are summarized by charge question (with EPA charge questions shown in italic font, for reference) and by tier. For the full text of all review post-meeting comments and for additional details on many of the comments summarized here, see Section 3.0.

2.1 Systematic Review Methods Documentation

Charge Question 1. *The Toxicological Review for PFHxA describes and applies a systematic review protocol for identifying and screening pertinent studies. The protocol is described in brief detail in Section 1.2.1*

(Literature Searching and Screening) and in full detail in Appendix A (Systematic Review Protocol for the PFAS IRIS Assessments). Please comment on whether the search strategy and screening criteria for PFHxA literature are clearly described. If applicable, please identify additional peer-reviewed studies of PFHxA that the assessment should incorporate[1].

All reviewers agreed that the search strategy and criteria were appropriate and clearly described. One reviewer noted how inherently challenging it is to identify pertinent studies with the increasing interest in PFAS, which has led to an increasing rate of new publications. Several reviewers provided references to additional studies for EPA's consideration.

Tier 1 Necessary Revisions

- For clarity, Faustman recommended that EPA add text describing the major reasons for excluding the 194 articles during the screening process, as shown in Figure 2-1.
- Faustman recommended adding several sentences to Section 1 that describe the in-press paper EHP (DOI 10.1289/EHP 10343) shown in EPA's slides during the May 16, 2022, peer review. In particular, she noted that the evidence maps illustrating how EPA is going to synthesize evidence across the PFAS compounds would be a good addition to the text.
- Georgopoulos recommended updating HAWC for PFHxA to include assessments/evaluations of any more recent studies that will be considered in finalizing this Toxicological Review. As a Tier 2 suggested revision or a Tier 3 future consideration (as listed below), Haney, Faustman, and Georgopoulos also suggested additional literature for EPA to consider for the Toxicological Review.

Tier 2 Suggested Revisions

- Given that EPA is preparing IRIS reviews for multiple PFAS compounds, Faustman suggested that EPA summarize key points for the individual PFAS so a user of the IRIS materials could see similarities and differences in this family of related chemicals. She noted that users of the IRIS documents will usually be addressing mixtures of these compounds in the field, therefore, a common summary in one place would help the user community coordinate the information.
- Referencing the systematic review protocol in Appendix A (Table 5-2), Ng suggested that EPA clarify why dam health (e.g., weight gain, food consumption) was only considered in "Developmental" and not in "Reproductive" or tied to the specific effect on dam health observed (e.g., weight gain as an endpoint).
- Tier 2 suggested revisions related to consideration of additional literature included:
    - Georgopoulos recommended including a list of documents relevant to PFHxA risk characterization that have been developed by state and international regulatory agencies in the literature searches and in resulting databases. He also recommended compiling a summary of established or proposed values for metrics of reference doses/concentrations. He also provided a list of studies that he suggested EPA consider as a Tier 3 Future Consideration.

[1] Newly identified studies (i.e., studies identified by EPA or the public that meet PECO criteria but were not addressed in the external review draft, for example due to recent publication) will be characterized by EPA and presented to the peer review panel. This characterization will focus on EPA's judgment of whether the studies would have a material impact on the conclusions (i.e., identified hazards or toxicity values) in the external review draft. The peer review panel is asked to review EPA's characterization and provide tiered recommendations to EPA regarding which studies, if any, to incorporate into the assessment before finalizing.

- Faustman identified and listed numerous additional studies which she suggested EPA consider.
- Haney suggested that EPA evaluate additional studies identified by his fellow peer reviewers for potential inclusion in the Toxicological Review.

Tier 3 Future Considerations

- Faustman recommended that EPA clarify if HERO/HAWC will be available to the public. If not, she suggested that EPA consider clarifying how and at what level the public will be able access the publications within the database.
- Georgopoulos recommended that EPA develop and implement a plan for the systematic and "continuous" updating of databases (such as HERO and HAWC) that track information relevant to the Toxicological Review. He added that EPA consider specifying the criteria for new information that would require re-evaluation and updating of the contents and conclusions of the Toxicological Review.
- Georgopoulos provided a list of peer-reviewed studies that he suggested EPA consider and evaluate for the PFHxA Toxicological Review.

Charge Question 2. *The Toxicological Review provides an overview of individual study evaluations and the results of those evaluations are made available in the Health Assessment Workplace Collaborative (HAWC). Data from studies considered informative to the assessment are synthesized in the relevant health effect-specific sections, and study data are available in HAWC.*

a. *Please comment on whether the study confidence conclusions for the PFHxA studies are scientifically justified and clearly described, considering the important methodological features of the assessed outcomes. Please indicate any study confidence conclusions that are not justified and explain any alternative study evaluation decisions.*

b. *Results from individual PFHxA studies are presented and synthesized in the health system-specific sections. Please comment on whether the presentation and analysis of study results are clear, appropriate, and effective to allow for scientifically supported syntheses of the findings across sets of studies.*

Six of the seven reviewers agreed that the confidence conclusions for the PFHxA studies were scientifically justified and clearly described. For example, one reviewer noted that the visual presentation of the evaluation results for the animal studies was very effective and found the use of interactive graphics to be very convenient. The seventh reviewer (Savitz) commented that the considerations used in evaluating study quality should be in the main text rather than solely in the HAWC. He provided a Tier 1 Revision to improve the presentation. Haney made a similar suggestion as a Tier 2 Revision.

Reviewers generally found the presentation and analysis of the study results as they appear in the health system-specific sections to be clear but recommended several Tier 1 and Tier 2 revisions to improve the clarity and accuracy of the presentation.

Tier 1 Necessary Revisions

- Faustman recommended expanding the discussion in Section 1.2.4 (or an additional section) on the use of low confidence studies to support mechanistic evidence when the mechanistic evidence is used across health effects.

- For histopathology, Ng commented that while two studies did not report significant changes to histopathology, the results of these high confidence studies should be included in Table 3-28, otherwise only the one study with significant effects is being highlighted, painting an incomplete picture.
- To clarify how decisions were made for each health endpoint, Savitz recommended that EPA add a brief section on the considerations used in evaluating study quality and summarize the basis for assignments. He noted that including this information solely within the HAWC template does not enable the reader to readily identify the basis for judgments about individual studies or the rationale behind the assignments.
- Zoeller recommended that EPA enumerate the adaptations made to the structured evaluation considerations first introduced by Hill (Hill, 1965).

Tier 2 Suggested Revisions

- For increased transparency and ease of reference, Haney suggested that EPA consider adding the HWAC animal toxicity study evaluation figure to the main document in addition to including it in the HAWC.
- For hepatic effects (Table 3-11), Haney provided several suggestions for revisions*:* 1) Consider additional tables and/or figures to help readers visualize the coherence of liver histopathology with liver weight effects since these results are only presented in separate tables in the document; 2) reconsider whether to include decreases in bilirubin amongst the serum biomarkers of hepatic injury cited in Table 3-11 based on the Loveless (2009) and Hall (2012) studies; and 3) in characterizing the strength of this evidence, reconfirm that the significant variability of responses across studies and sexes was considered and weighed, as well as the magnitude (frequently modest) and direction of change in the cases where there was a change in one of the serum enzyme biomarkers (in many cases there were decreases).
- Regarding developmental effects, Haney suggested that EPA consider revisions to further characterize the mouse dose-response for decreases in postnatal body weight.
- For hematopoietic effects, Haney provided detailed suggestions to: 1) add a table and/or figure to help readers visualize the coherence of these effects since these results are presented in separate figures and tables in the document; and 2) add information on the results of several chronic studies which are an important exception to the cited "consistent treatment related effect on platelet levels."

Tier 3 Future Considerations

- Faustman recommended clarifying when evidence is integrated across the individual health effects. She noted that this systems-based integration is essential to predict organism-level responses, especially in humans.

2.2 Noncancer Hazard Identification

Charge Question 3. *For each health effect considered in the assessment and outlined below, please comment on whether the available data have been clearly and appropriately synthesized to describe the strengths and limitations. For each, please also comment on whether the weight-of-evidence decisions for hazard identification are scientifically justified and clearly described.*

Hepatic Effects

a. *For hepatic effects, the Toxicological Review concludes the available **evidence indicates** PFHxA likely causes hepatic effects in humans under relevant exposure circumstances. This conclusion is based on studies of rats showing increased liver weight, hepatocellular hypertrophy, increased serum enzymes, and decreased serum globulins. The hepatic findings for PFHxA were similar for other PFAS and determined to be adverse and relevant to humans.*

    i. *Additional considerations influenced the hepatic effects hazard identification decisions. Appendix A (Systematic Review Protocol for the PFAS IRIS Assessments) outlines the human relevance of hepatic effects in animals that involve PPARα receptors as a key science issue. To the extent supported by the PFHxA literature (and to a lesser extent, literature for other PFAS), the Toxicological Review evaluates the evidence relevant to the potential involvement of PPARα and non-PPARα pathways with respect to the reported hepatic effects. The Toxicological Review ultimately concludes evidence from in vivo (including genetic mouse models) and in vitro studies support a potential role for multiple pathways operant in the induction of hepatic effects from PFHxA exposure, but those pathways cannot be specifically determined. Please comment on whether the conclusions regarding the available animal and mechanistic studies are scientifically justified and clearly described. The hepatic findings for PFHxA were similar for other PFAS and determined to be adverse and relevant to humans.*

Tier 1 Necessary Revisions

- Reviewers had no Tier 1 comments.

Tier 2 Suggested Revisions

- To improve clarity, Haney suggested revising the text (page 2-3) stating, "All outcomes rated low confidence or higher were used for evidence synthesis and integration." Haney commented that it may be unclear how this statement can be consistent with the statement on page 1-12 that "no low confidence studies were used in the evidence syntheses for PFHxA included in the narrative," since low confidence studies may presumably have outcomes that would also be rated as low confidence, which might be assumed to be included in evidence synthesis and integration based on the first sentence cited.
- Haney suggested utilizing information on other PFAS compounds (e.g., PFBA) to supplement and bolster the evidence consistent with the adversity of PFHxA-induced hepatic effects.
- Haney noted an inconsistency in discussions of necrosis in rats and suggested that EPA revise the wording to be consistent.
- In the "Evidence from other PFAS" section, Ng suggested that EPA emphasize that the observations of PPARα independent and dependent pathways from the four other PFAS are consistent for both short-chain (e.g., PFBA) and long-chain (e.g., PFNA) substances, increasing the plausibility that it also applies to PFHxA.
- Savitz noted that the interpretation of both epidemiologic studies is reasonable, but he commented that it is not clear why the potential for confounding is considered to be so substantial without some indication of the rationale for expecting that serum PFHxA levels are associated with the confounding factors. Savitz suggested including stronger reasoning as to why such confounding

would be expected. He made a similar comment on each of the remaining health effects, stressing that it is not obvious why confounding is considered a fatal flaw, and suggested it could be explained early in the report to avoid repetition.

Tier 3 Future Considerations

- Reviewers had no Tier 3 comments.

Developmental Effects

*b. For developmental effects, the Toxicological Review concludes the available **evidence indicates** PFHxA likely causes developmental effects in humans under relevant exposure circumstances. This judgment is based primarily on gestational exposure experiments in mice, with supportive findings in rats exposed throughout gestation and lactation, showing increased perinatal mortality, decreased offspring body weight, and delayed eye opening. These effects are similar to those observed for other PFAS following developmental exposure and were determined to be adverse and relevant to humans.*

Tier 1 Necessary Revisions

- Reviewers had no Tier 1 comments.

Tier 2 Suggested Revisions

- Haney suggested that EPA improve the discussion of human relevance such as by adding information on the conserved biological processes or similarities in anatomy and physiology between rodents and humans that EPA considers relevant to the observed developmental effects, or whether rodents (particularly the mouse) have been shown to be good laboratory animal models for assessing potential human developmental effects.

Tier 3 Future Considerations

- Reviewers had no Tier 3 comments.

Hematopoietic Effects

*c. For hematopoietic effects, the Toxicological Review concludes the available **evidence indicates** PFHxA likely causes hematopoietic effects in humans under relevant exposure circumstances. This judgment is based on consistent findings, including decreased red blood cells [RBCs], hematocrit, and hemoglobin, across study designs that, when interpreted together, signifies PFHxA-related hematological effects such as anemia. These findings were determined to be adverse and relevant to humans.*

Tier 1 Necessary Revisions

- Ng recommended that EPA clarify why the animal evidence is “moderate” rather than “robust” given that all four animal studies were assessed high confidence and there was agreement across study findings and doses. Ng noted that this clarification would provide context for what drives the “moderate” decision, and it will help to align with the conclusion that “the currently available evidence indicates that PFHxA likely causes hematopoietic effects in humans.”

Tier 2 Suggested Revisions

- Haney suggested that EPA improve the discussion of human relevance such as by adding information on the conserved biological processes between rats and humans that EPA considers relevant to the observed hematopoietic effects, or whether rodents (particularly the mouse) have been shown to be good laboratory animal models for assessing potential human hematopoietic effects. Ng provided a similar Tier 2 revision, suggesting that in addition to the existing statement that "effects in rats are considered relevant to humans," EPA add a more nuanced statement in the specific context of hematopoietic effects.

Tier 3 Future Considerations

- Ng commented there is a noted lack of discussion of findings across other PFAS as supporting information.

Endocrine Effects

d. For endocrine effects, the Toxicological Review concludes the available evidence suggests, but is not sufficient to infer, that PFHxA may cause endocrine effects in humans under relevant exposure circumstances. This conclusion is based on some evidence of thyroid effects based on hormone and histopathological changes in two rat studies; however, the data is limited, lacking consistency across studies, and histopathological changes may be explained by non-thyroid related effects.

Tier 1 Necessary Revisions

- Three reviewers recommended EPA reconsider the conclusion on endocrine effects that states, "Overall, the currently available *evidence suggests*, but is not sufficient to infer, that PFHxA could cause endocrine effects in humans under relevant exposure circumstances." Specifically:
    - Zoeller recommended that EPA conclude that the available evidence indicates that PFHxA exposure is likely to cause thyroid toxicity in humans given relevant exposure circumstances, primarily based on short-term studies in rats reporting a consistent and coherent pattern of effects on thyroid hormones following PFHxA exposure, but also drawing from the consistency of effects when considering evidence from structurally related PFAS. Zoeller's full comments (see Section 3.3) provide additional details supporting this recommendation.
    - Leung recommended including the consideration that PFHxA exposure may be associated with decreased thyroid hormones levels in humans as informed by the NTP (2018) study.
    - Faustman recommended that EPA re-examine the part of the statement that says, "but is not sufficient to infer" that PFHxA could cause endocrine effects in humans.
- Ng recommended deleting or providing a better justification for the statement, "some of these inconsistencies could be explained by differences in the test article (i.e., PFHxA vs. PFHxA salts)". She noted that both the acids and salts will dissociate at biologically relevant pH to form the identical anion.

Tier 2 Suggested Revisions and Tier 3 Future Considerations

- Reviewers had no Tier 2 or Tier 3 comments.

All Other Potential Health Effects

e. *For all other potential health effects (i.e., renal, male and female reproductive, immune, and nervous system), the Toxicological Review concluded the available evidence is inadequate to assess whether PFHxA may cause effects in humans under relevant exposure circumstances. In general, these conclusions were driven by sparse evidence bases or data that were largely null.*

Tier 1 Necessary Revisions

- Faustman recommended improving transparency by including observations across other PFOS compounds for the broad list of potential endpoints in this section, either by each endpoint listed in charge question 3(e) or by providing an overall summary table of input from evaluation of other PFOS compounds for these endpoints.
- For renal effects, Savitz recommended noting reverse causality as a concern in the Seo et al. (2018) study. He also recommended providing a clearer justification for considering Zhang et al. (2019) as "uninformative."

Tier 2 Suggested Revisions

- For respiratory effects, Georgopoulos suggested re-examining the respiratory effects observed in the 28-day NTP (2018) study and the 90-day Loveless et al. (2009) study for potential incorporation in the Toxicological Review.
- For renal effects, Haney suggested several revisions to Table 3-19: 1) Consider noting the potential for reverse causality as a factor that decreases certainty for the association of PFHxA with decrease in estimated eGFR; 2) consider adding "weak, no, or inconsistent dose-response" as a factor that decreases certainty for organ weight; 3) as a factor that decreases certainty, consider adding that "blood biomarkers of renal function were inconsistent"; and 4) as another factor that decreases certainty, consider adding difficulty in interpreting the observed effects as adverse or non-adverse.
- For immune effects, Leung suggested improving clarity by moving asthma to its own Pulmonary Effects section, since the one human asthma study examined was mostly of non-immune mediated outcomes.
- In regard to nervous system effects, for consistency with Table 3-31, Haney suggested editing Table 3-37 to indicate that EPA's "preferred metric" for brain weight is absolute brain weight.
- For nervous system effects, Ng noted that zebrafish studies are common for PFAS and should be considered as useful supplemental data to inform evaluations. She also commented that this section could benefit from discussion of known impacts of other PFAS that might inform design of future studies.

Tier 3 Future Considerations

- Reviewers had no Tier 3 comments.

2.3 Noncancer Toxicity Values Data Selection

Charge Question 4*. For PFHxA, no RfC was derived. The study chosen for use in deriving the RfD is the Loveless et al. (2009) one-generation reproductive toxicity study based on decreased offspring body weight in rats exposed continuously throughout gestation and lactation to PFHxA sodium salt via the dam. Is the*

selection of this study and these effects for use in deriving the RfD for PFHxA scientifically justified and clearly described?

a. *If yes, please provide an explanation.*

b. *If no, please provide an alternative study(ies) or effect(s) that should be used to support the derivation of the RfD and detail the rationale for use of such an alternative.*

c. *As part of the responses in "a" or "b" above, please comment on whether the effects selected are appropriate for use in deriving the RfD, including considerations regarding adversity (or appropriateness in representing an adverse change) and the scientific support for their selection.*

d. *Given the lack of studies on inhalation exposure to PFHxA, no reference concentration (RfC) is derived. Please comment on this decision.*

Three reviewers concurred with the selection of the Loveless et al. (2009) study and the effect of decreased offspring body weight as scientifically justified for derivation of an RfD for PFHxA. Two reviewers recommended the NTP (2018) study with serum T4 as an endpoint be used as an alternative. Leung commented that the reasoning presented for RfD derivation appeared sound but noted that this topic is not her area of expertise. Savitz declined to comment, stating that this topic was not in his area of expertise. All reviewers who provided comments agreed with the decision to not derive a reference concentration.

Tier 1 Necessary Revisions

- Faustman recommended that EPA calculate the developmental osRfD using the T4 endpoint from the NTP (2018) study to determine if this has significant impact on the calculation of the RfD. If this does have a significant impact, then Faustman recommended prioritizing the use of the T4 endpoint. Although not categorized as a tiered recommendation, Zoeller commented that the NTP (2018) study with serum T4 as an endpoint should be used as an alternative to support the derivation of an RfD. He stated that this study was high confidence and showed robust response to PFHxA exposure in terms of T4 suppression, which is relevant for human health and predictive of adverse effects in humans.

Tier 2 Suggested Revisions and Tier 3 Future Considerations

- Reviewers had no Tier 2 or Tier 3 comments.

Charge Question 5. *In addition, for PFHxA, an RfD for less-than-lifetime ("subchronic") exposures is derived. No "subchronic" RfC was derived. The same study and outcome were chosen for use in deriving the RfD. Is the selection of this study and these effects for the derivation of the subchronic RfD for PFHxA scientifically justified and clearly described?*

a. *If yes, please provide an explanation.*

b. *If no, please provide an alternative study(ies) and/or effect(s) that should be used to support the derivation of the subchronic RfD and detail the rationale for use of such an alternative.*

c. *As part of the responses in "a" or "b" above, please comment on whether the effects selected are appropriate for use in deriving the RfD, including considerations regarding adversity (or appropriateness in representing an adverse change) and the scientific support for their selection.*

d. *Given the lack of studies on inhalation exposure to PFHxA, no "subchronic" RfC is derived. Please comment on this decision.*

Reviewers' comments on the charge questions related to the derivation of the subchronic RfD were similar to those made for the chronic RfD. Most reviewers concurred with the selection of the Loveless et al. (2009) study and the selected effect as scientifically justified for derivation of the subchronic RfD for PFHxA. As with the chronic RfD, Zoeller suggested using the NTP (2018) study with the endpoint of T4 suppression, although he did not include this comment as a tiered recommendation. Leung commented that the reasoning presented in this section appeared sound but noted that this topic is not her area of expertise. Savitz declined to comment, stating that this topic was not in his area of expertise. All reviewers who provided comments agreed with the decision to not derive a subchronic reference concentration.

Tier 1 Necessary Revisions

- Reviewers had no Tier 1 comments.

Tier 2 Suggested Revisions

- In reviewing the hepatic and developmental impacts, Faustman suggested text to add, to the organ-specific narrative for hepatic effects and for developmental impacts, regarding adversity versus adaptation that she noted may be relevant for the study selection justification. These studies were either medium or high confidence studies with good annotation and discussion of observations, and the quantitative estimates resulting from these calculations indicate that these are sensitive hence protective endpoints for use in the RfD development. For details, see her response to charge questions 3a and 3d in Section 3.3.
- In reviewing the hepatic and developmental impacts, Faustman suggested text to add regarding health impacts to the human population, noting that these endpoint choices for the RfD are highly relevant for human populations. For details, see her response to charge question 3a and 3d in Section 3.3.

Tier 3 Future Considerations

- Reviewers had no Tier 3 comments.

2.4 Noncancer Toxicity Value Derivation

Charge Question 6. *EPA used benchmark dose modeling (USEPA, 2012) to identify points-of-departure (PODs) for oral exposure to PFHxA. Are the modeling approaches used, selection and justification of benchmark response levels, and the selected models used to identify each POD for toxicity value derivation scientifically justified and clearly described?*

All reviewers who provided responses to this charge question concurred that the approaches used, and the identification of PODs were scientifically justified and clearly described. Faustman was impressed with the details provided to identify the PODs for exposure to PFHxA and found the tables very easy to use. Leung and Savitz declined to comment, stating that this topic was outside of their area of expertise.

Tier 1 Necessary Revisions

- If models that do not provide adequate fit are included in the tables summarizing benchmark dose modeling results for different endpoints (in Appendix B), Georgopoulos recommended that these

models should be marked/identified as such in these tables (e.g., by placing the model names and associated estimates in parentheses).

- Ng commented that in Table B-25, the selected model (indicated by bold type in the table and shown in the proceeding figure) has neither the lowest AIC nor lowest BMDL. While an explanation of this was provided by EPA during the peer review meeting, Ng recommended that the text would benefit from including this as an example of the utility of visual inspection.

Tier 2 Suggested Revisions

- Reviewers had no Tier 2 comments.

Tier 3 Future Considerations

- Georgopoulos suggested comparing the POD estimates in the current Toxicological Review with estimates calculated using the Bayesian continuous models available in BMDS 3.2.

Charge Question 7. *Appendix A identifies the potential for pharmacokinetic differences across species and sexes as a key science issue and lays out a hierarchy for using relevant pharmacokinetic data in extrapolating oral doses between laboratory animals and humans. Section 5.2.1 describes the various approaches considered and the rationale for the selected approach. Given what is known and not known about the potential interspecies differences in PFHxA pharmacokinetics, EPA used the ratio of human-to-animal serum clearance values assuming the volume of distribution (V_d) in humans is equivalent to that in monkeys to adjust the POD to estimate a human equivalent dose (HED) in the derivation of the respective RfDs.*

a. *Is applying the ratio of human-to-animal serum clearance values for PFHxA scientifically justified and clearly described? If not, please provide an explanation and detail the preferred alternative approach.*

b. *Does the Toxicological Review clearly describe the uncertainties in evaluating the pharmacokinetic differences between the experimental animal data and humans?*

Reviewers who provided responses to this charge question generally concurred that the approach used for potential interspecies differences in PFHxA pharmacokinetics was scientifically justified and clearly described. The same reviewers stated that the Toxicological Review clearly described the uncertainties. Several reviewers provided recommendations for improving the clarity. Leung and Savitz declined to comment, stating that this topic was outside of their area of expertise.

Tier 1 Necessary Revisions

- Georgopoulos recommended that the pharmacokinetic assumptions and parameterizations used by EPA in the *httk: High-Throughput Toxicokinetics* package should be briefly mentioned/discussed in the Toxicological Review (since httk is a publicly available EPA "product") and the context for making comparisons with the assumptions and parameterizations of the pharmacokinetic modeling performed for this Review should be clarified.

- Ng commented that the reasoning behind using CL as opposed to $t_{1/2}$ uses two conflicting lines of reasoning and clarification is needed.

Tier 2 Suggested Revisions

- Given the lack of sex differences observed in human studies, Ng suggested clarifying the text implying that female human and male human equivalent doses will be calculated on the basis of sex-specific PODs in animals.
- Ng commented that discussion of the Pérez et al. study should note that some of the results were called into question for PBFA[2] and some of these issues could also apply to PFHxA. She suggested either avoiding use of the Pérez study as supplemental information, or if used, to include a caveat per the additional studies she referenced.
- Ng commented that the reference to slower elimination at higher concentrations (Dzierlenga et al.) was noted as opposite the expectation of saturable renal absorption (mediated by Oatp1a1). She noted that Han et al. mentions other transporters that have been tested for activity with PFAS. Ng suggested a clarification be added such as: "While saturation of reabsorption transporters would lead to decreased half-life, there are also transporters responsible for elimination of PFAS to urine, and saturation of these transporters, such as Oat 1 and Oat3, could lead to an increase in observed half-life and could thereby help explain the observations of Dzierlenga et al."

Tier 3 Future Considerations

- Given the overall uncertainty in human clearance, Haney suggested that EPA seek data that may allow for animal-to-human extrapolation methods and/or dosimetric adjustment factors for PFHxA that are associated with greater confidence/less uncertainty as soon as practicable and consistent with applicable guidelines.

Charge Question 8. *EPA has evaluated and applied uncertainty factors to account for intraspecies variability (UFH), interspecies differences (UF_A), database limitations (UF_D), exposure duration (UF_S), and LOAEL-to-NOAEL extrapolation (UF_L) for PFHxA.*

a. Is uncertainty in the derivation of the toxicity values scientifically justified and clearly described? Please describe and provide comments, if needed.

All reviewers who provided a response to Charge Question 8(a) generally concurred that uncertainty had been adequately accounted for in the Toxicological Reviewer. Leung and Savitz declined to comment, stating that this topic was outside of their area of expertise.

b. For uncertainty in interspecies differences (UF_A), a value of 3 is applied to account for remaining uncertainty in characterizing the pharmacokinetic and pharmacodynamic differences between laboratory animals and humans after calculation of the HED. For developmental and hematopoietic outcomes, the evidence base lacked chemical-and species-specific information that would have been useful for informing the UFA; for hepatic outcomes, however, available mechanistic and supplemental information was useful for further evaluating the interspecies uncertainty factor. Some data indicate a PPARα-dependent pathway that might support a UF_A of 1. Evidence for non-PPARα modes of action, however, is available in the PFHxA (and larger PFAS) database. Thus, uncertainty remains regarding the potential differences in sensitivity across species due to the involvement of both PPARα-dependent and-independent pathways. Further, data are lacking to determine with confidence the relative contribution of each of these pathways. As such, the Toxicological Review concludes the available data are not adequate to determine if humans are likely to be equally or less

[2] https://www.sciencedirect.com/science/article/pii/S1438463921001450?via%3Dihubsciencedirect.com

sensitive than laboratory animals with respect to the observed hepatic effects and that a value of UF_A=3 is warranted to account for the residual uncertainty in pharmacodynamic differences across species. Please comment on whether the available animal and mechanistic studies support this conclusion and whether the analysis presented in the Toxicological Review is scientifically justified and clearly described.

Reviewers had mixed responses when commenting on the UF_A of 3:

- Faustman supported EPA's approach and Ng commented that the UF_A of 3 was well justified.
- Haney noted that in his experience, the application of a default UF_A of 3 for potential interspecies toxicodynamic (TD) differences is standard EPA practice when interspecies toxicokinetics adjustments have been performed and there is a lack of chemical-specific information on TD for a more data-informed approach, as is the case here.
- Zoeller, however, commented that the UF_A of 3 did not seem well described and he suggested a Tier 2 revision that EPA provide a more explicit description of the reasoning for choosing a UF_A of 3 instead of 1 or 10.
- Georgopoulos commented that a UF_A of 10 should be considered since the current understanding of interspecies differences in PFAS both pharmacokinetics and pharmacodynamics for PFHxA has very significant gaps. He did not, however, list his comment as a tiered revision.
- Leung and Savitz declined to comment, stating that this topic was outside of their area of expertise.

c. *To inform uncertainty in intraspecies variability (UF_H), the assessment evaluates and considers the available evidence on potential susceptibility to PFHxA within different populations or lifestages, including any potential human health impacts from early life exposure. Are the available information and data appropriately considered and the resultant UF_H values scientifically justified and clearly described?*

All reviewers who responded to Charge Question 8c concurred with a UF_H of 10. Leung and Savitz declined to comment, stating that this topic was outside of their area of expertise.

d. *Are the provided rationales for the remaining uncertainty factors (UF_L , UF_D , UF_S) scientifically justified and clearly described? If not, please explain.*

Reviewers provided several comments related to the remaining uncertainty factors:

- Faustman liked the detailed discussion of these factors provided in Table 5-6.
- Following up on his comment on the UF_A of 3 (under Charge Question 8[b]), Georgopoulos commented as a Tier 2 suggestion that if EPA decides to maintain a value of 3 for UF_A, then a value of 10 should be adopted for UF_D.
- Haney provided detailed comments on the remaining uncertainty factors and included two Tier 2 suggested revisions (see below).
- Zoeller commented that a UF_S of 1 does not appear to cover the uncertainty for development described in the document.
- Leung and Savitz declined to comment, stating that this topic was outside of their area of expertise.

Tier 1 Necessary Revisions

- Reviewers had no Tier 1 comments.

Tier 2 Suggested Revisions

- Georgopoulos commented that if EPA decides to maintain a value of 3 for UF_A, then a value of 10 should be adopted for UF_D.
- For the UF_S for hepatocellular hypertrophy, Haney suggested that EPA consider including a discussion of the specific study results justifying the specific UF_S value proposed (i.e., 3 instead of 10).
- For the UF_D, Haney recommended that Table 5-6 be modified to delete "the dose received by the pups is unclear and might be significantly less than that administered to the dams" as a cited factor that in a meaningful way diminishes confidence in the database relevant to deriving the RfD. Otherwise, since developing organism (e.g., pup) doses are commonly unknown, by EPA's reasoning a UF_D of 3 might automatically be applied any time the basis for an RfD or candidate RfD is developmental effects. Moreover, it is not needed as the EPA cites other considerations that are sufficient to support a UF_D of 3.
- Zoeller suggested that EPA consider a more explicit description of the reasoning for choosing a UF_A of 3 instead of 1 or 10.
- Zoeller suggested revising the UF_S of 1 to 10.

Tier 3 Future Considerations

- Reviewers had no Tier 3 comments.

2.5 Carcinogenicity Hazard Identification and Toxicity Value Derivation

Charge Question 9. *The Toxicological Review concludes that there is inadequate information to assess carcinogenic potential for PFHxA and that this descriptor applies to oral and inhalation routes of human exposure. Please comment on whether the available animal and mechanistic studies and the analysis presented in the Toxicological Review are scientifically justified and clearly described.*

All reviewers concurred that the analysis presented in the Toxicological Review was scientifically justified and clearly described.

Tier 1 Necessary Revisions, Tier 2 Suggested Revisions, and Tier 3 Future Considerations

- Reviewers had no Tier 1, Tier 2, or Tier 3 comments.

Charge Question 10. *Given the conclusion there was inadequate information to assess carcinogenic potential for PFHxA (Charge Question 5), the Toxicological Review does not derive quantitative estimates for cancer effects for either oral or inhalation exposures. Is this decision scientifically justified and clearly described?*

All reviewers concurred that the decision to not derive quantitative estimates for cancer effects was scientifically justified and clearly described in the Toxicological Review.

Tier 1 Necessary Revisions, Tier 2 Suggested Revisions, and Tier 3 Future Considerations

- Reviewers had no Tier 1, Tier 2, or Tier 3 comments.

2.6 Additional Comments

Two reviewers (Ng and Georgopoulos) provided additional comments separately from their responses to the charge questions. These included the following tiered comments not already covered in their responses to charge questions.

Tier 2 Suggested Revisions

- Ng suggested that EPA carefully consider how data from other PFAS either support or differ from PFHxA observations and how those could be explained by structure-activity relationships (e.g., chain length vs. half-live observations) as well as how data from other model systems (e.g., zebrafish) could help to fill data gaps.
- Ng suggested that EPA harmonize the discussion of supporting evidence across the different endpoints considered. For example, if structure-activity relationship information is available for hepatic effects and the document includes text on what should be expected for PFHxA based on observations for other PFAS, then under developmental effects, the document should state whether similar structure-activity relationships could be considered or if such information is not available.
- Ng suggested adding context on reliability for the information presented in Table 1-1 on the available physicochemical properties of PFHxA. She noted, for example, that water solubility of ammonium vs. sodium salts varies five orders of magnitude and stated that "clearly one of these values is wrong as once dissociated these should behave similarly." Similarly, Ng commented that the same is true for the bioconcentration factor.
- In the pharmacokinetics background (Section 3.1) of the Toxicological Review, Ng suggested clarifying how "substantial binding" to serum proteins is defined (see page 3-5, lines 6-7). She noted that PFHxA has been shown in in vitro studies to bind less strongly than long-chain PFAS.

Tier 3 Future Considerations

- Georgopoulos commented that future efforts and revisions of the assessment for PFHxA (and other PFAS) must consider cumulative risks and reasonable population exposure (and potential exposure) distributions.

3.0 REVIEWER RESPONSE TO CHARGE QUESTIONS

3.1 The Toxicological Review for PFHxA describes and applies a systematic review protocol for identifying and screening pertinent studies. The protocol is described in brief detail in Section 1.2.1 (Literature Searching and Screening) and in full detail in Appendix A (Systematic Review Protocol for the PFAS IRIS Assessments). Please comment on whether the search strategy and screening criteria for PFHxA literature are clearly described. If applicable, please identify additional peer-reviewed studies of PFHxA that the assessment should incorporate.[3]

[3] Newly identified studies (i.e., studies identified by EPA or the public that meet PECO criteria but were not addressed in the external review draft, for example due to recent publication) will be characterized by EPA and presented to the peer review panel. This characterization will focus on EPA's judgment of whether the studies would have a material impact on the conclusions (i.e., identified hazards or toxicity values) in the external review draft. The peer review panel is asked to review EPA's characterization and provide tiered recommendations to EPA regarding which studies, if any, to incorporate into the assessment before finalizing.

Reviewer	Comments
Faustman	The search criteria used by EPA in Section 1 and in Appendix A are appropriate and clear to follow. Details in Section 1.2.1 provide a summary of assessment methods. It was clear to this reviewer what types of literature were included and how individual studies were evaluated. In Section 1.2.2 the general approach for meeting PECO criteria was outlined and the domain specific study confidence classifications defined. In addition, this section provided information on how grey literature was listed and how other "potentially relevant supplemental material" was inventoried. Evidence synthesis was also documented. Figure 2-1 provided a very clear search and screening flow diagram for PFHxA. This diagram provided details on numbers of individual studies that went through the various screening processes. For clarity it would be useful to explain in the text the major reasons for exclusions in Figure 2-1 as this question is always asked. (After the Title and Abstract Screening section is most in need of this clarification). This **Tier 1 Necessary Revisions** requires addition of a few sentences to identify what category of exclusions occurred for the 194 articles that were excluded in this part of the screening flow. A **Tier 3 Future Consideration** would be to clarify just what database access will be accorded to the public. For example, will HERO/ HAWK be available to the public? If not please clarify how the public will access the publications within the database and at what level of access. **Tier 2 Suggested Revision** to review newly found literature. I have attached a literature search that I performed. I found quite a few additional exposure studies that could inform and provide additional confidence for the discussions in Section 1 Overview. There were several in vitro and in vivo studies that will be highlighted in the organ specific section in Section 3. (I have **bolded** those references that are of potential interest.) [please see Section 4.0 Additional Comments] This reviewer appreciated the footnote by USEPA (Page 1-10) about newly identified studies and how they would handle new information arriving after their external review draft. This reviewer will be very interested in how the individual IRIS reviews across the multiple PFAS compounds will evolve as this review series continues. A **Tier 2 Suggested Revision** would be to prepare a link across these agents which could pull key points across the individual reviews so a user of the IRIS materials could see similarities and differences in these related family of chemicals. I would envision that the users of these documents will usually be addressing mixtures of these compounds in the field. A "cross-talk" document that listed in one common place key summary info such as the information from the tables presented in the Executive Summaries from the individual agents would help the user community with coordination of this information. Please note that during the May 16, 2022 meeting with USEPA on the External Peer Review of the EPA Draft "IRIS toxicological Review of Perfluorohexanoic Acid and Related Salts" K. Thayer presented a USEPA ORD background Presentation and in that presentation further discussion about the IRIS PFAS Systematic Evidence Mapping was presented. A **Tier 1 Necessary Revision** by this reviewer is to add several sentences to Section 1 that describes the in-press paper EHP (DOI 10.1289/EHP 10343) shown in the

	slides and available on line as these post- meeting comments were compiled. In particular the evidence maps such as shown in Figures 5 and 6 illustrate how EPA is going to do evidence synthesis across the PFAS compounds and would be a good addition to describe in text what will be occurring across the expanding PFAS database.
Georgopoulos	The search strategy and screening criteria for PFHxA literature are clearly described in Section 1.2.1 and Appendix A: the methods used were appropriate and consistent with scientific standards and practices. However, the review process for identifying and screening pertinent studies for the PFHxA Toxicological Review (as well as for the Toxicological Reviews for other PFAS) is inherently challenging. Historically, the number of both animal and epidemiological studies focusing on PFHxA has been relatively limited but the recognition of the fact that multiple health effects are associated (or are potentially associated) with PFAS exposures in general, as well as the increasing interest in short-chain compounds as alternatives to legacy PFAS, has led to numerous new (and on-going) studies, with multiple related publications appearing at an increasing rate. This is creating the need to regularly reassess and update the information contained in these Toxicological Reviews. The current draft of the PFHxA Toxicological Review already needs substantial updating. Though in the document it is stated (page 1-9, lines 14-15) that "the literature fully considered in the assessment was until April 2021," it is not clear if and how studies published after early 2020 have been incorporated/integrated in the Review. USEPA's Health & Environmental Research Online (HERO) database for PFHxA is maintained current with new publications but the process of incorporating them in a timely manner in the Toxicological Review is not clear. Even if no new toxicological studies are judged at this time as appropriate for incorporating in the Review, an updating of basic information is still needed. This is particularly true for the first Chapter (Overview of Background Information and Assessment Methods) of the Review. (It should be noted that the current Toxicological Review and the associated Supplemental Information document only cite references until 2020 [actually, the Toxicological Review only cites two references from 2020, i.e. U.S. EPA (2020) and Goodrow et al. (2020)]. As an example of outdated information, on page 1-6 (lines 14-16) one reads that PFHxA has not been reported in studies of human breast milk in the US, although a study that was widely publicized last year, reported that PFHxA was detected at a high frequency (64% of samples) in breast milk in the US general population (Zheng et al., 2021). In fact, Zheng et al. (2021) reported concentrations of PFHxA (median 9.69 pg/ml) that were higher than for the three short-chain PFAS that were detected, and the authors stated that the concentrations of PFHxA "were comparable to those of PFOA." Beyond the Zheng et al. (2021) study, references to large scale biomonitoring efforts (see, e.g., Calafat et al., 2019) should also be included. Furthermore, representative documents reporting risk assessment efforts of regulatory agencies, both in the US and internationally (e.g. ECHA, 2019) relevant to PFHxA, should be mentioned and cited in the Toxicological Review. An issue that requires clarification is the consistency of information provided regarding the literature searches (and associated outcomes) conducted for this Toxicological Review. Though, as mentioned above, the Review document asserts that "the literature fully considered in the assessment was until April 2021," Figure 2-1 (on page 2-2 of the

	Review) states that information considered is from "Literature Searches (through Feb 2020)." According to the flowchart in that figure, these searches resulted in "339 records after duplicate removal" after "Title & Abstract Screening," and, from these 339 records, 194 were excluded as "Not relevant to PECO." After "Full-Text Screening" off 77 studies, 7 more were excluded (3 as not relevant to PECO, 2 as being a review/commentary/letter, and 2 as "Other"), thus resulting to 26 "studies Meeting PECO" and 118 studies "Tagged as Supplemental." It should be pointed out that the HAWC (Health Assessment Workplace Collaborative) website for PFHxA, in the "Study List" tab, lists 27 (instead of 26) studies. Furthermore, the table of studies currently available for downloading (as an Excel spreadsheet) from HAWC lists a total of 335 (instead of the 339 in Figure 2-1) study records, 226 of which (instead of the 194+7=201 in Figure 2-1) are flagged as "excluded" and 68 (instead of 118) are flagged as "Supplemental Material." Though it is stated in Figure 2-1 that "some studies were assigned multiple tags," it appears that there are various discrepancies between the numbers in Figure 2-1 and the downloadable HAWC table of studies considered/tagged, as further explained below, that should be explained (or reconciled): Specifically, the "Studies Meeting PECO (n = 26)" according to Figure 2-1 are classified as follows: Human health effects studies (n = 14); Animal health effect studies (n = 6); Genotoxicity studies (n = 3); PK studies (n = 3). On the other hand, 36 of the 335 studies listed in the downloadable HAWC table (Excel spreadsheet) were assigned the "Inclusion" tag; the numbers of studies tagged within the "Meeting PECO" categories in that table are: Human Study (n = 19); Animal Study (n = 14); in vitro (genotox) (n = 5). One specific issue, that was in fact identified in the Public Comments provided by NRDC, is the inclusion of the study of Maekawa et al. (2017), with "inclusion" tag, in the HAWC table. Table 1 of the Maekawa et al., (2017) study lists "Perfluorohexanoic acid", but the abbreviation in the table is provided as PFHxS and the authors refer to the chemical in all other occurrences within the article as PFHxS. *A clarification is needed for this issue.* The 118 studies "Tagged as Supplemental" in Figure 2-1 of the PFHxA Toxicological Review are classified as follows: ADME (n = 40); Background/exposure (n = 42); Case report or case study (n = 2); Mechanistic or MOA (n = 9); Mixture-only (n = 3); Non-PECO route of exposure (n = 2); Qualitative exposure only (n = 12); Susceptible population (n = 4); Other (n = 15). Again, there are various discrepancies with some of the numbers referring to studies in different categories tagged as "Supplemental Material" in the HAWC table; examples are: ADME (n = 16); Mechanistic or MOA (n = 7); Mixture-only (n = 1); and Susceptible population (n = 2). Another issue that also requires explanation/reconciliation is the discrepancy of the number of pre- and up to-2020 literature records involving PFHxA, that were examined (339 according to Figure 2-1 or 335 according to the HAWC table) and the numbers of literature records in EPA's PFHxA HERO database. Specifically, HERO currently contains 865 such records, but 763 of them are in fact dated from 2020 and before, and 657 records are from 2019 and before: this is almost double the number of records considered for the Toxicological Review. An explanation regarding this discrepancy (i.e. why and with what criteria studies were not considered/reviewed) is needed. It should also be pointed out that in the HERO database there are 567 PFHxA-relevant references dated from 2018 and before, while the HAWC website for PFHxA, in the "Literature

	Review" tab, under the header "References for PFHxA (2018)," mentions 1,071 "total references" (782 "tagged" and 589 "untagged"). On page 1-10 (lines 26-28) PFHxA Toxicological Review it is stated that "Literature inventories for studies meeting PECO criteria and studies tagged as "potentially relevant supplemental material" during screening were created to facilitate subsequent review of individual studies or sets of studies by topic-specific experts." An open and flexible framework is therefore necessary for identifying and systematically organizing and screening Supplemental PFHxA-related studies with respect to both the methods used (in vitro, in silico, in vivo, etc.) and the toxicity (or, in general, bioactivity) endpoints considered. In silico, in vitro and non-mammalian model organism studies may not have the same weight as rodent laboratory and human epidemiological studies, but they can provide mechanistic insights, both for hypothesis development and for animal study evaluation, as well as corroborate hypotheses derived from rodent and epidemiological studies, when their results consistently "point to the same direction". It should also be recognized that real-world exposures to PFHxA are in most cases occurring as co-exposures to mixtures of PFAS, and that PFHxA most probably shares multiple AOPs (Adverse Outcome Pathways) with other PFAS; these facts will continue to pose challenges in the interpretation and evaluation of epidemiological studies. Furthermore, screening of PFHxA studies will also need to consider concerns regarding available (past and current) analytical methods for short-chain PFAS, and to address and evaluate potential contradictions between published studies: an example would be the critique of the analytical methods used in the study of Pérez et al. (2013), which is a study included in the Supplemental Information for this Toxicological Review, presented in the recent publication by Abraham et al. (2021). Another example of potential inconsistencies in the literature seems to be the characterization of PPAR binding affinities of PFHxA (and of other short chain PFAS), as described in the studies of Khazae et al. (2021) and Ishibashi et al. (2019). It should be noted that these peer reviewed articles are not cited/mentioned in the current draft document of the PFHxA Toxicological Review, though they are included in the 865 references listed under PFHxA in the HERO database. It should also be noted that Khazaee et al. (2021) concluded that the "relatively strong [PPAR-alpha] binding affinity suggested by the KD of 0.097µM for PFHxA could have implications for short-chain PFAS safety," a finding that is potentially relevant to the present Toxicological Review. Another example of studies with potential inconsistencies (regarding binding affinities with serum proteins) are presented in Chen et al. (2020) and Allendorf (2021); the latter does not appear to be included in HERO, although Chen et al. (2020) is included. The above comments provide the rationale for the following recommendations and suggestions: **Tier 1 Necessary Revision:** Clarify and (if possible) reconcile discrepancies existing in study numbers that are quoted/listed in the Toxicological Review document and in the HAWC website for PFHxA **Tier 1 Necessary Revision:** Resolve ambiguities regarding the Maekawa et al. (2017) study.

	Tier 1 Necessary Revision: Update HAWC for PFHxA, to include assessments/evaluations of any more recent studies that will be considered in finalizing this Toxicological Review. **Tier 2 Suggested Revision:** Include a list of documents relevant to PFHxA risk characterization that have been developed by State and international regulatory agencies in the literature searches and in resulting databases; compile a summary (e.g. in the form of a table or a brief Appendix) of established or proposed values for metrics of reference doses/concentrations. **Tier 3 Future Consideration:** Develop and implement a plan for the systematic and "continuous" updating of databases (such as HERO and HAWC) tracking information relevant to the Toxicological Review; furthermore, specify criteria for new information that would require re-evaluation and updating of the contents and conclusions of the Toxicological Review. **Tier 3 Future Consideration:** The following is a list peer-reviewed studies (one is currently under review) that EPA should consider and evaluate for the PFHxA Toxicological Review: **ALTERNATIVE (NON-MAMMALIAN) MODEL ORGANISM STUDIES** Wasel, O., Thompson, K. M., Gao, Y., Godfrey, A. E., Gao, J., Mahapatra, C. T., ... & Freeman, J. L. (2021). Comparison of zebrafish in vitro and in vivo developmental toxicity assessments of perfluoroalkyl acids (PFAAs). Journal of Toxicology and Environmental Health, Part A, 84(3), 125-136. Zhang, S., Guo, X., Lu, S., He, J., Wu, Q., Liu, X., ... & Xie, P. (2022). Perfluorohexanoic acid caused disruption of the hypothalamus-pituitary-thyroid axis in zebrafish larvae. Ecotoxicology and Environmental Safety, 232, 113283. **IN VITRO STUDIES** Allendorf, F., Berger, U., Goss, K. U., & Ulrich, N. (2019). Partition coefficients of four perfluoroalkyl acid alternatives between bovine serum albumin (BSA) and water in comparison to ten classical perfluoroalkyl acids. Environmental Science: Processes & Impacts, 21(11), 1852-1863 Allendorf, F. (2021). Equilibrium sorption of perfluoroalkyl acids (PFAAs) and four of their alternatives in mammals (Doctoral dissertation, Dissertation, Halle (Saale), Martin-Luther-Universität Halle-Wittenberg, 2021). Amstutz, V. H., Cengo, A., Sijm, D. T. H. M., & Vrolijk, M. F. (2022). The impact of legacy and novel perfluoroalkyl substances on human cytochrome P450: An in vitro study on the inhibitory potential and underlying mechanisms. Toxicology, 468, 153116. Chen, H., Wang, Q., Cai, Y., Yuan, R., Wang, F., & Zhou, B. (2020). Investigation of the interaction mechanism of perfluoroalkyl carboxylic acids with human serum albumin by spectroscopic methods. International Journal of Environmental Research and Public Health, 17(4), 1319. Coperchini, F., De Marco, G., Croce, L., Denegri, M., Greco, A., Magri, F., ... & Chiovato, L. (2022) The Per-and Poly-Fluoroalkyl Substances, Pfoa, Pfhxa and C6o4 Differently

Modulate the Expression of the Pro-Tumorigenic Chemokine Cxcl8 in Normal Thyroid Cells and in Thyroid Cancer Cell Lines. SSRN PREPRINT http://dx.doi.org/10.2139/ssrn.4060867

Ishibashi, H., Hirano, M., Kim, E. Y., & Iwata, H. (2019). In vitro and in silico evaluations of binding affinities of perfluoroalkyl substances to Baikal seal and human peroxisome proliferator-activated receptor α. Environmental Science & Technology, 53(4), 2181-2188.

Khazaee, M., Christie, E., Cheng, W., Michalsen, M., Field, J., & Ng, C. (2021). Perfluoroalkyl acid binding with peroxisome proliferator-activated receptors α, γ, and δ, and fatty acid binding proteins by equilibrium dialysis with a comparison of methods. Toxics, 9(3), 45.

Modaresi, S. M. S., Wei, W., Emily, M., DaSilva, N. A., & Slitt, A. L. (2022). Per-and polyfluoroalkyl substances (PFAS) augment adipogenesis and shift the proteome in murine 3T3-L1 adipocytes. Toxicology, 465, 153044.

Pierozan, P., Cattani, D., & Karlsson, O. (2022). Tumorigenic activity of alternative per-and polyfluoroalkyl substances (PFAS): Mechanistic in vitro studies. Science of The Total Environment, 808, 151945.

Xie, M. Y., Lin, Z. Y., Liu, L. Y., Wu, C. C., Liu, Y. W., Huang, G. L., & Zeng, E. Y. (2022). Use of glioma to assess the distribution patterns of perfluoroalkyl and polyfluoroalkyl substances in human brain. Environmental Research, 204, 112011.

IN SILICO STUDIES

Yu, S., Ren, J., Lv, Z., Li, R., Zhong, Y., Yao, W., & Yuan, J. (2022). Prediction of the endocrine-disrupting ability of 49 per-and polyfluoroalkyl substances: In silico and epidemiological evidence. Chemosphere, 290, 133366.

ANIMAL STUDIES - TOXICOKINETICS (MICE)

Jia, Y., Zhu, Y., Xu, D., Feng, X., Yu, X., Shan, G., & Zhu, L. (2022). Insights into the Competitive Mechanisms of Per-and Polyfluoroalkyl Substances Partition in Liver and Blood. Environmental Science & Technology.

ANIMAL STUDIES - HEPATOTOXICITY (MICE)

Jiang, L., Hong, Y., Xie, G., Zhang, J., Zhang, H., & Cai, Z. (2021). Comprehensive multi-omics approaches reveal the hepatotoxic mechanism of perfluorohexanoic acid (PFHxA) in mice. Science of The Total Environment, 790, 148160.

HUMAN EXPOSURE STUDIES THAT MEASURED BIOMARKERS

Babayev, M., Capozzi, S. L., Miller, P., McLaughlin, K. R., Medina, S. S., Byrne, S., ... & Salamova, A. (2022). PFAS in drinking water and serum of the people of a southeast Alaska community: A pilot study. Environmental Pollution, 119246.

Bao, J., Shao, L. X., Liu, Y., Cui, S. W., Wang, X., Lu, G. L., ... & Jin, Y. H. Target Analysis and Suspect Screening of Per-and Poly-Fluoroalkyl Substances in Paired Samples of

	Maternal Serum, Umbilical Cord Serum, and Placenta Near Fluorochemical Plants in Fuxin, China. SSRN http://dx.doi.org/10.2139/ssrn.4091456 Chen, X., Feng, X., Sun, X., Li, Y., Yang, Y., Shan, G., & Zhu, L. (2022). Quantifying Indirect Contribution from Precursors to Human Body Burden of Legacy PFASs Based on Paired Blood and One-Week Duplicate Diet. Environmental Science & Technology. Claessens, J., Pirard, C., & Charlier, C. (2022). Determination of contamination levels for multiple endocrine disruptors in hair from a non-occupationally exposed population living in Liege (Belgium). Science of The Total Environment, 815, 152734. Fiedler, H., Sadia, M., Krauss, T., Baabish, A., & Yeung, L. W. (2022). Perfluoroalkane acids in human milk under the global monitoring plan of the Stockholm Convention on Persistent Organic Pollutants (2008–2019). Frontiers of Environmental Science & Engineering, 16(10), 1-14. Li, N., Ying, G. G., Hong, H., & Deng, W. J. (2021). Perfluoroalkyl substances in the urine and hair of preschool children, airborne particles in kindergartens, and drinking water in Hong Kong. Environmental Pollution, 270, 116219. Macheka, L. R., Abafe, O. A., Mugivhisa, L. L., & Olowoyo, J. O. (2022). Occurrence and infant exposure assessment of per and polyfluoroalkyl substances in breast milk from South Africa. Chemosphere, 288, 132601. Peng, L., Xu, W., Zeng, Q., Sun, F., Guo, Y., Zhong, S., ... & Chen, D. (2022). Exposure to perfluoroalkyl substances in waste recycling workers: Distributions in paired human serum and urine. Environment international, 158, 106963. Xu, Y., Fletcher, T., Pineda, D., Lindh, C. H., Nilsson, C., Glynn, A., ... & Li, Y. (2020). Serum half-lives for short-and long-chain perfluoroalkyl acids after ceasing exposure from drinking water contaminated by firefighting foam. Environmental health perspectives, 128(7), 077004. Xu, Y., Nielsen, C., Li, Y., Hammarstrand, S., Andersson, E. M., Li, H., ... & Jakobsson, K. (2021). Serum perfluoroalkyl substances in residents following long-term drinking water contamination from firefighting foam in Ronneby, Sweden. Environment international, 147, 106333. Rawn, D. F., Dufresne, G., Clément, G., Fraser, W. D., & Arbuckle, T. E. (2022). Perfluorinated alkyl substances in Canadian human milk as part of the Maternal-Infant Research on Environmental Chemicals (MIREC) study. Science of The Total Environment, 154888. Zheng, G., Schreder, E., Dempsey, J. C., Uding, N., Chu, V., Andres, G., ... & Salamova, A. (2021). Per-and polyfluoroalkyl substances (PFAS) in breast milk: Concerning trends for current-use PFAS. Environmental Science & Technology, 55(11), 7510-7520. HUMAN EXPOSURE/RISK STUDIES THAT ONLY MONITOR ENVIRONMENTAL AND MICROENVIRONMENTAL MEDIA

Li, B. J., Chen, J. Y., Liu, Z. Z., Wang, J., & He, S. C. (2022). Pollution Characteristics and Health Risk Assessment of Perfluorinated Compounds in PM 2.5 in Zhejiang Province. Huan jing ke xue= Huanjing kexue, 43(2), 639-648. ARTICLE IN CHINESE

Lin, H., Taniyasu, S., Yamashita, N., Khan, M. K., Masood, S. S., Saied, S., & Khwaja, H. A. (2022). Per-and polyfluoroalkyl substances in the atmospheric total suspended particles in Karachi, Pakistan: Profiles, potential sources, and daily intake estimates. Chemosphere, 288, 132432.

Sonego, E., Simonetti, G., Di Filippo, P., Riccardi, C., Buiarelli, F., Fresta, A., ... & Pomata, D. (2022). Characterization of organophosphate esters (OPEs) and polyfluoralkyl substances (PFASs) in settled dust in specific workplaces. Environmental Science and Pollution Research, 1-15.

Tang, J., Zhu, Y., Li, Y., Xiang, B., Tan, T., Lv, L., & Luo, Q. (2022). Occurrence characteristics and health risk assessment of per-and polyfluoroalkyl substances from water in residential areas around fluorine chemical industrial areas, China. Environmental Science and Pollution Research, 1-11.

Wang, F., Wang, W., Zhao, D., Liu, J., Lu, P., Rose, N. L., & Zhang, G. (2022). Source apportionment and wet deposition of atmospheric poly-and per-fluoroalkyl substances in a metropolitan city centre of southwest China. Atmospheric Environment, 273, 118983.

Wang, Y., Gao, X., Liu, J., Lyu, B., Li, J., Zhao, Y., & Wu, Y. (2022). Exposure to Emerging and Legacy Polyfluoroalkyl Substances in the Sixth Total Diet Study—China, 2016–2019. China CDC Weekly, 4(9), 168.

Zhou, J., Baumann, K., Chang, N., Morrison, G., Bodnar, W., Zhang, Z., ... & Turpin, B. J. (2022). Per-and polyfluoroalkyl substances (PFASs) in airborne particulate matter (PM2. 0) emitted during floor waxing: A pilot study. Atmospheric Environment, 268, 118845.

HUMAN POPULATION HEALTH STUDIES

Cao, L., Guo, Y., Chen, Y., Hong, J., Wu, J., & Hangbiao, J. (2022). Per-/polyfluoroalkyl substance concentrations in human serum and their associations with liver cancer. Chemosphere, 296, 134083.

Duan, Y., Sun, H., Yao, Y., Li, Y., Meng, Y., Lu, Y., ... & Chen, L. (2021). Serum concentrations of per-/polyfluoroalkyl substances and risk of type 2 diabetes: A case-control study. Science of The Total Environment, 787, 147476.

Jin, H., Mao, L., Xie, J., Zhao, M., Bai, X., Wen, J., ... & Wu, P. (2020). Poly-and perfluoroalkyl substance concentrations in human breast milk and their associations with postnatal infant growth. Science of the Total Environment, 713, 136417.

Liu, J. J., Cui, X. X., Tan, Y. W., Dong, P. X., Ou, Y. Q., Li, Q. Q., ... & Zhao, X. M. (2022). Per-and perfluoroalkyl substances alternatives, mixtures and liver function in adults: A community-based population study in China. Environment International, 163, 107179.

Nian, M., Zhou, W., Feng, Y., Wang, Y., Chen, Q., & Zhang, J. (2022). Emerging And Legacy PFAS And Cytokine Homeostasis In Women of Childbearing Age. (In Review, Scientific Reports) https://doi.org/10.21203/rs.3.rs-1217618/v1)

Velarde, M. C., Chan, A. F. O., Sajo, M. E. J. V., Zakharevich, I., Melamed, J., Uy, G. L. B., ... & Gerona, R. R. (2022). Elevated levels of perfluoroalkyl substances in breast cancer patients within the Greater Manila Area. Chemosphere, 286, 131545

Zhang, Y. T., Zeeshan, M., Su, F., Qian, Z. M., Geiger, S. D., McMillin, S. E., ... & Dong, G. H. (2022). Associations between both legacy and alternative per-and polyfluoroalkyl substances and glucose-homeostasis: The Isomers of C8 health project in China. Environment international, 158, 106913.

REVIEWS

Zhu, Q., Li, H., Wen, Z., Wang, Y., Li, X., Huang, T., ... & Ge, R. S. (2020). Perfluoroalkyl substances cause Leydig cell dysfunction as endocrine disruptors. Chemosphere, 253, 126764.

Reviews rodent experiments and human epidemiological studies

Appel, M., Forsthuber, M., Ramos, R., Widhalm, R., Granitzer, S., Uhl, M., ... & Gundacker, C. (2022). The transplacental transfer efficiency of per-and polyfluoroalkyl substances (PFAS): a first meta-analysis. Journal of Toxicology and Environmental Health, Part B, 25(1), 23-42.

Reviews rodent and human toxicokinetics

GENERAL METHODS FOR PFAS RISK ASSESSMENTS (NOT SPECIFIC TO PFHxA)

Chou, W. C., & Lin, Z. (2020). Probabilistic human health risk assessment of perfluorooctane sulfonate (PFOS) by integrating in vitro, in vivo toxicity, and human epidemiological studies using a Bayesian-based dose-response assessment coupled with physiologically based pharmacokinetic (PBPK) modeling approach. Environment International, 137, 105581.

Neagu et al. (2021). Adverse outcome pathway in immunotoxicity of perfluoroalkyls. Current Opinion in Toxicology, 25, 23-29.

LARGE SCALE BIOMONITORING PROGRAMS, FRAMEWORKS and PROTOCOLS THAT INCLUDE PFHxA

Calafat, A. M., Kato, K., Hubbard, K., Jia, T., Botelho, J. C., & Wong, L. Y. (2019). Legacy and alternative per-and polyfluoroalkyl substances in the US general population: paired serum-urine data from the 2013–2014 National Health and Nutrition Examination Survey. Environment international, 131, 105048.

Cousins, I. T., DeWitt, J. C., Glüge, J., Goldenman, G., Herzke, D., Lohmann, R., ... & Wang, Z. (2020). Strategies for grouping per-and polyfluoroalkyl substances (PFAS) to protect human and environmental health. Environmental Science: Processes & Impacts, 22(7), 1444-1460.

	DeLuca, N. M., Angrish, M., Wilkins, A., Thayer, K., & Hubal, E. A. C. (2021). Human exposure pathways to poly-and perfluoroalkyl substances (PFAS) from indoor media: A systematic review protocol. Environment international, 146, 106308. ECHA (European Chemicals Agency). (2019). Annex XV Restriction Report. Proposal for a Restriction. Substance Names: Undecafluorohexanoic acid (PFHxA), its salts and related substances. BAuA Federal Institute for Occupational Safety and Health Division 5 - Federal Office for Chemicals Friedrich-Henkel-Weg 1-25. D-44149 Dortmund, Germany. EFSA Panel on Contaminants in the Food Chain (EFSA CONTAM Panel), Schrenk, D., Bignami, M., Bodin, L., Chipman, J. K., del Mazo, J., ... & Schwerdtle, T. (2020). Risk to human health related to the presence of perfluoroalkyl substances in food. EFSA Journal, 18(9), e06223. López, M. E., Göen, T., Mol, H., Nübler, S., Haji-Abbas-Zarrabi, K., Koch, H. M., ... & Castaño, A. (2021). The European human biomonitoring platform-Design and implementation of a laboratory quality assurance/quality control (QA/QC) programme for selected priority chemicals. International Journal of Hygiene and Environmental Health, 234, 113740.
Haney	The literature search strategy and screening criteria for PFHxA appear appropriate and clearly described. This reviewer personally knows of no additional peer-reviewed studies of PFHxA that the assessment should incorporate, although public comments such as those from the New Jersey Department of Environmental Protection (NJ DEP) and others contain several recommendations regarding various studies (e.g., Zheng et al. 2021, Jiang et al. 2021, Modaresi et al. 2022, Pierozan et al. 2022) to be considered for inclusion by EPA in the final assessment. In regard to such studies, the EPA has applied reasonable and practical rationales in the document entitled "EPA characterization of studies identified after the public release of the draft IRIS Toxicological Review of Perfluorohexanoic Acid (PFHxA) and Related Salts" regarding the importance of study inclusion in the final assessment based on the potential impact (if any) of study results on data gaps, draft assessment conclusions, etc. As a **Tier 2 Suggested Revision**, additional study suggestions from fellow external expert peer reviewers should similarly be evaluated by EPA for potential inclusion based on these types of relevant considerations.
Leung	The search strategy and screening criteria used to evaluate PFHxA literature was systematic and is clearly described. Initial searching was performed in 2017 and yearly thereafter until 2021, adhered to PECO criteria, and the output independently reviewed in accordance with a prespecified protocol. The entire process of the literature search and screening algorithm is captured nicely in Figure 2-1.
Ng	The description of the systematic review approach and inclusion parameters is generally clear. In Appendix A, Table 3-1 describing the PECO criteria is clear and choices are well justified. I am not aware of new studies that meet these criteria and would material change the outcome of the assessments presented.

	Tier 1 Necessary Revision: Chapter 2, p. 2-2, Figure 2-1: It is unclear why there is a line connecting "Studies Meeting PECO" (bottom blue box) to "Tagged as Supplemental" (bottom green box). Are there PECO studies with non-PECO components that feed to Supplemental data? If correct, the version shown in the revised Appendix A (Figure 4-10 Panel B) is clearer and should be used in the review document as well. **Tier 2 Suggested Revision:** Please clarify under Appendix A, Table 5-2, page 5-6 why "Dam health (e.g. weight gain, food consumption)" was only considered in "Developmental" and not in "Reproductive" or tied to the specific effect on dam health observed (e.g. weight gain as endpoint). Or is this meant to be included under the Table's footnote b? [This is relevant to the general systematic review approach as described in Appendix A and not only PFHxA-specific.]" **Editorial Comment:** The brief description provided in Chapter 2 of the Review is generally clear, although some rewording may improve readability/clarity. For example, page 2-1 line 1 states "18 records identified from… (NTP) study tables and review of reference lists…" – the majority of these (17/18) are from ATSDR and only one from NTP. Also, both NTP and ATSDR are listed on Figure 2-1 in the "other" category, but the other two entries under "Other" are not mentioned in that first paragraph of section 2.1, which seems inconsistent.
Savitz	**Editorial Comment**: The literature search process was clearly described in Section 1.2.1 and reflects state-of-the-art methods and was quite thorough. It is extremely unlikely that any relevant information would not have been identified and I do not know of any published material that was missed.
Zoeller	Section 1.2.1 contains the details of the literature search and screening and they appear to be appropriate and detailed so that the methods used were understandable. Although the protocol for this systematic review was updated in response to public comments, the literature being evaluated appears to have been updated in 2020. No recommendation.

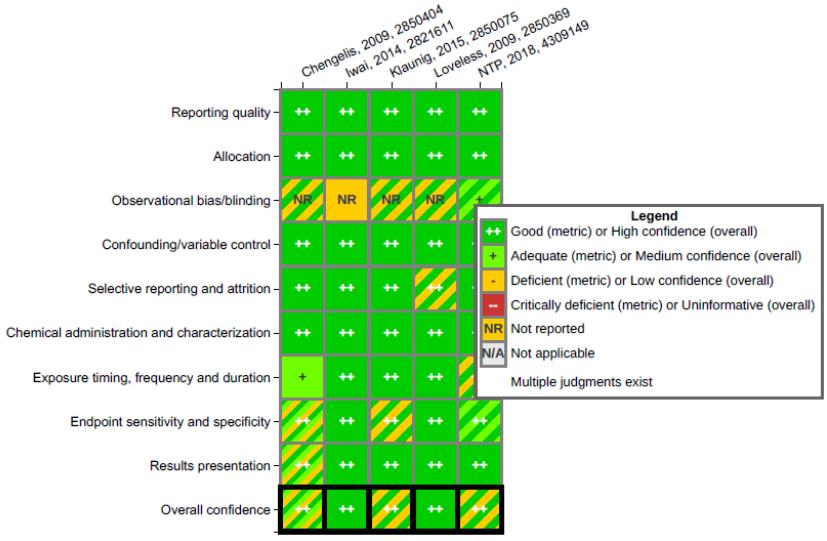
3.2. The Toxicological Review provides an overview of individual study evaluations and the results of those evaluations are made available in the Health Assessment Workplace Collaborative linked here HAWC. Note that a "HAWC FAQ for assessment readers" document, linked here (scroll to the bottom of the page, and the document is available for download under "attachments"), is intended to help the reviewer navigate this on-line resource. Data from studies considered informative to the assessment are synthesized in the relevant health effect-specific sections, and study data are available in HAWC.

a. Please comment on whether the study confidence conclusions for the PFHxA studies are scientifically justified and clearly described, considering the important methodological

features of the assessed outcomes. Please indicate any study confidence conclusions that are not justified and explain any alternative study evaluation decisions.

b. Results from individual PFHxA studies are presented and synthesized in the health system-specific sections. Please comment on whether the presentation and analysis of study results are clear, appropriate, and effective to allow for scientifically supported syntheses of the findings across sets of studies.

Reviewer	Comments
Faustman	**2a:** This reviewer felt that the study confidence conclusions for the PFHxA studies are scientifically justified. Section 1.2.2 details the evaluation of individual studies and provides definitions for the study confidence classifications from High to Low. Also included is the category "Uninformative". This later category of study confidence means that serious flaws exist for that study being evaluated and it also means that that study will not be used nor undergo further data extraction. The descriptions also describe in evidence synthesis and integration that "…evidence for human and animal health effects are based primarily on studies of high and medium confidence." (line 25, page 1-12) This section says that low confidence studies can be used to "evaluate consistency" or address uncertainties within the high to medium confidence studies but were not used in evidence syntheses but were included in the synthesis of mechanistic evidence and other supplemental information. This reviewer would agree with these approaches. **2b:** This reviewer felt that the presentation and synthesis of the individual PFHxA studies was well done and appreciated the review of these studies within each of the health effects and in that context appreciated the inclusion of classification of the study confidence levels. Where this reviewer felt there could be additional clarity and specificity is when evidence is integrated across the individual health effects as this reviewer feels strongly that to predict organism level responses, especially in humans, this systems-based integration is essential. This integration is a **Tier 3 Future Considerations**. For this review, Section 1.2.4 (or an additional section) could expand on the use of low confidence studies to support mechanistic evidence when the mechanistic evidence is used cross health effects. This is a **Tier 1 Necessary Revision** that could be addressed by adding an additional paragraph to this section that links with cross health effects discussions, especially for the mechanistic discussions.
Georgopoulos	The confidence conclusions for the studies that were included in the PFHxA Toxicological Review are scientifically justified. The results from individual PFHxA studies (which are presented and synthesized in the health system-specific sections) are concisely and clearly summarized. The summary visual presentation of the evaluation results for the animal studies (https://hawc.epa.gov/summary/visual/assessment/100500070/animal-toxicology-study-evaluation/ and for the epidemiological studies (https://hawc.epa.gov/summary/visual/assessment/100500070/pfhxa-human-epidemiology-study-evaluation/) is very effective and the use of the interactive graphics

	for accessing the summaries is functional and very convenient. (This is in fact true for all the studies available in the "Visualizations" tab of the PFHxA (2018) HAWC website). Further specific comments on whether (a) study confidence conclusions are scientifically justified and (b) the analysis of study results is clear and appropriate, are included, for specific endpoints, in the responses to the Charge Questions that follow.
Haney	**2a:** Yes, the study confidence conclusions appear scientifically justified and clearly described, giving appropriate consideration to important study attributes such as methodological features of the assessed outcomes. However, for increased transparency and ease of reference, as a **Tier 2 Suggested Revision**, the EPA should consider adding the figure summary below to the main document in addition to it appearing in the Health Assessment Workplace Collaborative (HAWC), which is more difficult to navigate than the draft assessment itself. Also, the specific meanings of striped cells in this figure should be explicitly explained within the text. Additionally, it is noted that for the *Human Study Evaluation Hepatic Effects* visualization figure in HAWC, the word "evaluation" is misspelled.  **Figure 1 Animal Toxicology Study Evaluation** Briefly, as shown above in my Figure 1 above (downloaded from HAWC), EPA's assessment summarizes the relevant scientific considerations utilized and applied to animal studies in determining the overall confidence in each animal study. There are nine such considerations, each concerning an aspect of overall study quality. Similarly, as shown in Figure 3-1 (p. 3-19) of the draft document, for example, EPA's assessment summarizes the relevant scientific considerations utilized and applied to epidemiological studies in determining the overall confidence in each epidemiological study. There are seven considerations, each concerning an aspect of overall study quality. As might be expected, the considerations differ somewhat between animal and epidemiological studies, although there are some general areas of overlap (e.g., confounding, exposure characterization, reporting quality/selective reporting). For each study, each consideration is essentially rated (i.e., graded) from good (++) down to deficient (-) or even critically deficient (--), and if not reported in the study or not

	applicable receives an "NR" or "N/A", respectively. The overall confidence rating (high down to low or even uninformative) essentially depends upon the balance of the ratings across the relevant considerations or a critical deficiency (e.g., confounding in a human study). For each study, the consideration ratings provide the scientific justification needed for the overall study confidence level rating. **2b:** This question is related to question 3 below, which asks whether the available data for the effects considered (i.e., hepatic, developmental, hematopoietic, endocrine and other noncancer effects) have been clearly and appropriately synthesized to describe the strengths and limitations. However, this question (2b) concerns whether the presentation and analysis of study results is clear, appropriate, and effective, a step just upstream of synthesis that if well done, then allows for appropriate syntheses of the endpoint-specific findings. In this reviewer's opinion, by and large, the presentation and analysis of study results appears clear, appropriate, and effective. However, I do have several specific recommendations to improve the clarity and/or accuracy of the presentation of results. In regard to hepatic effects, in Tables 3-3 (p. 3-21) and 3-5 (p. 3-25), it was not as easy as desirable, for me as a reader, to discern the bolded numbers indicating a statistically significant increase. This comment also applies to Tables 3-6 and 3-7 on pp. 3-26 and 3-27, respectively, as well as Tables 3-8 and 3-9 on pp. 3-30 and 3-31, respectively. As a **Tier 2 Suggested Revision**, the EPA should consider additional font effects, superscripts, or other designations (e.g., symbols, asterisks) to help more clearly denote and document statistically significant results. Table 3-11 (p. 3-39) cites coherence of cellular hypertrophy with liver weight. As a **Tier 2 Suggested Revision**, the EPA should consider additional tables and/or figures that would help readers visualize the coherence of liver histopathology with liver weight effects since these results are only presented in separate tables within the document (i.e., Table 3-4 for hepatocellular hypertrophy and Table 3-3/Figure 3-2 for liver weight changes). Also, Table 3-11 (p. 3-40) cites decreases in bilirubin as "serum biomarkers of hepatic injury." However, Loveless et al. (2009) indicate that these changes were considered non-adverse (see Section 3.2.6 of that study), and furthermore, the draft EPA assessment states that "lower than normal bilirubin levels are usually not a concern and can be reduced in response to increased conjugation rates after hepatic enzyme induction and excretion into bile (Hall et al., 2012)" (p. 3-32, lines 9-11). Accordingly, the EPA should reconsider (**Tier 2 Suggested Revision**) whether to include decreases in bilirubin amongst the serum biomarkers of hepatic injury cited in Table 3-11. Lastly, Table 3-11 cites "strong support for liver injury from serum biomarkers." In characterizing the strength of this evidence, the EPA should reconfirm (**Tier 2 Suggested Revision**) that they have considered and weighed the significant variability of responses across studies and sexes (as summarized in Tables 3-5 (ALT), 3-6 (AST), and 3-7 (ALP)), including the lack of any type of dose-response for ALT/AST/ALP in the chronic study (Klaunig et al. 2015), as well as the magnitude (frequently modest) and direction of change in the cases where there was a change in one of these serum enzyme biomarkers (in many cases there were decreases). Regarding developmental effects, examination of the data in Table 3-14 (p. 3-47) reveals that in an appreciable number of instances, the decreases in postnatal body

	weight are not monotonic, including in mice. However, this does not appear to be mentioned in the relevant section of the assessment (*Offspring Body Weight*). Additionally, in terms of evidence integration, Table 3-16 (p. 3-51) indicates for body weight that there was a "dose-response observed in mouse study." However, not even in the best case (perhaps for PND 0, male and female (combined) mice or PND 4, male and female (combined) mice; Table 3-14) does the response in mice increase (i.e., body weight decrease more) with every increase in dose. Generally, the dose-responses for postnatal body weight decreases in mice are non-monotonic. The EPA should consider revisions to further characterize the mouse dose-response for decreases in postnatal body weight (**Tier 2 Suggested Revision**). Lastly, for Tables 3-13 (pp. 3-44 and 3-45), 3-14 (p. 3-47), and 3-15 (p. 3-49), it was not as easy as desirable, for me as a reader, to discern the bolded numbers indicating a statistically significant result. As a **Tier 2 Suggested Revision**, the EPA should consider additional font effects, superscripts, or other designations (e.g., symbols, asterisks) to help more clearly denote and document statistically significant results. In regard to hematopoietic effects, Table 3-25 (p. 3-74) cites coherence of red blood cells, hematocrit (HCT), and hemoglobin (HGB) and reticulocytes. As a **Tier 2 Suggested Revision**, the EPA should consider an additional table and/or figure that would help readers visualize the coherence of these effects since these results are presented in separate figures and tables within the document (i.e., Figure 3-15/Table 3-24 for reticulocytes and Figure 3-13/Tables 3-21, 3-22, and 3-23 for the other effects). Table 3-25 (p. 3-75) also cites "consistent treatment related effect on platelet levels." However, examination of Figure 3-16 (p. 3-72) shows that while statistically increased platelet count occurred in males at relatively low doses in one 90-day rat study (Chengelis et al. 2009b), inconsistent results were obtained for similar and higher doses for similar and longer duration rat studies (Loveless et al. 2009, Klaunig et al. 2015). There were not statistical differences in platelet count at any of the three time points (i.e., weeks 25, 51, 104) at any dose in the chronic study (Klaunig et al. 2015), wherein two doses exceeded that causing increased platelet counts in male rats in the 90-day Chengelis et al. (2009b) study. These chronic study results are an important exception to the cited "consistent treatment related effect on platelet levels", especially since chronic studies, often with lower, more environmentally-relevant doses, are particularly relevant/well-suited for chronic reference dose (RfD) derivation. The EPA should consider (**Tier 2 Suggested Revision**) adding such information to the *Hemostasis* section (p. 3-71) of the draft assessment and adding a lack of positive findings in the chronic study as a "factors that decrease certainty" in Table 3-25 (p. 3-75), in addition to adding a data table specifically for the platelet endpoint. Lastly, consistent with previous comments on other tables, for Tables 3-21 (p. 3-67), 3-22 (p. 3-69), 3-23 (pp. 3-69 and 3-70), and 3-24 (p. 3-71), it was not as easy as desirable, for me as a reader, to discern the bolded numbers indicating a statistically significant result. As a **Tier 2 Suggested Revision**, the EPA should consider additional font effects, superscripts, or other designations (e.g., symbols, asterisks) to help more clearly denote and document statistically significant results. Regarding kidney weight and endocrine effects (i.e., on thyroid hormones and follicular epithelial cell hypertrophy), consistent with previous comments on various tables for other effects, it was not as easy as desirable to discern the bolded numbers indicating a statistically significant result in Table 3-18 (p. 3-56) and Tables 3-27 (p. 3-78) and 3-28

	(p. 3-79), respectively. As a **Tier 2 Suggested Revision**, the EPA should consider additional font effects, superscripts, or other designations (e.g., symbols, asterisks) to help more clearly denote and document statistically significant results. Finally, a bullet in Table 3-29 (p. 3-83) for *Organ Weights* states [*emphasis added*], "*right* adrenal weights decreased but no other adrenal effects were reported." However, "right" appears to be a misspelling considering the discussion in the *Organ Weights* section of the draft assessment (p. 3-79, lines 6-14), and should perhaps read "absolute". The EPA's consideration of the comments above in conjunction with any public comments on the clarity of EPA's presentation (e.g., NJ DEP comments regarding potential clarifications on pp. 41-46 of EPA's *Public Comments Received on Draft IRIS Toxicological Review of Perfluorohexanoic Acid (PFHxA) and Related Salts*) will help ensure that the presentation and analysis of study results is as clear, appropriate, and effective as possible.
Leung	**2a:** The conclusions drawn to estimate confidence of the screened literature adhere to the specified PECO criteria, appear scientifically justified, and have been clearly described in Section 2.2. **2b:** The presentation and analysis of the study results are clear, effective, and appropriately ordered as they appear in the health system-specific sections. The processes used for evidence synthesis and integration of literature findings are logically outlined.
Ng	The analysis of study results is laid out in a logical manner with both graphical and tabular data presented to support the review conclusions. **<u>Hepatic Toxicity</u>** For **organ weight**, the study confidence conclusions are scientifically justified and clearly presented in the health-system-specific section of the review. **Editorial Comment:** Figure 3-2 legend the symbols and text overlap. For **histopathology**, the study confidence conclusions are scientifically justified and clearly presented in HAWC. For **clinical chemistry**, the study confidence conclusions are scientifically justified and clearly presented in the health-system-specific section of the review. **Editorial Comment:** Figure 3-3 is missing legend to explain symbols and Figure 3-4 legend has text/symbol overlapping. **Tier 2 Suggested Revision:** It is unclear why the section on **peroxisomal beta oxidation** comes only after the subheading "Mechanistic Evidence and Supplemental Information", since beta oxidation was part of the results included in Table 3-2 and is summarized in the "available evidence base" at the start of the Mechanistic Evidence section. Move up? **<u>Developmental Toxicity</u>**

	For **offspring mortality**, the study confidence conclusions are scientifically justified and clearly presented in the health-system-specific section of the review. For **body weight**, the study confidence conclusions are scientifically justified and clearly presented in the health-system-specific section of the review. For **eye opening**, the study confidence conclusions are scientifically justified and clearly presented in the health-system-specific section of the review. **Editorial Comment:** Page 3-48, line 7: significant. For **malformations and variations**, the endpoint was evaluated in only one study and no effects were reported. **Hematopoietic Effects** For **hematology and hemostasis**, the single human study by Jiang et al. 2014 was considered uninformative and this was clearly justified in HAWC based on lack of consideration of confounding. The four animal studies presented were all judged to be high quality for blood counts, an agreement with the HAWC assessments and clearly justified. Agreement across studies on findings such as decreased red blood cell count, hematocrit, and hemoglobin provide added confidence in these study confidence conclusions. Study results were summarized clearly in the results document with informative tables and graphs. **Editorial comment:** As previously noted, revise (widen) the figure legends to avoid overlapping text and symbols and clarify which symbols belong with which labels. **Endocrine Effects** For **thyroid hormones**, the two available human studies are evaluated as uninformative and low confidence, based on lack of consideration of confounding and concerns around study design and decreased sensitivity. These considerations are clearly explained in the review, consistent with the HAWC evaluations, and scientifically justified. Regarding the reduced sensitivity, this is due to low levels of PFHxA detected in the participants (though with moderate detection frequency of 53%), and may be difficult to address in a human study for regular (e.g. non occupationally exposed) populations for a substance with rapid clearance. Of the four available animal studies, only one addressed thyroid hormones, and is a high confidence study that showed clear and statistically significant dose-response but only in male rats. Study results were summarized clearly with informative tables and graphs. One potential concern is that male rats showed significant effects at all levels tested relative to control. For **histopathology**, three animal studies were high confidence and one was low confidence (consistent with previous evaluations, e.g. hepatic effects discussed above). Significant effects were observed mainly only at the highest dose levels in the results presented for the Loveless et al. 2009 study. **Tier 1 Necessary Revision:** While the NTP and Klaunig studies did not report significant changes to histopathology, the results of these high confidence studies should be

	included in table 3-28, otherwise only the one study with significant effects is being highlighted, and paints an incomplete picture. For **organ weights**, three high confidence animal study results are available. Of these the Loveless et al. study shows a significant effect (increased relative thyroid weight for female rats at highest dose) and the NTP 2018 study shows a significant trend of decreased absolute adrenal gland weight in male rats.
Savitz	**2a: Tier 1 Necessary Revision:** Burying this solely within the HAWC template does not enable the reader to readily identify the basis for judgments about individual studies or the rationale behind the assignments. A brief section, perhaps a page or two, could provide the considerations used in evaluating study quality and summarize the basis for assignments. It might be noted, for example, that the epidemiologic studies designated as uninformative tended to have particular shortcomings, or what the main dividing line was between low and medium confidence. It's appropriate that the study-by-study details are embedded deeper in the document where few people will be likely to go, but the logic should be in the main text. This general orientation would help to clarify how decisions were made for each of the health endpoints for which there were epidemiologic studies available. **2b: Editorial Recommendation:** If the recommendation in response to 2a above were provided, then a brief notation of what made specific studies particularly strong or weak could be indicated with sufficient context to interpret the basis for assignment. The assignments themselves seem reasonable (though as a reviewer, I did not conduct a complete audit) so the main issue is simply transparency.
Zoeller	**2a:** The document presents evaluations of individual studies using a standardized set of criteria to evaluate quality, observational bias, confounding variable control, etc. These considerations are compiled to facilitate study versus study comparisons, and they provide the basis for an open process to assign overall confidence rating. This reviewer did not agree with all study confidence conclusions and these remarks are clarified below under organ-specific discussions. **No Recommendations.** **2b:** The Agency is clear and transparent in their evaluation of results for the studies identified for the PFHxA IRIS. As a result, it was clear to this reviewer exactly how the Agency assessed the information provided by studies identified in the review. Detailed responses to these assessments are found in the individual health-system specific sections. **Tier 1 Necessary Revision:** On page 1-13, Line 6, the Agency states that, "Building from the separate syntheses of the human and animal evidence, the strength of the evidence from the available human and animal health effect studies was summarized in parallel, but separately, using a structured evaluation of an adapted set of considerations first introduced by Bradford Hill (Hill, 1965)." The "Hill views" articulated in this 1965 address are important but are seriously outdated and it is important that the Agency has adapted these for the current setting. However, these adaptations should be

	enumerated here. A separate adaptation by members of a UNEP/WHO writing group was enumerated earlier and may be useful to the Agency [1] (see Zoeller footnote below) **Zoeller Footnote 1:** Zoeller RT, Bergman A, Becher G, et al. A path forward in the debate over health impacts of endocrine disrupting chemicals. Environ Health 2015;14:118.

3.3 For each health effect considered in the assessment and outlined below, please comment on whether the available data have been clearly and appropriately synthesized to describe the strengths and limitations. For each, please also comment on whether the weight-of-evidence decisions for hazard identification are scientifically justified and clearly described.

a. For hepatic effects, the Toxicological Review concludes the available evidence indicates PFHxA likely causes hepatic effects in humans under relevant exposure circumstances. This conclusion is based on studies of rats showing increased liver weight, hepatocellular hypertrophy, increased serum enzymes, and decreased serum globulins. The hepatic findings for PFHxA were similar for other PFAS and determined to be adverse and relevant to humans.

i. Additional considerations influenced the hepatic effects hazard identification decisions. Appendix A (Systematic Review Protocol for the PFAS IRIS Assessments) outlines the human relevance of hepatic effects in animals that involve PPARα receptors as a key science issue. To the extent supported by the PFHxA literature (and to a lesser extent, literature for other PFAS), the Toxicological Review evaluates the evidence relevant to the potential involvement of PPARα and non-PPARα pathways with respect to the reported hepatic effects. The Toxicological Review ultimately concludes evidence from in vivo (including genetic mouse models) and in vitro studies support a potential role for multiple pathways operant in the induction of hepatic effects from PFHxA exposure but those pathways cannot be specifically determined. Please comment on whether the conclusions regarding the available animal and mechanistic studies are scientifically justified and clearly described. The hepatic findings for PFHxA were similar for other PFAS and determined to be adverse and relevant to humans.

b. For developmental effects, the Toxicological Review concludes the available evidence indicates PFHxA likely causes developmental effects in humans under relevant exposure circumstances. This judgment is based primarily on gestational exposure experiments in mice, with supportive findings in rats exposed throughout gestation and lactation, showing increased perinatal mortality, decreased offspring body weight, and delayed eye opening. These effects are similar to those observed for other PFAS following developmental exposure and were determined to be adverse and relevant to humans.

c. For hematopoietic effects, the Toxicological Review concludes the available evidence indicates PFHxA likely causes hematopoietic effects in humans under relevant exposure circumstances. This judgment is based on consistent findings, including decreased red blood cells [RBCs], hematocrit, and hemoglobin, across study designs

that, when interpreted together, signifies PFHxA-related hematological effects such as anemia. These findings were determined to be adverse and relevant to humans.

d. For endocrine effects, the Toxicological Review concludes the available evidence suggests, but is not sufficient to infer, that PFHxA may cause endocrine effects in humans under relevant exposure circumstances. This conclusion is based on some evidence of thyroid effects based on hormone and histopathological changes in two rat studies; however, the data is limited, lacking consistency across studies, and histopathological changes may be explained by non-thyroid related effects.

e. For all other potential health effects (i.e., renal, male and female reproductive, immune, and nervous system), the Toxicological Review concluded the available evidence is inadequate to assess whether PFHxA may cause effects in humans under relevant exposure circumstances. In general, these conclusions were driven by sparse evidence bases or data that were largely null.

Reviewer	Comments
Faustman	**3a:** This reviewer agreed that the data has been "clearly and appropriately synthesized in order to describe the strengths and limitations of the data" for hepatic effects from PFHxA. These results are summarized in Section 3.2.1 where two human epidemiological studies and four rodent studies are summarized. This reviewer appreciated Tables such as 3.2 that showed the details of these animal studies, and provided exposure details as well as outcome rating for four of the key hepatic endpoints of concern in this section of the hazard identification. Such findings were then supplemented by figures like 3-2 and 3-3 where the specific hepatic results from both males and females are presented by study type and endpoint response across dose. These figures also showed the statistical evaluation and direction of impact of PFHxA after both acute and chronic exposures when data was available. For the human studies only one was included in further analysis (Nian et al 2019). Because the exposure levels were considered "low" with minimal variability across the participants in this cohort, the absence of seeing significant liver enzyme changes was noted in these study results but was listed with deficient sensitivity. For the rodent studies relative liver weights were significantly increased for the 28 and 90-day studies and were dose responsive. The histopathology of the four rodent studies showed dose dependent hepatocellular hypertrophy across both the high confidence short term and sub chronic rat studies with male rats being more responsive than female rats. These hepatic findings "correlated with changes in clinical chemistry (e.g. serum enzymes, blood proteins) and necrosis". Hematopoietic effects were reported for decreased RBCs, decreased hematocrit values, and increased reticulocyte counts Detailed tables and figures reported study, sex and dose related details. This reviewer also felt that the weight-of evidence decisions used for hazard identification were "scientifically justified and clearly described" for the hepatic effects. Mechanistic data was included in this assessment and was obtained by examining measurement of peroxisomal beta oxidation in two high confidence sub chronic studies and significant dose related increases were seen for both males and females with the

	former being more sensitive. It was noted that in both the males and females increased activity persisted after a 30-day recovery period with greater sensitivity in the males. In vitro studies showed that PPAR alpha activation was seen in COS 1 cells transfected with PPAR alpha receptor transfected with mouse or human PPAR alpha reporter genes. PFHxA was more potent in inducing the human PPAR alpha transfected cells than the mouse. High throughput ToxCast data for PFHxA effects on 19 assay targets from human liver cell-based assays (Table 3-10) were reported. Assays in HepG2 cells showed that PFHxA exposure activated PPAR alpha and HIF 1 in vitro. The IRIS draft report included a section that discussed "evidence from other PFAS" and this reviewer felt this discussion about results across other perfluorinated compounds exposures (such as PFAS, PROA, PRNA and PFBA) was especially important for interpreting the PFHxA results and by structural analogy that PFHxA would also work via both PPAR alpha and non PPAR alpha response pathways. These comparisons showed that the involvement of other non PPAR alpha receptors in the response to PFAS and by structural relationship relevance for PFHxA. A section on assessment of adaptive versus adverse responses was included and it discussed the hepatic results from PFHxA using guidance from Hall et al 2012. A compelling narrative, which compares point by point the PFHxA responses against this guide concludes that these responses are adverse, human relevant and of concern for such biological effects of necrosis. This reviewer applauded inclusion of this discussion and outcome. Informative Table 3-22 provides an excellent and easy to use synthesis of the evidence for PFHxA causing human relevant adverse hepatic effects via both PPAR alpha and non PPAR alpha dependent response pathways. **3b:** This reviewer agreed that the data has been "clearly and appropriately synthesized in order to describe the strengths and limitations of the data". This reviewer also felt that the weight-of evidence decisions used for hazard identification were "scientifically justified and clearly described". In particular, for developmental effects, there were no human studies available but three relevant rodent studies (in two papers) were identified. The rat study looked at PFHxA exposure (sodium salt) in both a reproductive and developmental study using 3 exposure groups in addition to control. The mouse study used the Ammonium salt and conducted a development study as well with two phases and included six different doses. These high confidence study results are presented in Table 3-16. The conclusion from these evaluations are that PFHxA causes developmental impacts based on observations of significantly reduced offspring body weight and increased perinatal mortality in both rats and mice. This report clearly discusses how the results in mice (with rats as secondary) support these conclusions. Table 3-12 summarizes the study outcomes and lists high confidence of three endpoints: offspring viability, offspring body weight and developmental milestones (eye opening). This section includes a discussion on the significance of delayed eye opening and mentions that this developmental milestone is one of a set of milestones that indicate developmental stage and provides one assessment along the developmental trajectory (reviewer addition). Delayed eye opening denies the offspring of early sensory input

	and this depravation is taken seriously by developmental toxicologists in interpreting potential impacts. The section discusses potential issues such as how decreases in maternal body weight in the Loveless et al 2009 study might have contributed to the effects on the offspring. Dams that were exposure to PFHxA at 500 mg/kg-day from GD 6-20 showed a significant 5% decrease in total net body weight and a body weight gain on GD 21. These same investigators also reported a significant reduction in maternal body weight in early gestation from GD 0-7 at the highest dose of 500 mg/kg-day but no significant change in maternal body weight gain from GD 0 to 21. The report states that "the effects on offspring body weight in this study are not expected to be driven by maternal toxicity". This reviewer would agree and add to this discussion that developmental toxicologist don't completely discount the adverse impacts in offspring even in the presence of some maternal toxicity given general consensus that the offspring would be less able to recover from such impacts and would be more susceptible to such changes during developmental trajectories whereas the maternal dam alterations in weight might more frequently be reversible. **3c:** This reviewer agreed that the data has been "clearly and appropriately synthesized in order to describe the strengths and limitations of the data". This reviewer also felt that the weight-of evidence decisions used for hazard identification were "scientifically justified and clearly described". Table 3-20 shows four high confidence repeated dose animal studies and their experimental and exposure details. All of these studies were conducted in rats and have different exposure durations: 28d, 90d (2) and a 2-year cancer bioassay. Both males and females were assessed in these rat studies. Note that one human low confidence study was available however it was correctly identified as "indeterminate". When the rat studies are examined as a collective of study results, they provide compelling evidence for PFHxA causing "macrocytic anemia" (low hemoglobin and large RBC) and could be expected to cause serious harm in humans. Table 3-25 shows these study details and assigns an evidence category of Likely. **3d:** This reviewer agreed that the data has been "clearly and appropriately synthesized in order to describe the strengths and limitations of the data: and would in general agree with the report recommendations that the data is suggestive but not sufficient to infer that PFHxA exposures would result in endocrine effects in human populations. The report lists two human studies that were deficient and were labeled "uninformative". This reviewer agreed with these assessments with the first study (Seo et al, 2018) not accounting for multiple possible confounding factors and with the second study (Li et al 2017) having almost 50% of the exposure samples below the level of exposure detection. Four rat studies (28d, 90d (2) and a 2 yr cancer bioassay) were identified that looked at endpoints relevant for endocrine assessments. In all four of these studies, organ weight, histopathology and thyroid hormones were examined. The sections that describe the observations from these studies are detailed and convey evidence for thyroid impacts. The NTP, 2018 28day study showed "clear dose-dependent decreases in thyroid hormones" in males but no significant changes in TSH for either male or female rodents.

	Histopathological changes were also reported however the linkage of thyroid hormonal changes and reported histopathological changes and/or organ weight changes were not consistently linked across the studies. The evidence integration section addresses these inconsistencies by providing a series of possible explanations of these differences including identifying similarities with other perfluorinated compounds. In fact, this reviewer agreed with the report when it states that a "significant data gap for PFHxA" resulted from these assessments. This reviewer was convinced by the discussion of male versus female pharmacokinetics in describing the sex difference but less clear about the alignment of secondary impacts from hepatic damage causing the thyroid impacts. This reviewer would agree with the report's statement that "evidence suggests" the PFHxA could cause endocrine effects in humans but not the second part of this statement ". but is not sufficient to infer" that PFHxA could cause endocrine effects in humans. **Tier 1 Necessary Revision** is to ask USEPA to re-examine the second part of their statement (page 3-80, lines 37, 38) where "is not sufficient to infer" is used. This could be done by adding a few more sentences that clarify if the suggestions about indirect hepatic impacts is plausible with quantitation of when these impacts align. This discussion should include a discussion of whether TSH changes are required for serum thyroid changes to occur as references Hood et al 1999a; Hood et al 1999b and Hood and Klaassen, 2000 suggest otherwise. **3e:** This reviewer agreed that the data has been "clearly and appropriately synthesized in order to describe the strengths and limitations of the data" and would in general agree with the comment that these endpoints did not have adequate data to determine impact or not. This reviewer also felt that the weight-of evidence decisions used for hazard identification were "scientifically justified and clearly described". However, this reviewer feels that several other aspects of the review would provide more transparent input on the evidence stream if observations across other PFOS compounds was done for these broad list of potential endpoints (renal, male and female reproductive, immune and nervous system (as they were reviewed in the Hepatic section and discussed above). My **Tier 1 Necessary Revision** is to put in such a section or section header either by each endpoint listed in 3 e or to provide an overall summary table of input from evaluation of other PFOS compounds for these endpoints. For example, on Page 3-80 lines 28-36 provides a small example of this for endocrine effects as does Table 3-29, page 3-83 but it is not presented in the same manner as it was for hepatic. Also, in the Immune section 3.2.8 page 3-104 lines 20- 25 are hidden in the evidence integration section.
Georgopoulos	**3a: Hepatic Effects** The available data (and their strengths and limitations) for effects on the hepatic system were appropriately synthesized and discussed in Section 3.2.1 of the Toxicological Review. The weight-of-evidence decisions for hazard identification have been adequately described and justified. The relevant animal data include multiple multiple short-term, subchronic, and chronic studies in rats and mice; studies were generally rated as medium or high confidence for the hepatic outcomes, but some outcome-specific considerations for study evaluation were influential on the overall confidence ratings for hepatic effects. The PFHxA Toxicological Review reasonably concludes that the evidence from in vivo and in vitro studies potentially supports multiple pathways

	operating in the induction of hepatic effects from PFHxA exposure but that those pathways cannot be specifically determined. The conclusions regarding the available animal and mechanistic studies are scientifically justified and clearly described. Specifically, the Review correctly states that the currently available evidence indicates that "PFHxA likely causes hepatic effects in humans under relevant exposure circumstances" (page 3-38, lines 3,4), having arrived to that conclusion based on four primarily high confidence studies of short-term, subchronic, and chronic PFHxA exposure in (primarily male) rats showing increased liver weight, hepatocellular hypertrophy, increased serum enzymes and decreased serum globulins. These findings were determined to be adverse and relevant to humans, with the likely involvement of both PPARα-dependent and -independent pathways. The hepatic findings for PFHxA were similar to those known for other PFAS and determined to be adverse and relevant to humans. **3b: Developmental Effects** The available data for developmental effects were appropriately discussed and synthesized in Section 3.2.2. The Toxicological Review reasonably concludes that the available evidence indicates PFHxA likely causes developmental effects in humans under relevant exposure circumstances. The Review reached this conclusion based primarily on gestational exposure experiments in mice, with supportive findings in rats exposed throughout gestation and lactation, showing increased perinatal mortality, decreased offspring body weight, and delayed eye opening. These effects are similar to those observed for other PFAS following developmental exposure and were reasonably determined to be adverse and relevant to humans. **3c: Hematopoietic Fffects** The available information for hematopoietic effects is appropriately discussed and synthesized in Section 3.2.4. The PFHxA Toxicological Review reasonably concludes that the available evidence indicates PFHxA likely causes hematopoietic effects in humans under relevant exposure circumstances. This conclusion is reasonably based on consistent findings, including decreased red blood cells [RBCs], hematocrit, and hemoglobin, across study designs that, when interpreted together, suggest PFHxA-related hematological effects such as anemia. These findings are consistent with similar effects for multiple other PFAS and are reasonably determined to be adverse and relevant to humans. **3d: Endocrine Effects** The available information for endocrine effects is appropriately discussed and synthesized in Section 3.2.5. The PFHxA Toxicological Review reasonably concludes that the available evidence for endocrine effects suggests, but is not sufficient to infer, that PFHxA may cause endocrine effects in humans, under relevant exposure circumstances. The Review reaches this conclusion based on limited data from two rat studies, based on hormone and histopathological changes, acknowledging, however, that histopathological changes may be explained by non-thyroid related effects. It is plausible that evidence from recent and on-going studies might strengthen this

	conclusion, but at this point the assessment presented in the review represents the most reasonable judgment. **3e: Other Potential Non-Cancer Effects** The PFHxA Toxicological Review reasonably concludes that the available data do not provide sufficient evidence for assessing whether exposures to PFHxA have the potential to cause renal, male and female reproductive, immune, nervous system or other health effects in humans. The relevant available information is adequately discussed and synthesized in Sections 3.2.6 to 3.2.9 of the document. Note: The Public Comments provided by the NJDEP suggest the re-consideration by EPA of the respiratory effects (dose-related increase in nasal lesions) that were observed in the 28-day NTP (2018) study and the 90-day Loveless et al. (2009) study; this is a reasonable suggestion. **Tier 2 Suggested Revision:** Re-examine the respiratory effects (dose-related increase in nasal lesions) that were observed in the 28-day NTP (2018) study and the 90-day Loveless et al. (2009) study for potential incorporation in the Toxicological Review.
Haney	p. 2-3 lines 24-25 state [*emphasis added*] that "All *outcomes rated low confidence or higher* were used for evidence synthesis and integration." While this statement can be consistent with [*emphasis added*] "syntheses of the evidence for human and animal health effects are based *primarily* on studies of high and medium confidence" (p. 1-12, lines 24-26), it is less clear to the reader exactly how it is consistent with p. 1-12 lines 31-21 [*emphasis added*] that indicate "*no low confidence studies were used in the evidence syntheses* for PFHxA included in the narrative", since low confidence studies may presumably have outcomes that would also be rated as low confidence, which might be assumed to be included in evidence synthesis and integration based on the first sentenced cited above. The answer may be that outcome confidence ratings are limited to only outcomes from studies with medium or high confidence ratings, but in any event, it is not abundantly clear to the reader and the consistency of these two statements should be clarified (**Tier 2 Suggested Revision**). For example, if accurate, perhaps the EPA could simply revise the first cited sentence to [*emphasis added*]… "All outcomes rated low confidence or higher*, from studies rated medium or high confidence*, were used for evidence synthesis and integration." **3a:** Yes, it appears that by and large, the available data on hepatic effects are clearly and appropriately synthesized to describe the strengths and limitations. P. 3-38, lines 3-4 state that "overall, the currently available evidence indicates that PFHxA likely causes hepatic effects in humans under relevant exposure circumstances." Table 3-11 (pp. 3-39 through 3-41) is the evidence profile table for hepatic effects, which among other information contains factors that increase certainty (e.g., consistent increases in organ weight for all studies and sexes with a dose-response in all studies and coherence with cellular hypertrophy) and no factors that decrease certainty (although limitations of relevant mechanistic evidence are mentioned) along with evidence stream (i.e., human, animal, mechanistic/supplemental) judgments/rationales, including biologically plausible support for PPARα-dependent and independent pathways contributing to hepatic effects of PFHxA, and a summary judgment. Obviously, the text of the document (Section 3.2.1) also contains information relevant to and supporting the

	weight-of-evidence for hepatic effects, and comments below are intended to help improve that documentation. Importantly, recommendations of the Hall et al. (2012) paper were considered by the EPA in assessing the adversity of observed hepatic effects. That is, in the absence of a known mechanism leading to increased liver weight, hepatocellular hypertrophy, and necrosis, the draft assessment (*Considerations for Potentially Adaptive Versus Adverse Responses*, pp. 3-35 and 3-36) evaluates the evidence for PFHxA-mediated hepatotoxicity to inform interpretations regarding adversity utilizing guidance from Hall et al. (2012). Criteria are summarized on p. 3-36, lines 3-15. The section concludes, "Considering the Hall et al. (2012) criteria above, the observed increase in relative liver weight and hepatocellular hypertrophy in rats exposed to PFHxA are interpreted as adverse, human relevant, and potentially leading to increasingly severe outcomes such as necrosis." However, the interpretation as "adverse" appears to be based on somewhat limited PFHxA-specific information to fulfill the cited criteria. For example, necrosis only occurred in female rats at the highest dose in a chronic study but not in males or in shorter duration studies, and while a 2.37-fold increase in alanine aminotransferase (ALT) in male rats in one study might be construed to satisfy one liver parameter criterion in conjunction with more clear dose-responses in male rats in other studies (NTP 2018, Loveless et al. 2009), the EPA does not characterize as biologically significant either the observed increase in alkaline phosphatase (ALP) or aspartate aminotransferase (AST) to satisfy the second liver parameter criterion. Although liver effects are not the proposed key effects for derivation of either the final chronic or subchronic RfD, the EPA should consider (**Tier 2 Suggested Revision**) utilizing information on other PFAS compounds (e.g., PFBA) to supplement and bolster the evidence consistent with the adversity of PFHxA-induced hepatic effects (e.g., as the EPA did in discussing PPARα on p. 3-35, lines 23-26). Also note that while this section (*Considerations for Potentially Adaptive Versus Adverse Responses*) states that the "incidence of necrosis were not observed in rats (male or female) from the short-term study (NTP, 2018)" (p. 3-36, lines 21-22), the next section indicates "hepatocellular necrosis was observed in male rats in a high confidence short term study (NTP, 2018) at 1,000 mg/kg-day" (p. 3-36, lines 11-12), as does the "histopathology" section of Table 3-11 (pp. 3-39 and 3-40) more generally (i.e., "necrosis (with *short term*, subchronic, and chronic exposure)"). These statements appear inconsistent. Since elsewhere the characterization is "a slight increase in male rats (n = 1/10 reported in a short term study at 1,000 mg/kg-day PFHxA (NTP, 2018)" (p. 3-23, lines 2-3), perhaps the latter text should be revised [*emphasis added*] to read… "hepatocellular necrosis was observed in *a single male rat* in a high confidence short term study (NTP, 2018) at 1,000 mg/kg-day". In any event, the EPA should revise the assessment (**Tier 2 Suggested Revision**) to appear consistent in regard to the discussion and characterization of hepatocellular necrosis in male rats in the short-term study NTP (2018). This being said, taken together, the weight of the available scientific information presented (e.g., effects in humanized PPARα mice, evidence of PPARα-independent pathways) reasonably supports that assuming sufficiently high exposure over a sufficiently long duration (i.e., "given relevant exposure circumstances"), PFHxA exposure is likely to cause hepatic toxicity in the general human population, which

	includes potentially susceptible subpopulations (e.g., those with pre-existing liver disease).[1 (see Haney footnote below)] Though there is some room for improvement, as documented in various comments, the bases for this weight-of-evidence decision are clearly described in the text (*Evidence Integration* on pp. 3-37 and 3-38) and Table 3-11 (pp. 3-39 through 3-41) of the document. **3b:** Yes, it appears that overall, the available data on developmental effects are clearly and appropriately synthesized to describe the strengths and limitations. P. 3-50, lines 17-18 state that "overall, the currently available evidence indicates that PFHxA likely causes developmental effects in humans under relevant exposure circumstances." Table 3-16 (pp. 3-51 and 3-52) is the evidence profile table for developmental effects, which among other information contains both factors that increase certainty (e.g., consistency across high confidence studies and species in body weight effects) and factors that decrease certainty (e.g., unexplained inconsistency across species in offspring mortality) along with evidence stream (i.e., human, animal) judgments/rationales and a summary judgment. Obviously, the text of the document (Section 3.2.2) also contains information relevant to and supporting the weight-of-evidence for developmental effects. Human relevance, however, is an area of potential improvement. While p. 3-50 (lines 20-22) states that "findings are interpreted as relevant to humans based on similarities in the anatomy and physiology of the developmental system across rodents and humans", and Table 3-16 states that "without evidence to the contrary, effects in rats and mice are considered relevant to humans", the text of the document contains little-to-no discussion of: the conserved biological processes or similarities in anatomy and physiology between rodents and humans that the EPA considers relevant to the observed developmental effects, or whether rodents (particularly the mouse) have been shown to be good laboratory animal models for assessing potential human developmental effects. The EPA should consider adding additional information supporting the human relevance of developmental effects (**Tier 2 Suggested Revision**). This being said, taken together, the weight of the available scientific information presented (e.g., body weight decreases in mouse fetuses and pups) reasonably supports that assuming sufficiently high exposure over a sufficiently long duration (i.e., "given relevant exposure circumstances"), PFHxA exposure is likely to cause developmental toxicity in the general human population, which includes potentially susceptible subpopulations (e.g., developing fetuses of pregnant women).[2 (see Haney footnote below)] **3c:** On the whole, the available data on hematopoietic effects are clearly and appropriately synthesized to describe the strengths and limitations. P. 3-72, lines 22-23 state that "overall, the currently available evidence indicates that PFHxA likely causes hematopoietic effects in humans under relevant exposure circumstances." Table 3-25 (pp.3-72 and 3-73) is the evidence profile table for hematopoietic effects and contains factors that increase certainty (e.g., consistent changes (decreases in hematocrit, hemoglobin, red blood cells, and MCHC and increases in reticulocytes, MCV, and MCH) across studies) with no factors that decrease certainty along with evidence stream (i.e., animal) judgments/rationales and a summary judgment. While Table 3-25 states that "without evidence to the contrary, effects in rats are considered relevant to humans", the text of the document contains little-to-no discussion of: the conserved biological processes between rats and humans that the EPA considers relevant to the observed hematopoietic effects, or whether the rat has been shown to be a good laboratory

	animal model for assessing potential human hematopoietic effects. The EPA should consider adding additional information supporting the human relevance of hematopoietic effects observed in rats (**Tier 2 Suggested Revision**). While there is some room for improvement (e.g., additional discussion on human relevance), the weight of the available scientific information reasonably supports that assuming sufficiently high exposure over a sufficiently long duration (i.e., "given relevant exposure circumstances"), PFHxA exposure is likely to cause hematopoietic toxicity in the general human population, which includes potentially susceptible subpopulations (e.g., those with pre-existing hematopoietic conditions).[3] (See Haney footnote below) **3d:** Overall, the critical available data on endocrine effects are clearly and appropriately synthesized to describe the strengths and limitations. For endocrine effects, p. 3-80 (lines 37-38) states that "overall, the currently available evidence suggests, but is not sufficient to infer, that PFHxA could cause endocrine effects in humans under relevant exposure circumstances." Table 3-29 (pp. 3-82 and 3-83) is the evidence profile table for endocrine effects, which among other information documents factors that increase or decrease certainty (e.g., lack of coherence across related thyroid hormone measures in the human study, unexplained inconsistency across animal studies in organ weight effects and histopathology results) along with evidence stream (i.e., human, animal) judgments/ rationales and a summary judgment. Notably, there are no mechanistic data or supplemental information to inform a potential mode of action (MOA) for the observed effects to help support the suggestive evidence. Available data have been clearly and appropriately synthesized to describe the strengths and limitations in the *Evidence Integration* section (pp. 3-79 through 3-81), which among other statements and information supporting the weight-of-evidence decision indicates that, "No clear pattern of treatment-related effects were reported for endocrine organ weights… The availability of only one short-term study of thyroid hormones represents a significant data gap for PFHxA… It is possible that the observed changes in thyroid histopathology are secondary to hepatic effects... Based on the results, there is *slight* animal evidence of endocrine effects." In this reviewer's opinion, the weight-of-evidence decision for endocrine effects is scientifically justified. **3e:** Again, overall, the critical available data on other potential health effects (i.e., renal, male and female reproductive, immune, and nervous system) are clearly and appropriately synthesized to describe the strengths and limitations. For these effects, the draft assessment contains the following: P. 3-60 (lines 6-7) states that "Overall, the currently available evidence is inadequate to assess whether PFHxA may causes renal effects in humans under relevant exposure circumstances (see Table 3-19)." P. 3-89 (lines 25-27) states that "Overall, the currently available evidence is inadequate to assess whether PFHxA might cause male reproductive effects in human under relevant exposure circumstances (see Table 3-31)."

	P. 3-97 (lines 5-7) states that "Overall, the currently available evidence is inadequate to assess whether PFHxA might cause female reproductive effects in humans under relevant exposure circumstances (see Table 3-33)." P. 3-104 (lines 26-28) states that "Overall, the currently available evidence is inadequate to determine whether PFHxA exposure might cause immune system effects in humans under relevant exposure conditions (see Table 3-35)." P. 3-108 (lines 30-31) states that "Overall, the currently available evidence is inadequate to assess whether PFHxA might cause nervous system effects in humans under relevant exposure circumstances", with Table 3-37 (pp. 3-109 and 3-110) being the evidence profile table for nervous system effects. For renal effects, the human evidence was limited to a single low confidence study reporting an inverse association between PFHxA exposure and estimated glomerular filtration rate (eGFR), although there is a potential for reverse causality. Based on these data, there is *indeterminate* human evidence for renal effects. The *Evidence Integration* (pp. 3-59 and 3-60) discusses the evaluation of laboratory animal study results that led to a determination of *slight* animal evidence of renal effects (e.g., findings from high confidence studies were generally null except for histopathology and some urinary biomarkers, and findings of adversity were considered uncertain based on lack of coherence between effects (organ weight, histopathology, blood and urine biomarkers), inconsistency between sexes, and lack of coherence across exposure designs). In regard to potential male reproductive effects, while two low confidence human studies provided some indication of an association between PFHxA exposure and sperm motility (Song et al. 2018) and reproductive hormone levels (Zhou et al. 2016), the results are difficult to interpret due to a high risk of bias for these single studies (i.e., there are limited data). Accordingly, there is *indeterminate* human evidence of male reproductive effects. Similarly, the *Evidence Integration* (pp. 3-88 and 3-89) discusses the evaluation of laboratory animal study results that led to a determination of *indeterminate* animal evidence of male reproductive effects (e.g., reproductive hormone levels were reduced only at the 26-week time point in the chronic study (Klaunig et al. 2015), but the effect was small in magnitude and not dose-dependent, and similar effects on testosterone were not observed in the short-term high confidence study (NTP 2018)). For female reproductive effects, based on results from the single low confidence human study (Zhou et al. 2016), there is *indeterminate* human evidence of female reproductive effects. Similarly, as discussed by the EPA in the *Evidence Integration* (pp. 3-96 and 3-97), there is *indeterminate* evidence of female reproductive effects from laboratory animal studies (e.g., no biologically significant (> 10%) effects on maternal weight or weight gain in rodents). Regarding immunotoxicity, the human evidence was limited to one study that showed no clear association between PFHxA exposure and immune-related health outcomes. The animal evidence supporting the potential immunotoxicity to humans is likewise limited (e.g., lack of consistency across studies for outcomes), as are the outcomes

	evaluated. Based on relevant results, there is *indeterminate* human and animal evidence of immune effects. For nervous system effects, no human studies were identified to inform the potential nervous system effects of PFHxA or PFHxA salts. The available animal toxicity data are largely null and derived from low risk of bias studies (although some uncertainties and data gaps remain). Additionally, no mechanistic data were identified to inform the potential for health effects. Based on relevant results, there is i*ndeterminate* human and animal evidence of nervous system effects. Getting back to renal effects, the EPA should consider (**Tier 2 Suggested Revision**) noting the potential for reverse causality as a factor that decreases certainty for the association of PFHxA with decrease in estimated eGFR in Table 3-19 (p. 3-61). The draft assessment states (p. 3-55, lines 5-7) that "with the exception of the results from Chengelis et al. (2009b), effects on relative kidney weights generally showed a weak or no dose-response gradient (see Table 3-18)", and that "absolute kidney weight was increased, but only in one of the three studies reporting on this endpoint (NTP, 2018), and only in female rats at the highest dose group (1,000 mg/kg-day)" (p. 3-55, lines 10-12). EPA should consider (**Tier 2 Suggested Revision**) adding "weak, no, or inconsistent dose-response" as a factor that decreases certainty for organ weight in Table 3-19 (p. 3-61), especially since "consistent increases, all studies" under "factors that increase certainty" in the same table may imply a consistent dose-response for increased organ weight to some readers. As "blood biomarkers of renal function were inconsistent" (p. 3-57, line 6), the EPA should consider (**Tier 2 Suggested Revision**) adding this to Table 3-19 (p. 3-62) as a factor that decreases certainty in addition to the current draft "lack of coherence with other histopathological findings; chronic study." Similarly, as [*emphasis added*] "the urinalysis findings were more consistent than the blood biomarkers, but *still difficult to interpret as adverse or nonadverse*" (p. 3-58, lines 2-3), difficulty in interpreting the observed effects as adverse or nonadverse appears to be a factor that decreases certainty for EPA consideration (**Tier 2 Suggested Revision**) as an addition to Table 3-19 (p. 3-62). Finally, in regard to nervous system effects, for consistency with Table 3-31, Table 3-37 (p. 3-109) could indicate that EPA's "preferred metric" for brain weight is absolute brain weight. (**Tier 2 Suggested Revision**). While the comments above may provide for some room for improvement, overall, the critical available data on potential health effects (i.e., renal, male and female reproductive, immune, and nervous system) are clearly and appropriately synthesized to describe the strengths and limitations. **Haney Footnote 1:** To help prevent misinterpretation or an overly broad interpretation of this comment, note that my interpretation of "given relevant exposure circumstances" in this context means that [*emphasis added*] given that hepatic effects have been demonstrated in laboratory animals, *sufficiently high exposure over a sufficiently long duration will likely produce hepatic effects in humans at some point as dose and duration rise*, but will not necessarily begin to occur at the same doses/lowest-observed-adverse-effect-levels (LOAELs) that caused such effects in laboratory animals when extrapolated to estimated human equivalent doses (HEDs) as there is uncertainty associated with these extrapolations and there are potential interspecies toxicodynamic differences relative to the most sensitive laboratory animal species,

	Haney Footnote 2: To help prevent misinterpretation or an overly broad interpretation of this comment, note that my interpretation of "given relevant exposure circumstances" in this context means that [*emphasis added*] given that developmental effects have been demonstrated in laboratory animals, *sufficiently high exposure over a critical duration(s) will likely produce developmental effects in humans at some point as dose rises* for durations of critical windows of development, but will not necessarily begin to occur at the same doses/LOAELs that caused such effects in laboratory animals when extrapolated to estimated HEDs as there is uncertainty associated with these extrapolations and there are potential interspecies toxicodynamic differences relative to the most sensitive laboratory animal species. **Haney Footnote 3:** To help prevent misinterpretation or an overly broad interpretation of this comment, note that my interpretation of "given relevant exposure circumstances" in this context means that [*emphasis added*] given that hematopoietic effects have been demonstrated in laboratory animals, *sufficiently high exposure over a sufficiently long duration will likely produce hematopoietic effects in humans at some point as dose and duration rise*, but will not necessarily begin to occur at the same doses/LOAELs that caused such effects in laboratory animals when extrapolated to estimated HEDs as there is uncertainty associated with these extrapolations and there are potential interspecies toxicodynamic differences relative to the most sensitive laboratory animal species.
Leung	**3a:** It is noted that the two human studies assessing the hepatic effects of PFHxA exposure were deemed uninformative and of medium confidence, respectively, with the latter further limited by low PHFxA exposure levels, and thus neither was used to inform human risks. The human risks were estimated from preclinical studies that were all either medium or high confidence and generally consistent in demonstrating increased liver weight, hepatocellular hypertrophy, increased serum liver function enzymes, and decreased concentrations of various serum binding globulins. Although corroborative human data are not available, the findings and weight-of-evidence supporting adverse hepatic effects of PFHxA exposure from these animal studies have been clearly and appropriately synthesized. **3a(i):** The conclusions formed from the *in vitro* and *in vivo* studies appear scientifically justified and their reasoning is well-described. The conclusions are further strengthened by examination of similar effects from other chemicals in the PFAS class, as well as the consideration of reasonable mechanisms linking the preclinical data with potential human risks. **3b:** Similarly, there were no available studies assessing developmental effects of PFHxA exposure in humans. However, the animal data presented of three studies are reasonable to support these likely risks related to offspring mortality, offspring weight, and developmental milestones, as has been clearly summarized. It appears that the only developmental milestone that was studied is eye opening though, thus the report may consider rephrasing the term "milestone" as this single metric instead (**Editorial Comment**; **Tier 2 Suggested Revision**). **3c:** The estimated adverse hematopoietic effects of PFHxA exposure are drawn from four rat studies of high confidence; there were no information human studies available. The conclusions presented from the animal data are reasonable and clearly summarized.

	3d: There were identified only two animal studies regarding endocrine effects (both high confidence); informative human studies are absent on this topic. I agree with the astute points raised by Dr. Zoeller in the public meeting on 5/17/22, including the limitations of the Li 2017 study, and that only the NTP 2018 study measured circulating thyroid hormones. Although it is very limited data, the NTP 2018 is the only study available on this endpoint and showed decreased circulating thyroid hormone levels in males but not females. Thus, I support including the consideration that PFHxA exposure may be associated with decreased thyroid hormones levels in humans as informed by these animal data (**Tier 1 Necessary Revision**). **Clarification:** I affirm that the recommendation has been made with the information contained in the IRIS PFAS systematic review protocol (Tables 10-3, 10-4, 10-5) in mind. **3e:** The findings presented for the ability to evaluate potential renal, male and female reproductive, immune, and nervous system effects of PFHxA exposure are reasonable and clearly presented to include the reasons for this position. For Section 3.2.8 (Immune Effects), it may be more clear to separate out asthma into its own Pulmonary Effects section, since the one human asthma study examined were mostly of non-immune mediated outcomes (**Tier 2 Suggested Revision**).
Ng	**3a: Hepatic effects** The discussion of relevance to humans and synthesis of available animal data is logically laid out and generally well-justified, with clear explanation of integrative evidence of both PPARα dependent and independent pathways of hepatic effects in both rodents and humans. **Tier 2 Suggested Revisions:** In the "Evidence from other PFAS" section (p. 3-35, lines 22-29) it may be helpful to highlight/emphasize that these observations of PPARα independent and dependent pathways from the four other PFAS are consistent for both short-chain (e.g. PFBA) and long-chain (e.g. PFNA) substances, increasing the plausibility that it also applies to PFHxA (since toxicological evidence is often mostly available for long-chain PFAS with substantially longer half-lives). **Editorial Comment:** p. 3.35 line 22: "…SV and PPARα null ~~and~~ mice"; on line 34 there is a space in the word "in". p. 3-36, line 1: missing word: "conclude whether the adverse or not"; line 5: "proliferation" **3b: Developmental effects** The integration of available animal data, based on two high quality animal studies (with three experiments) and on plausibility for human relevance, supports the finding that PFHxA likely causes developmental effects in humans. **Editorial Comment:** Page 3-50 lines 3 and 8 seem to be missing words.

	3c: Hematopoietic effects The integration of available evidence (from animal studies only) provide high confidence in anemia as an outcome of PFHxA exposure, and also show consistency across dose ranges (of effects seen as low as 200 mg/kg-d, two studies). **Tier 1 Necessary Revision:** Based on the assessment of all four animal studies as high confidence and the agreement across study findings and doses, it is not clear why the animal evidence lands on "moderate" rather than "robust"—what additional information, endpoint, or consideration would be needed to qualify as robust? Context here for what drives the "moderate" decision would be helpful. It would also help to align with the conclusion that "the currently available evidence indicates that PFHxA likely causes hematopoietic effects in humans". **Tier 2 Suggested Revisions:** The only reference to "human relevance" is given in Table 3-25 with the statement "Without evidence to the contrary, effects in rats are considered relevant to humans". While this is a (potentially) useful blanket statement, is there not a more nuanced statement that can be made in the specific context of hematopoietic effects? For example, there are many studies that use rats as models of different types of anemia. This comment also applies to tables in further sections using this statement. **Tier 3 Future Considerations:** Given the agreement across studies, this is not necessarily needed to strengthen the current evaluation, but there is a noted lack of discussion of findings across other PFAS as supporting information. **3d: Endocrine effects** Integration of evidence from the four animal studies paints in unclear picture, with a single study showing a high magnitude of effect on thyroid hormones and inconsistent findings across three high confidence studies on histopathology. Based on this and only one uninformative and one low confidence study available for humans, the evaluation of "evidence suggests" endocrine effects based on slight animal evidence is appropriate. The use of supplemental information from studies for other PFAS (p. 3-80, lines 14-16) and studies on PFHxA binding to thyroid receptors and transport proteins helps in the interpretation of these complex data. **Tier 1 Necessary Revision:** On p. 3-80, lines 21-22, the statement: "some of these inconsistencies could be explained by differences in the test article (i.e. PFHxA vs. PFHxA salts)" should be deleted or better justified. Both the acids and salts will dissociate at biologically relevant pH to form the identical anion. If the authors refer to the difference in dose due to differences in molecular weight, this should be specified, but this would result in a quite small adjustment. **3e: Other potential effects** Renal effects: Evidence is inadequate. No concerns. Male reproductive effects: Evidence is inadequate. No concerns.

	Editorial Comment: Figures 3-20 and 3-21: add explanation for blue dotted line in figure caption (p. 3-87,88). Female reproductive effects: Evidence is inadequate. No concerns. Immune effects: Evidence is inadequate. No concerns. Nervous system effects: Evidence is inadequate. **Tier 2 Suggested Revisions:** For nervous system effects, zebrafish studies are common for PFAS and should be considered as useful supplemental data to inform evaluations. The study by Gaballah et al. (2020) [https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7228129/] provides evidence of a "unique hyperactivity signature" associated with PFHxA exposure. This section could have benefited from discussion of known impacts of other PFAS that might inform design of future studies (see e.g. the review by Cao & Ng, https://pubs.rsc.org/en/content/articlelanding/2021/em/d1em00228g).
Savitz	**3a: Tier 2 Suggested Revisions:** The interpretation of both epidemiologic studies is reasonable, with Jiang et al. (2014) weaker than Nian et al. (2019) but I would note very limited analysis and the potential for confounding in Jiang et al. (2014). It is not clear why the potential for confounding is considered to be so substantial without some indication of the rationale for expecting that serum PFHxA levels are associated with the confounding factors. There are predictors of liver enzymes among the unadjusted predictors noted in text, which is part of what would drive confounding, but in order to confound the association between PFHxA and liver enzymes, the confounding factor would need to be related to both. This concern applies to other places where the failure to consider confounding is a limitation but in order to consider it a "fatal flaw" that renders the study as "inadequate," some stronger reasoning as to why such confounding would be expected is needed. Any changes from "inadequate" to "low confidence" would not affect the overall conclusion though. **3b: Editorial Comment:** No comments, agree with assessment. **3c: Editorial comment:** I agree Jiang et al. (2014) is uninformative, but the same point about alleged confounding noted above is applicable here. This would not affect the overall conclusion. **3d: Editorial comment:** This automatic invocation of confounding to assign "inadequate" is consistent throughout the report. It is not obvious to this reviewer why this is considered a fatal flaw, but if a case is made, perhaps it could be done early and suffice for all the decisions that followed. I recognize it would be repetitive to go through the logic each time. **3e: Tier 1 Necessary Revision for Renal Effects:** I would note reverse causality as a concern in the Seo et al. (2018) study, but it is not clear what the problem with confounding is in the Zhang et al. (2019) study which compared populations with and without elevated exposure through electronic waste. A clearer justification for considering Zhang et al. (2019) as "uninformative" is needed.

	Male and Female Reproductive Effects: No comments, agree with assessments and rationale. Immune Effects: No comments, agree with assessments and rationale. Nervous System Effects: No comments, agree with assessments and rationale.
Zoeller	**3a(i):** The review of data focused on liver effects of PFHxA concludes that this chemical likely causes hepatic effects in humans under relevant exposure circumstances, that the effects are indicative of adversity and are relevant to humans. The Agency's weight-of-evidence analysis is scientifically justified and clearly described. The Agency identified one study in humans that met criteria for analysis, but this was a low confidence study that did not provide actionable information. In contrast, the Agency identified several studies in animals that provided relevant information. The most relevant observations of these high confidence animal studies were that the *in vivo* activation of CAR, PPARα, PPARγ, and Era. In addition, there was increased peroxisomal beta oxidation activity that was persistent, and indirect evidence of fatty liver, hepatocellular hypertrophy and hepatomegaly in PPARα KO mice. Finally, there was evidence that PPARα activity was induced by PFHxA in vitro. These effects were deemed relevant to human populations according to accepted criteria. **No Recommendations.** **3b**: The Agency identified no human studies to inform the potential developmental effects of PFHxA exposure. However, there were 6 high confidence studies in rats and mice identified that contained relevant information. Key findings included increased perinatal mortality, fetal and postnatal body weight decrease, and delayed eye opening, and these were observed without overt symptoms of maternal toxicity. The Agency's logic was clear and transparent, and their conclusions scientifically justified. **No Recommendations.** **3c:** The Agency did not identify informative human studies in relation to hematopoietic effects. However, several high confidence animal studies were identified that described consistent effects of PFHxA exposure to decrease hematocrit, RBCs, and hemoglobin, and increases in reticulocytes with potential findings of compensatory erythroid responses. These findings were clear and transparent and scientifically justified. One minor comment: **Tier 2 Suggested Revisions:** Page 3-64, Line 4: "The CBC measures three primary types of blood cells (red blood cells, white blood cells, and erythrocytes)…". RBCs = erythrocytes, so this should be revised. **3d:** The Agency identified two publications in humans that were deemed deficient or critically deficient in terms of their ability to inform the Agency on the relationship between PFHxA exposure and thyroid hormone system disruption in humans. The study by Li et al., (2017) was a complex design that included euthyroid, hypothyroid and hyperthyroid groups. In addition to these studies, four animal studies rated high confidence were identified to inform the Agency on the relationship of interest. Only one study (NTP, 2018) included measures of circulating concentrations of thyroid

	hormones and PFHxA exposure. The other studies reported measures of thyroid weight and/or histopathology. The Agency's data integration may conflate what appears to be inconsistent effects of HPHxA on measure of the thyroid gland with effects of HPHxA on circulating levels of thyroid hormones. In the first case, the pathway by which thyroid hormones affect measures of thyroid weight and histology/histopathology is through an increase in serum TSH. This did not happen. While it is a serious limitation in our understanding of the frequent observation that some chemicals can reduce serum thyroid hormones without increasing TSH, it is a well-known phenomenon [2-4] (see Zoeller footnotes below) Considering this, it seems reasonable to conclude that the effect of PFHxA on the thyroid gland was not due to changes in circulating thyroid hormones and that the "inconsistency" in the findings may only be inconsistent from the point of view of a presumed AOP-like pathway whereby a reduction in serum thyroid hormones triggers elevated serum TSH which then affects the thyroid gland. Thus, the high confidence NTP study showing a robust, dose-related suppression of serum thyroid hormones by PFHxA should be viewed separately from the other studies. It is perplexing that PFHxA did not reduce serum hormones in females, and the Agency discussed the possibility of sex differences in PFHxA metabolism that may account for this. Two other possibilities may be germane. First, the immunoassay employed for thyroid hormones showed that control males had total T4 levels of 4.26±0.15 µg/dL where it was 3.62±0.30 µg/dL for females. This reviewer could not find the specific "kit" that was employed, but many commercial kits have a LOD of 2.0 µg/dL. Thus, the low level in controls may mask a decrease by treatment. In addition, a recent review of the pathways by which microsomal enzyme inducers can reduce serum thyroid hormones[5] (see Zoeller footnote below) provides additional information that may account for sex differences. Importantly, the failure of PFHxA to reduce serum thyroid hormones in females in the NTP, 2018 study should not discount the significant effect of PFHxA on thyroid hormones in males. Given the observation that several other PFAS produce this same effect should allay the concern that this is only a single study of PFHxA and thyroid hormone levels. As the Agency points out, it is well known that thyroid hormone insufficiency during pregnancy or during the perinatal period can have life-long adverse effects on the health of the offspring, including cognitive deficits [6]. Moreover, it is important to recognize that even transient thyroid hormone insufficiency during the perinatal period can have life-long adverse effects. This is true for development in humans [6-9] (see Zoeller footnotes below) and for development in rodents [9-14] (see Zoeller footnotes below). Thus, this high-quality endpoint should be incorporated into the risk assessment. **Tier 1 Necessary Revision:** Considering these elements, the Agency should conclude that the available evidence indicates that PFHxA exposure is likely to cause thyroid toxicity in humans given relevant exposure circumstances, primarily based on short-term studies in rats reporting a consistent and coherent pattern of effects on thyroid hormones following PFHxA exposure, but also drawing from the consistency of effects when considering evidence from structurally related PFAS.

	3e: The Agency identified several low confidence studies focused on the ability of PFHxA on the renal system, male and female reproduction, immune and nervous systems. The Agency clearly characterized both strength and weaknesses of these studies and the conclusion that there is inadequate information to assess whether PFHxA affects these physiological domains is scientifically justified. **No Recommendations.** **Zoeller Footnote 2:** Hood A, Klaassen CD. Differential effects of microsomal enzyme inducers on in vitro thyroxine (T(4)) and triiodothyronine (T(3)) glucuronidation. Toxicol Sci 2000;55:78-84. **Zoeller Footnote 3:** Hood A, Liu YP, Gattone VH, 2nd, Klaassen CD. Sensitivity of thyroid gland growth to thyroid stimulating hormone (TSH) in rats treated with antithyroid drugs. Toxicol Sci 1999;49:263-71. **Zoeller Footnote 4:** Hood A, Hashmi R, Klaassen CD. Effects of microsomal enzyme inducers on thyroid-follicular cell proliferation, hyperplasia, and hypertrophy. Toxicol Appl Pharmacol 1999;160:163-70. **Zoeller Footnote 5:** Vansell NR. Mechanisms by Which Inducers of Drug Metabolizing Enzymes Alter Thyroid Hormones in Rats. Drug Metab Dispos 2022;50:508-17. **Zoeller Footnote 6:** Rovet JF. The role of thyroid hormones for brain development and cognitive function. Endocrine development 2014;26:26-43. **Zoeller Footnote 7:** Stagnaro-Green A, Rovet J. Pregnancy: Maternal thyroid function in pregnancy - a tale of two tails. Nat Rev Endocrinol 2016;12:10-1. **Zoeller Footnote 8:** Rovet JF. Children with congenital hypothyroidism and their siblings: do they really differ? Pediatrics 2005;115:e52-7. **Zoeller Footnote 9:** Zoeller RT, Rovet J. Timing of thyroid hormone action in the developing brain: clinical observations and experimental findings. J Neuroendocrinol 2004;16:809-18. **Zoeller Footnote 10:** Dong H, You S-H, Williams A, Wade MG, Yauk CL, Thomas Zoeller R. Transient Maternal Hypothyroxinemia Potentiates the Transcriptional Response to Exogenous Thyroid Hormone in the Fetal Cerebral Cortex Before the Onset of Fetal Thyroid Function: A Messenger and MicroRNA Profiling Study. Cereb Cortex 2014. **Zoeller Footnote 11:** Navarro D, Alvarado M, Morte B, et al. Late maternal hypothyroidism alters the expression of Camk4 in neocortical subplate neurons: a comparison with Nurr1 labeling. Cereb Cortex 2014;24:2694-706. **Zoeller Footnote 12:** Berbel P, Navarro D, Auso E, et al. Role of late maternal thyroid hormones in cerebral cortex development: an experimental model for human prematurity. Cereb Cortex 2010;20:1462-75. **Zoeller Footnote 13:** de Escobar GM, Ares S, Berbel P, Obregon MJ, del Rey FE. The changing role of maternal thyroid hormone in fetal brain development. Semin Perinatol 2008;32:380-6.

	Zoeller Footnote 14: Cuevas E, Auso E, Telefont M, Morreale de Escobar G, Sotelo C, Berbel P. Transient maternal hypothyroxinemia at onset of corticogenesis alters tangential migration of medial ganglionic eminence-derived neurons. Eur J Neurosci 2005;22:541-51.

3.4 For PFHxA, no RfC was derived. The study chosen for use in deriving the RfD is the Loveless et al. (2009) one-generation reproductive toxicity study based on decreased offspring body weight in rats exposed continuously throughout gestation and lactation to PFHxA sodium salt via the dam. Is the selection of this study and these effects for use in deriving the RfD for PFHxA scientifically justified and clearly described?

a. If yes, please provide an explanation.

b. If no, please provide an alternative study(ies) or effect(s) that should be used to support the derivation of the RfD and detail the rationale for use of such an alternative.

c. As part of the responses in "a" or "b" above, please comment on whether the effects selected are appropriate for use in deriving the RfD, including considerations regarding adversity (or appropriateness in representing an adverse change) and the scientific support for their selection.

d. Given the lack of studies on inhalation exposure to PFHxA, no reference concentration (RfC) is derived. Please comment on this decision.

Reviewer	Comments
Faustman	**4a:** This reviewer agreed with the choice of the Loveless et al (2009) study for the calculation of the RfD. Table ES-1 provides the summary cross endpoint comparisons for PFHxA. It shows that the Developmental studies have medium confidence values for the posted os RfD and these listed values at among the lowest presented on this table. **Tier 1 Necessary Revision** is to also calculate this value using the T4 endpoint from the NTP, 2018 study and to determine if this has significant impact on the calculation of the RfD. If this dose have a significant impact then this reviewer would prioritize the use of the T4 endpoint. **4c:** Please see my responses about adversity in my hepatic discussion section above and my discussion in the developmental impacts section on maternal toxicity and offspring impacts. **4d:** There is a great amount of uncertainty in extrapolating from RfD to RfC and with this data set I would not recommend that an RfC be developed with the data in hand.
Georgopoulos	**4a:** From the identified human health effects of PFHxA and derived osRfDs for hepatic, hematopoietic, and developmental effects (summarized in Table 5-9, page 5-25), a chronic RfD of 5×10^{-4} mg/kg-day PFHxA was selected based on decreased postnatal body weight in rats. Using data from the high-confidence one-generation reproductive toxicity study of Loveless et al. (2009) to derive the RfD for PFHxA is scientifically justified and adequately described. The overall study size, design, and test species were reasonably considered relevant for deriving toxicity values. Confidence in the RfD

	is medium, based on medium confidence in the developmental RfD. The decision to select the developmental RfD was based on all available osRfDs in addition to overall confidence and composite uncertainty for those osRfDs (page 5-26 of the Toxicological Review). **4b:** N/A **4c:** The effect considered for deriving the RfD, i.e., decreased offspring body weight in rats exposed continuously throughout gestation and lactation to PFHxA sodium salt via the dam, is appropriate for representing adverse change. **4d:** Finally, the decision to not derive a reference concentration (RfC) is justified, given the lack of studies on inhalation exposures to PFHxA.
Haney	**4a:** In brief, selection of the Loveless et al. (2009) one-generation reproductive toxicity study is scientifically justified for derivation of the RfD. Loveless et al. (2009) is a high confidence study, and although an RfD is typically a chronic/lifetime value, it must be protective of effects that can occur over a shorter, less-than-chronic exposure duration (e.g., developmental effects). As documented in Table 5-8 (p. 5-24), characteristics that make it suitable for deriving toxicity values include relevance of the exposure paradigm (route, duration, and exposure levels), use of a relevant species, and the study size and design. In regard to critical effect, decreased offspring (i.e., F_1 postnatal day 0) body weight in rats exposed continuously throughout gestation and lactation is judged by the EPA to be relevant to human health "based on similarities in the anatomy and physiology of the developmental system across rodents and humans" (p. 3-50, lines 20-22), which is scientifically reasonable but could be better supported within the document (consistent with a previous comment in 3b). Additionally, there was decreased F_1 postnatal day 0 (PND 0) body weight (critical effect) in the key study (Table 3-14, p. 3-47) above the standard 5% reduction in developmental body weight generally considered to be a minimally biologically significant response (i.e., the study observed effects greater than a minimally biologically significant response level above the lowest dose), which is a consideration relevant to the adversity of the observed weight decreases. Finally, important for selection and justification of the critical effect point of departure (POD), it is noted that as the NJ DEP comments, the POD and human equivalent dose POD (POD_{HED}) values for decreased F_1 postnatal body weight in rats (Loveless et al. 2009) are lower than these respective POD values for mice (Iwai and Hoberman 2014) (see Table 5-5, p. 5-18 of the draft assessment). In summary, the selection of the Loveless et al. (2009) study and critical effect (i.e., decreased F_1 PND 0 body weight) for deriving the RfD for PFHxA is scientifically defensible. **4b:** N/A **4c:** As stated above, the selection of the Loveless et al. (2009) study as well as the critical effect (i.e., decreased F_1 PND 0 body weight) is scientifically defensible for deriving the RfD for PFHxA. Concerning adverse considerations, there was decreased F_1 PND 0 body weight in the key study (Table 3-14, p. 3-47) above the standard 5% reduction in developmental body weight generally considered to be a minimally biologically significant response. That is, the key study observed effects greater than a

	minimally biologically significant response level (i.e., decreased F_1 body weight > 5% at the mid and high doses), which is a consideration that suggests adversity of these observed weight decreases. **4d:** The decision not to derive an RfC is justified. As stated on p. 5-33, lines 5-7, "No published studies investigating the inhalation effects of subchronic, chronic, or gestational exposure to PFHxA in humans or animals have been identified. Therefore, an RfC is not derived." In addition to the lack of these inhalation studies, there is an apparent lack of a validated physiologically based pharmacokinetic (PBPK) model for PFHxA for consideration of route-to-route (i.e., ingestion-to-inhalation) extrapolation. P. 1-1, lines 4-5 state, "no physiologically based pharmacokinetic (PBPK) models are available to support route-to-route extrapolation." Consequently, the EPA has provided rationale for not deriving an RfC for PFHxA. Additionally, oral exposure to PFHxA (i.e., through drinking water and food preparation) is likely to represent the far greater potential concern for most people, and any actions required to mitigate exposure through the oral route pursuant to the RfD (e.g., reducing drinking water concentrations) will also serve to reduce potential inhalation exposure.
Leung	**4(a/b/c):** The process for RfD derivation is not my specific area of expertise, but the reasoning as presented, based on the one selected study to conclude that an RfD for PFHxA was unable to be derived, appears overall sound. **4d:** This appears to be a sound decision, as there were no available inhalation exposure studies for PFHxA identified.
Ng	**4(a/b/c):** The studies selected for each organ or system-specific candidate RfD derivation are based on first identifying the relevant endpoints and then the studies selected for each specific endpoint's point of departure (POD). For **hepatic effects**, hepatocellular hypertrophy using results from the studies of Chengelis et al. (low confidence, males only) and Loveless et al. (high confidence, both sexes) is selected as the specific and reliable endpoint to use for POD derivation. This is driven by the high confidence Loveless study with support from the Chengelis et al. study and is well justified by the preceding discussion of noncancer hazards. For **hematopoietic effects**, hemoglobin and red blood cell counts are both considered for POD derivation with the Chengelis et al., Loveless et al., and Klaunig et al. data (all high confidence) contributing to the benchmark dose (BMD) modeling. This is well justified by the preceding analysis of this noncancer hazard. For **developmental effects**, the postnatal pup body weight endpoint prioritizes early postnatal day (PND) results from the Loveless et al. and Iwai and Hoberman studies (both high confidence), while perinatal mortality uses pooled data from Iwai and Hoberman across two cohorts which is justified based on similar experiments differing only by their dose ranges (an initial higher dose range was used as range finding for the second study). These selections are appropriately justified. Finally, the Loveless et al. study for decreased offspring body weight was selected as the basis for deriving the RfD. This was based on high confidence in the study, the

	lowest overall POD, and is considered protective of all life stages. It also has a lower uncertainty factor that the slightly lower hepatic RfD which was based on a higher POD. The selection of this study is well justified based on these considerations. **4d:** This is well justified based on the lack of exposure studies to PFHxA via the air pathway. It is likely that human exposure to PFHxA precursors is most relevant for the air pathway (e.g. to volatile fluorotelomer alcohols that degrade to PFHxA in the organism). Therefore, PFHxA serum levels may reflect an inhalation exposure to a precursor, but that is outside the scope of this review.
Savitz	Lacking necessary expertise to comment
Zoeller	**4b:** The NTP, 2018 study with serum T4 as an endpoint should be used as an alternative to support the derivation of an RfD. This study was high confidence, showed robust response to PFHxA exposure in terms of T4 suppression, which is relevant for human health and predictive of adverse effects in humans. Scientific justification for this study as an alternative is discussed above. [see recommendation under 3d] **4c: No Recommendation.** **4d:** The Agency is reasonable in their decision not to derive an RfC because of lack of data. **4e: No Recommendation.**

3.5 In addition, for PFHxA, an RfD for less-than-lifetime ("subchronic") exposures is derived. No "subchronic" RfC was derived. The same study and outcome were chosen for use in deriving the RfD. Is the selection of this study and these effects for the derivation of the subchronic RfD for PFHxA scientifically justified and clearly described?

a. If yes, please provide an explanation.

b. If no, please provide an alternative study(ies) and/or effect(s) that should be used to support the derivation of the subchronic RfD and detail the rationale for use of such an alternative.

c. As part of the responses in "a" or "b" above, please comment on whether the effects selected are appropriate for use in deriving the RfD, including considerations regarding adversity (or appropriateness in representing an adverse change) and the scientific support for their selection.

d. Given the lack of studies on inhalation exposure to PFHxA, no "subchronic" RfC is derived. Please comment on this decision.

Reviewer	Comments
Faustman	**5a**: Yes, this choice was a good one. Table 5-1 provides a structure for this decision which I support. Information is presented on sex, duration and rationale for the

	available studies. **Tier 2 Suggested Revisions**--I added text to the organ specific narrative for hepatic as well as development on adversity versus adaptation that maybe relevant for this justification. These studies were either medium or high confidence studies with good annotation and discussion of observations and the quantitative estimates that result from these calculations indicate that these are sensitive (hence protective endpoints for use in the RfD development). **5c:** This reviewer would state that this is an appropriate choice. Please see my comments above for the choice of the RfD for chronic assessment (see above responses for 5a). Table 5-1 provides an excellent summary of the rationale for choosing between endpoints. **Tier 2 Suggested Revisions**-- For my review of the hepatic and developmental impacts I provided text on what the health impact meant for human population. These endpoint choices for the RfD are highly relevant for human populations. **5d:** There is a great amount of uncertainty in extrapolating from RfD to RfC and with this data set I would not recommend that an RfC be developed with the data in hand.
Georgopoulos	**5a:** From the identified targets of PFHxA toxicity and derived subchronic osRfDs (Table 5-13 on page 5-32), the Toxicological Review selected a subchronic RfD of 5×10^{-4} mg/kg-day based on decreased postnatal body weight for less-than-lifetime exposure. Confidence in the RfD is medium, based on medium confidence in the developmental RfD. The data were from the same Loveless et al. (2009) one-generation reproductive toxicity study that was used to derive the chronic RfD and are scientifically justified and adequately described. Confidence in the study is high based on the study evaluation results (i.e., rated high confidence overall) and characteristics that make it suitable for deriving toxicity values, including relevance of the exposure paradigm (route, duration, and exposure levels), use of a relevant species, and the study size and design (Table 5-12 on page 5-31 of the Toxicological Review). **5b:** N/A **5c:** The effect considered for deriving the RfD, i.e., decreased offspring body weight in rats exposed continuously throughout gestation and lactation to PFHxA sodium salt via the dam, is appropriate for representing adverse change. **5d:** The decision to not derive a subchronic RfC is the only reasonable option since no available inhalation exposure studies to PFHxA have been identified.
Haney	**5a:** Yes, in brief, selection of the Loveless et al. (2009) one-generation reproductive toxicity study is scientifically justified for derivation of the subchronic RfD. Loveless et al. (2009) is a high confidence study, and both chronic and subchronic RfDs must also be protective of effects that can occur due to a relatively short exposure duration (e.g., developmental effects), such as during any critical windows of development. More generally, subchronic RfDs provide a useful risk assessment complement to chronic RfDs, furthering risk assessment and risk communication. As documented in Table 5-8 (p. 5-24), characteristics that make it suitable for deriving toxicity values include

	relevance of the exposure paradigm (route, duration, and exposure levels), use of a relevant species, and the study size and design. In regard to critical effect, decreased offspring (i.e., F_1 postnatal day 0) body weight in rats exposed continuously throughout gestation and lactation is judged by the EPA to be relevant to human health "based on similarities in the anatomy and physiology of the developmental system across rodents and humans" (p. 3-50, lines 20-22). This is scientifically reasonable although it could be better supported within the document (consistent with a previous comment in 3b). Finally, the key study observed decreased F_1 PND 0 body weight (critical effect; Table 3-14, p. 3-47) above the standard 5% reduction in developmental weight generally considered to be a minimally biologically significant response (i.e., the study observed effects greater than a minimally biologically significant response level at the mid and high dose), which is a consideration relevant to the adversity of the observed weight decreases. In summary, the selection of the Loveless et al. (2009) study and critical effect (i.e., decreased F_1 PND 0 body weight) for deriving the subchronic RfD for PFHxA is scientifically defensible. **5b:** N/A **5c:** As stated above, the selection of the Loveless et al. (2009) study as well as the critical effect (i.e., decreased F_1 PND 0 body weight) is scientifically defensible for deriving the subchronic RfD for PFHxA. Concerning adversity considerations, there was decreased F_1 PND 0 body weight in the key study (Table 3-14, p. 3-47) above the standard 5% reduction in developmental body weight generally considered to be a minimally biologically significant response. That is, the key study observed effects greater than a minimally biologically significant response level (i.e., decreased F_1 body weight > 5% at the mid and high dose), which is a consideration that suggests adversity of these observed weight decreases. **5d:** The decision not to derive a subchronic RfC is justified. As stated on p. 5-33, lines 5-7, "No published studies investigating the inhalation effects of subchronic, chronic, or gestational exposure to PFHxA in humans or animals have been identified." In addition to the lack of these inhalation studies, there is an apparent lack of a validated PBPK model for PFHxA for consideration of route-to-route (i.e., ingestion-to-inhalation) extrapolation. P. 1-1, lines 4-5 state, "no physiologically based pharmacokinetic (PBPK) models are available to support route-to-route extrapolation." Consequently, the EPA has provided rationale for not deriving a subchronic RfC for PFHxA. Additionally, oral exposure (i.e., through drinking water and food preparation) is likely to represent the far greater potential concern for most people, and any actions required to mitigate PFHxA exposure through the oral route pursuant to the RfD (e.g., reducing drinking water concentrations) will also serve to reduce potential inhalation exposure.
Leung	**5(a/b/c):** The process for subchronic RfD derivation is not my specific area of expertise, but the reasoning as presented, based on the one selected study to conclude that a subchronic RfD for PFHxA was unable to be derived, appears overall sound. **5d:** This appears to be a sound decision, as there were no available inhalation exposure studies for PFHxA identified.

Ng	**5(a/b/c):** The rationale for the subchronic RfD derivation follows the same logic as was discussed for the chronic RfD above, with the addition that sub-chronic studies (Loveless, Chengelis) were prioritized over chronic (Klaunig et al., relevant for hematopoietic effects). The conclusions were therefore the same as for the chronic RfD selection of Loveless et al. for derivation. **5d:** The same justification and rationale applies as discussed above for the chronic RfC.
Savitz	Lacking necessary expertise to comment
Zoeller	**5b:** The Agency was clear in their reasoning for choosing the Loveless 2009 study to support the subchronic RfD. However, the same reasoning described above argues to use the NTP, 2018 study with the endpoint of T4 suppression. **5d:** The Agency is justified in not deriving an RfC given the lack of studies on inhalation exposure to PFHxA. **No Recommendation**.

3.6 EPA used benchmark dose modeling (USEPA, 2012) to identify points-of-departure (PODs) for oral exposure to PFHxA. Are the modeling approaches used, selection and justification of benchmark response levels, and the selected models used to identify each POD for toxicity value derivation scientifically justified and clearly described?

Reviewer	Comments
Faustman	This reviewer was impressed with the details provided to identify the PODs for exposure to PFHxA. These were described in Section 5 and the considerations by specific endpoint were impressive. The tables in this section also provided specific rationale for what was chosen as an appropriate POD. The rationale included consideration of sensitive life stage, consistency as a measure, sex related differences, etc. These tables were very easy to use and review steps in the part of the decision-making process. Strong verification of options and methods to apply.
Georgopoulos	The BDM (benchmark dose modeling) approaches used to calculate PODs for toxicity value derivation (including model and benchmark response level selection) are scientifically justified and clearly presented in Chapter 5 (Derivation of Toxicity Values) of the PFHxA Toxicological Review. As discussed in Appendix B of the Toxicological Review, the endpoints selected for BMD modeling were hepatocellular hypertrophy [from Chengelis et al. (2009a) and Loveless et al. (2009)]; hemoglobin and red blood cells [from Chengelis et al. (2009a), Loveless et al. (2009), and Klaunig et al. (2015)]; postnatal body weight decreases [from Loveless et al. (2009) and Iwai and Hoberman (2014)]; and perinatal mortality [from Iwai and Hoberman (2014)]. The animal doses were used in the BMD modeling and then converted to human equivalent doses (HEDs) using the ratio of animal-to-human serum

	half-lives. For endpoints with successful BMD model fit, the modeling results were e presented in that Appendix. BMD modeling of continuous noncancer data was conducted using EPA's Benchmark Dose Software (BMDS, Version 3.2). For these data, the Exponential, Hill, Polynomial, and Power models were fit using a benchmark response (BMR) of one standard deviation (SD) when no toxicological information was available to determine an adverse level of response; when toxicological information was available, the BMR was based on relative deviation, as outlined in the USEPA (2012) Benchmark Dose Technical Guidance. **Note (directly related to the Tier 1 Necessary Revision that follows it):** Though the discussion of benchmark dose modeling in Chapter 5 and Appendix B of the Toxicological Review is very thorough. However, the statement summarizing model selection on page 5-7 (lines 10 to 14), i.e. "[a]mong all models providing adequate fit for a given endpoint, the benchmark dose lower confidence limit (BMDL) from the model with the lowest Akaike's information criterion (AIC) was selected as a potential POD when BMDL values were sufficiently close (within 3-fold). Otherwise, the lowest BMDL was selected as a potential POD for each endpoint[,]" appeared to contradict the selection of the Multistage Degree 3 model in Table B-25 (page B-25) that lists "Benchmark dose results for hepatocellular hypertrophy in female rats—nonconstant variance, BMR = 10% Extra Risk," where the logistic model has a lower AIC value. If the fit of the Logistic model did not provide an adequate fit in this case, it would be helpful if this is marked/identified appropriately in Table B-25. **Tier 1 Necessary Revision:** If models that do not provide adequate fit are included in the Tables summarizing benchmark dose modeling results for different endpoints (in Appendix B), these models should be marked/identified in these tables (e.g. by placing the model names and associated estimates in parentheses). **Note (directly related to the Tier 3 Future Consideration that follows it):** Since BMDS 3.2 also includes "preview" versions of Bayesian continuous models (and a model averaging option for calculating PODs) it would be very informative to re-calculate the PODs for the PFHxA data sets with the Bayesian models and compare with the values presented in the current version of the Toxicological Review. I do realize that the BMDS 3.2 software release is accompanied by the statement "The preview Bayesian continuous models have not been formally reviewed and approved by the EPA for risk assessment purposes," but application to the PFHxA data sets offers an opportunity to test and evaluate these models, which would eventually be a necessary step in their formal approval process. It should be recognized that implementing integrative Bayesian frameworks combining toxicokinetic modeling with benchmark dose calculations (e.g. Chou & Lin, 2020) will gradually progress from the research realm to the regulatory realm, and testing user-oriented tools such as BMDS will facilitate this process. **Tier 3 Future Consideration:** Compare the POD estimates contained in the current Toxicological Review with estimates calculated using the Bayesian continuous models available in BMDS 3.2
Haney	Generally, the modeling approaches, model selection process, and benchmark response levels used to derive PODs for toxicity value derivation are scientifically justified. Use of

	benchmark dose modeling to the extent possible, guided by standard statistical model fit criteria (+ visual inspection) for model selection, is essentially standard scientific procedure inside (and outside) EPA.[4 (see Haney footnote below)] Additionally, overall, reasonable scientific justifications for the benchmark responses (BMRs) utilized are provided in Table 5-2 (pp. 5-6 and 5-7). For example, a 5% relative deviation in markers of growth/development in gestational studies (e.g., fetal weight) has generally been considered a minimally biologically significant response level and has historical precedence for use as a BMR (EPA 2012b, 2004, 2003), while the severe effect of offspring mortality justifies use of a 1% extra risk, and a BMR equal to 1 standard deviation is generally used for continuous endpoints when biological information is not sufficient to identify the BMR (e.g., for decreases in red blood cells). **Haney Footnote 4:** An adequate fit is judged on the basis of a $\chi 2$ goodness-of-fit p-value ($p > 0.1$), scaled residuals at the data point (except the control) closest to the predefined BMR (absolute value <2.0), and visual inspection of the model fit. Among all models providing adequate fit, the benchmark dose lower confidence limit (BMDL) from the model with the lowest Akaike's information criterion (AIC) was selected as a potential POD when BMDL estimates differed by less than threefold. When BMDL estimates differed by greater than threefold, the model with the lowest BMDL was selected to account for model uncertainty (p. B-1).
Leung	This is not my area of expertise; I am unable to comment.
Ng	The benchmark dose modeling procedure is generally well described on pages 5-6 to 5-7 and in Appendix B. However, two discrepancies were noted in the data tables in Appendix B relative to the described procedure that "Among all models providing adequate fit, the benchmark dose… from the model with the lowest Akakike's information criterion (AIC) was selected… When BMDL estimates differed by greater than threefold, the model with the lowest BMDL was selected to account for model uncertainty." **Tier 1 Necessary Revisions:** In Table B-25, the selected model (indicated by bold type in the table and shown in the proceeding figure) has neither the lowest AIC nor lowest BMDL. A good explanation of this was given during the in person discussions, and the text would benefit from including this as an example of the utility of visual inspection.
Savitz	Lacking necessary expertise to comment
Zoeller	The use of benchmark dose modeling according to the Agency's 2012 technical guide was well justified in the PFHxA review. Their justification and analysis were clearly described and preferable to other approaches to identify the PODs. **No Recommendation.**

3.7 Appendix A identifies the potential for pharmacokinetic differences across species and sexes as a key science issue and lays out a hierarchy for using relevant pharmacokinetic data in extrapolating oral doses between laboratory animals and humans. Section 5.2.1 describes the various approaches considered and the rationale for the selected approach.

Given what is known and not known about the potential interspecies differences in PFHxA pharmacokinetics, EPA used the ratio of human-to-animal serum clearance values assuming the volume of distribution (Vd) in humans is equivalent to that in monkeys to adjust the POD to estimate a human equivalent dose (HED) in the derivation of the respective RfDs.

a. Is applying the ratio of human-to-animal serum clearance values for PFHxA scientifically justified and clearly described? If not, please provide an explanation and detail the preferred alternative approach.

b. Does the Toxicological Review clearly describe the uncertainties in evaluating the pharmacokinetic differences between the experimental animal data and humans?

Reviewer	Comments
Faustman	**7a:** Yes, this is justified. It is important to remember that there is not a kinetic model for PFHxA however, there are data on the chemistry of this compound and there are elimination rates. C max has also been estimated as another parameter. Table 5-3 on page 5-13 summarizes the serum half-lives across studies. Section 3.1 on pharmacokinetics provides an excellent discussion on ADME It has a large and pertinent narrative that could be useful for our assessments. There is also a great deal of scientific observations that can be shared across the PFOS compounds. Section 3.1 provides some excellent background and identification of chemical related process. **7b:** Yes, this section was very helpful and was great in providing details on the parameters for the Kinetic models across the PFAS compounds.
Georgopoulos	**7a:** Pharmacokinetics of PFAS in general are primarily driven by processes such as serum protein binding and renal reabsorption, and these processes can differ dramatically between animal models (rodents) and humans. The importance of interspecies differences in pharmacokinetic processes pf PFHxA is correctly recognized in the Toxicological Review and applying the ratio of human-to-animal serum clearance values for PFHxA is scientifically justified and definitely a more appropriate approach than scaling doses allometrically, using body weight $(BW)^{3/4}$ methods. **7b:** The Toxicological Review provides a discussion of the pharmacokinetic differences between the experimental animal data and humans that should convey to the reader the significance of the uncertainties associated with these differences. In fact, it appears that there are important knowledge gaps in the pharmacokinetic processes (and the values of pharmacokinetic parameters) of PFHxA that are more extensive than for other PFAS. These gaps include not only uncertainties in binding affinities to serum proteins and renal transporters but also biomonitoring challenges, with measurements of blood PFHxA levels not being consistent with measurements of serum/plasma levels. In the face of these uncertainties EPA's use of empirically observed pharmacokinetic parameters (e.g., distribution and elimination rates) rather than parameters predicted based on in vitro measured binding affinities is appropriate. EPA's approach of evaluating PFHxA elimination in rats and mice (described in section C.1 of Appendix C) by estimating pharmacokinetic parameters separately for male and female rats and mice using a

	hierarchical, Bayesian framework to allow for the partial pooling of time-course concentration data across multiple studies is appropriate. Also, EPA's approach of evaluating PFHxA elimination in humans (described in section C.2 of Appendix C), using a Bayesian inference model to estimate parameters for each of the eleven subjects of the Nilsson et al. (2013) study is appropriate. Some further clarity is needed regarding the presumed linearity of PFHxA pharmacokinetics. It should be mentioned that in the pharmacokinetic analysis of Gomis et al. PFHxA did not display the biphasic elimination pattern typical of many PFAS, with a rapid decline in an initial (α) phase and a slower decline in a second (β) phase; instead PFHxA pharmacokinetics were consistent with a single phase β decline pattern. This behavior is expected to be true in most cases, when exposures to PFHxA (and co-occurring PFAS) are low; however, it should not necessarily lead to the conclusion that there is a "universal" PFHxA biological half-life value for a given species/gender, as predicated by linear kinetics. In fact, PFAS kinetics in general involve saturable processes, with high interspecies and interindividual variabilities, that may induce nonlinearities in situations of high exposures (or significant co-exposures with other PFAS that have common molecular targets). In general, discussion of pharmacokinetics and metabolism in Chapter 3 (Pharmacokinetics, Evidence Synthesis, and Evidence Integration) and in Chapter 5 (Derivation of Toxicity Values) of the Toxicological Review needs careful editing to resolve certain ambiguities and inconsistencies. As an example, on page 3-7 (line 31) it is stated that "PFHxA is not readily metabolized," potentially leading the reader to assume that under certain conditions PFHxA might be metabolized; however, such conditions have not been documented. As another example, on page 5-8 (lines 21-13) it is correctly stated that "comparison of $BW^{3/4}$ scaling to the available PK data in rats and humans indicates that use of $BW^{3/4}$ would overpredict human clearance, and hence underpredict risk, by 1–2 orders of magnitude. Thus, $BW^{3/4}$ scaling was not considered appropriate for this assessment." However, earlier, In Chapter 3 (page 3-15, lines 12-13) one reads that "based on the PFHxA-specific PK data, use of $BW^{0.75}$ for dosimetric extrapolation could lead to an underprediction of human elimination by 1–2 orders of magnitude." Clearly, "underprediction" should be replaced with "overprediction" in this sentence. **Tier 1 Necessary Revision:** Edit sections on metabolism and pharmacokinetics in Chapters 3 and 5, to ensure clarity and consistency. One final note regarding PFHxA pharmacokinetics: US EPA has derived and included PFHxA-specific pharmacokinetic parameters in the *httk: High-Throughput Toxicokinetics* R package (Pearce et al., 2017); however, this is not mentioned in the Toxicological Review. It would be useful to clarify how pharmacokinetic modeling for PFHxA using httk (and the assumptions inherent in httk approaches) compare with the pharmacokinetic modeling performed and assumptions used for this Toxicological Review. **Tier 1 Necessary Revision:** The pharmacokinetic assumptions and parameterizations used by US EPA in the httk: High-Throughput Toxicokinetics package should be briefly mentioned/discussed in the Toxicological Review (since httk is a publicly available US EPA "product") and the context for making comparisons with the assumptions and

	parameterizations of the pharmacokinetic modeling performed for this Review should be clarified. ***Note:*** I performed simple simulations with httk and it was not clear whether the httk presumed half-life of 88 hours was consistent with apparent half-lives calculated for scenarios of continuous long-term (multi-year) exposures to PFHxA. It would be useful if US EPA could further examine this point.
Haney	**7a:** Yes, the EPA appears to have used a reasonable, scientifically-informed approach given what is known, and unknown, about potential interspecies differences in the toxicokinetics (TK) of PFHxA. In brief, the draft assessment examined multiple options for estimating clearance (*CL*) in humans and essentially selected the option associated with the least uncertainty. The human clearance (*CL*) value selected by the EPA (Table 5-4 , p. 5-14) is based on the reasonable expectation, considering data from multiple chemicals, that the volume of distribution (V_d) in humans does not substantially differ from that in experimental animals (i.e., monkeys), and the resulting dosimetric adjustment factors (DAFs) are apparently consistent with data for other PFAS. This appears to be a reasonable and scientifically supportable choice given the available data, its limitations, and other considerations (e.g., general preference for chemical-specific data and primate if not human data, the greater potential uncertainty of alternatives such as generic allometric scaling based on body weight$^{0.75}$). I acknowledge, however, that given their training, experience, and expertise, PBPK modelers are likely to have much more specific and insightful comments than the general comments provided here. Discussion relevant to scientific justification of the EPA's approach (e.g., hierarchy of scientific approaches; chemical-specific, data-informed approach details and comparison to default BW$^{0.75}$ scaling) is provided in the *Approach for Animal-Human Extrapolation of PFHxA Dosimetry* subsection (pp. 5-8 through 5-15) of the draft assessment. **7b:** Yes, the *Uncertainty of animal-human extrapolation of PFHxA dosimetry* section (pp. 5-15 through 5-17) of the draft assessment clearly describes uncertainties in evaluating the TK differences between the experimental animals and humans. Additionally, the method used by the EPA for interspecies TK adjustments in deriving RfDs appears to account for associated uncertainties by limiting them. That is, it appears that the TK extrapolation method associated with the least uncertainty was used. Discussion relevant to the uncertainties associated with TK extrapolation options is provided by the EPA in the *Approach for Animal-Human Extrapolation of PFHxA Dosimetry* subsection (pp. 5-8 through 5-15) of the draft assessment, and consideration of the alternatives provides justification for the method utilized by the EPA. Additionally, use of the ratio of clearance values is consistent with a more conservative approach compared to body weight scaling in the face of appreciable uncertainty. Consequently, in this reviewer's opinion and as documented in the draft assessment, the methods selected by the EPA to derive toxicity values for PFHxA are intended to limit uncertainty in interspecies TK extrapolation given the options available, which is the most scientifically appropriate approach. However, in regard to the options available for estimating human clearance (*CL*), p. 5-11 (lines 1-4) seems to acknowledge appreciable uncertainty, and p. 5-17 (line 1) indicates that the overall uncertainty in human clearance is estimated to be ≈16-fold. As a **Tier 3 Future Consideration**, since as to human clearance the current draft seems to be

	implementing the notion of "doing the best you can with what you currently have" within a scientific context, as soon as practicable and consistent with applicable guidelines, the EPA should seek to obtain data (e.g., on human clearance) that may allow for animal-to-human extrapolation methods and/or DAFs for PFHxA that are associated with greater confidence/less uncertainty. If significantly different, greater confidence DAFs could have important implications for the accuracy of the HED and the RfD better meeting its definition (i.e., an estimate, *with uncertainty spanning perhaps an order of magnitude*, of an exposure to the human population (including susceptible subgroups) that is likely without an appreciable risk of deleterious health effects over a lifetime), as well as important risk assessment implications.
Leung	This is not my area of expertise; I am unable to comment.
Ng	**7a:** The prioritization of PBPK modeling/data and decision to not consider the $BW^{3/4}$ scaling factor for dosimetry extrapolation is appropriate and well supported by the discussions provided in section 3.1 of the review. Unfortunately, no PBPK model is available for PFHxA, thus necessitating the analysis of PK data to determine a suitable approach for developing the human equivalent dose (HED). The analysis of available data led to selection of human-to-animal clearance ratio, rather than half-life, as the more reliable metric for this extrapolation. This is well justified but some clarification is suggested in the description of the findings. **Tier 2 Suggested Revisions:** Suggest clarification. On p. 5-10 lines 16-18 imply that female human and male human equivalent doses (HED) will be calculated on the basis of sex-specific PODs in animals. Is this the case, given the lack of sex differences observed in human studies? Does this match final derivations of RfDs? **Tier 2 Suggested Revisions:** Any discussion of the Pérez et al. study should make note of the fact that the analytical method to detect PFAS used is subject to error, especially for PFBA but to some extent also for PFHxA. See: https://www.sciencedirect.com/science/article/pii/S1438463921001450?via%3Dihubsciencedirect.com. Based on this, it is appropriate to either avoid using Pérez as supplemental information to contextualize study results, or to always include the caveat of this recent response to their study (relevant also to Chapter 3 of the review). **Clarification:** Thank you for following up on this. My comment came out of a discussion with colleagues when the response paper by Abraham et al. cited above was first published. They noted that the issue discussed in that paper on PFBA could also apply to some extent to PFHxA (I had a specific interest in PFHxA concentration reported in brain tissue) so caution on interpreting those results was also needed. I dug in a bit more with respect to PFHxA specifically to better explain the comment above. The issue is potential for co-elution and/or ion suppression for the more polar/water soluble PFAAs, and is strongest for PFBA but also notable for PFHxA. It is discussed to some extent here: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7725277/

	However, given that PFHxA is not directly discussed in the Abraham paper, I would downscale my previous comment to a suggested revision, noting that some of the results of Perez et al. were called into question (specifically for PFBA) and that some of these issues could also apply to PFHxA as reported by Sanan and Magnuson. **Tier 1 Necessary Revision:** The reasoning behind using CL as opposed to t1/2 uses two conflicting lines of reasoning. Perhaps need to clarify between "significant" and "substantial" differences in Vd? On p. 5-15, lines 18-20: "the reasonable expectation, based on data from multiple chemicals, is the volume of distribution in humans does not substantially differ from that in experimental animals" followed by on lines 28-30: "use of half-life makes an intrinsic assumption that Vd is the same in the test species as in humans. There is a significant difference between rats and monkeys, which leads to the expectation of a difference between rats and humans." Perhaps add a line that analysis of the data would suggest some difference, but not more than an order of magnitude. **Tier 2 Suggested Revisions:** On p. 5-9 lines 12-18, the review makes reference to the Dzierlenga et al. finding of slower elimination at higher concentrations. This is also mentioned in Chapter 3, and noted as opposite the expectation of saturable renal reabsorption (mediated by Oatp1a1). While it is true that much of the work on transporters, particularly PFOA, has focused on reabsorption in the kidney, the reference by Han et al. cited in chapter 3 also mentions other transporters that have been tested for activity with PFAS. Not only Oatp1a1 but also transporters responsible for elimination of PFAS to urine may play a role in observed clearance rates, and therefore saturation of such elimination-facilitating transporters could explain slower clearance at higher doses. For example, Oat1 and Oat3 have been proposed to mediate excretion of PFOA based on an in vitro study (see Weaver et al. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2807038/). **Clarification:** Suggest adding something along the lines of: "While saturation of reabsorption transporters would lead to decreased half-life, there are also transporters responsible for elimination of PFAS to urine, and saturation of these transporters, such as Oat 1 and Oat3, could lead to an increase in observed half-life and could thereby help explain the observations of Dzierlenga et al." **Editorial Comment:** p. 5-14, Table 5-4 and lines 7-21: for easier interpretation, suggest using same units for CL in table and text. On p. 5-15 line 9, should be PFHxA (not PFHxS). **7b:** The sources of uncertainty are well described. **Editorial Comment:** It's unclear on p. 5-17, line 1 whether the authors meant 16-fold±4 fold (12-20 fold) or 16-fold meaning x4 or ÷4. Clarify.
Savitz	Lacking necessary expertise to comment
Zoeller	**7a:** Given the data available to the Agency, applying the ratio of human-to-animal serum clearance values for PBFA is reasonable and the Agency has clearly articulated the scientific justification of this approach.

	No Recommendation. **7b:** The Review does a good job of describing the uncertainties in evaluating the pharmacokinetic differences between the experimental animal data and humans. The Agency had identified and evaluated several studies in humans and animals that provides insight into clearance rates. **No Recommendation**.

3.8 EPA has evaluated and applied uncertainty factors to account for intraspecies variability (UFH), interspecies differences (UFA), database limitations (UFD), exposure duration (UFS), and LOAEL-to-NOAEL extrapolation (UFL) for PFHxA.

a. Is uncertainty in the derivation of the toxicity values scientifically justified and clearly described? Please describe and provide comments, if needed.

b. For uncertainty in interspecies differences (UFA), a value of 3 is applied to account for remaining uncertainty in characterizing the pharmacokinetic and pharmacodynamic differences between laboratory animals and humans after calculation of the HED. For developmental and hematopoietic outcomes, the evidence base lacked chemical-and species-specific information that would have been useful for informing the UFA; for hepatic outcomes, however, available mechanistic and supplemental information was useful for further evaluating the interspecies uncertainty factor. Some data indicate a PPARα-dependent pathway that might support a UFA of 1. Evidence for non-PPARα modes of action, however, is available in the PFHxA (and larger PFAS) database. Thus, uncertainty remains regarding the potential differences in sensitivity across species due to the involvement of both PPARα-dependent and-independent pathways. Further, data are lacking to determine with confidence the relative contribution of each of these pathways. As such, the Toxicological Review concludes the available data are not adequate to determine if humans are likely to be equally or less sensitive than laboratory animals with respect to the observed hepatic effects and that a value of UFA=3 is warranted to account for the residual uncertainty in pharmacodynamic differences across species. Please comment on whether the available animal and mechanistic studies support this conclusion and whether the analysis presented in the Toxicological Review is scientifically justified and clearly described.

c. To inform uncertainty in intraspecies variability (UFH), the assessment evaluates and considers the available evidence on potential susceptibility to PFHxA within different populations or lifestages, including any potential human health impacts from early life exposure. Are the available information and data appropriately considered and the resultant UFH values scientifically justified and clearly described?

d. Are the provided rationales for the remaining uncertainty factors (UFL, UFD, UFS) scientifically justified and clearly described? If not, please explain.

Reviewer	Comments
Faustman	**8a:** Yes. Section 5 was an excellent example of methodically going through endpoint by endpoint and study by study the choices for related uncertainty value. For example, Table 5-6 was very helpful! **8b:** Yes this is justifiable. The answer to whether these numbers is justified goes across several of the sections in the current review document. In Section 3.1 there is discussion about pharmacokinetics and that provides a basis for Section 3.2 non-cancer evidence synthesis on various organ and endpoint toxicity. There is also discussion on what endpoints and their relevance with be used to ensure adequate review across each organs system. This section informed the tables posted in Section 5 and provided the basis for POD determination and subsequent UF factor selection. Another example included conversations about ADME that were raised to ensure consideration in the selection of UF factors. There was discussion on what measured endpoints could inform the discussion on adverse versus adaptive response. Also, understanding the potential for PFHxA to work via PPAR alpha receptors is a good example of comments and review that can inform the selection of the TD component in the UF. Thus, this reviewer was very supportive of this methodical approach where the basis for choosing UFs and endpoints was more integrated. Well done. **8c:** This reviewer was very supportive of the way that various considerations were incorporated into the choice of the interspecies variability factor. Much of this integration was shown in Section 5. For example, Table 5-6 provided consideration of various lifestyle factors. **8d:** Section 5, Table 5-6 provided consideration of these factors and as stated above this reviewer like this detailed discussion of these factors.
Georgopoulos	**8a:** The uncertainty factors that were selected in the PFHxA Toxicological Review to account for interspecies differences (UF_A), interindividual variability (UF_H), duration (UF_S), database limitations (UF_D), and LOAEL-to-NOAEL extrapolation (UF_L), are generally reasonable and the rationale provided for their selection makes sense. Nevertheless, it should be recognized that the process of selecting uncertainty factors is as much art as it is science, and reasonable arguments could be made for assigning a different value to either (or both) the interspecies differences factor (UF_A) and the database factor (UF_D). **8b:** In my opinion, a value of 10 should be considered and evaluated as an alternative to selecting of a value of 3 for UF_A since our current understanding of interspecies differences in PFAS both pharmacokinetics and pharmacodynamics for PFHxA has very significant gaps. Of course, this could be also considered a database-related uncertainty: **Tier 2 Suggested Revision:** if EPA decides to keep a value of 3 for UF_A then a value of 10 should be adopted for UF_D. **8c:** The characterization of prenatal and early postnatal periods as potentially sensitive life stages for the effects of PFHxA is reasonable and appropriate.

	8d: Tier 2 Suggested Revision: If EPA decides to maintain a value of 3 for UF_A then a value of 10 should be adopted for $UF_{D.}$
Haney	**8a:** As clearly described in the draft assessment, uncertainty has been adequately accounted for in the derivation of the RfD values through the application of UFs based on various scientific considerations. **8b:** Application of a UF_A of 3 for potential interspecies toxicodynamic (TD) differences is standard EPA practice when interspecies TK adjustments have been performed, as in this case. The EPA indicates that while some aspects of the cross-species extrapolation of TK processes have been accounted for by calculating a HED through application of a DAF based on animal and human clearance, residual uncertainty related to potential interspecies TD differences remains (Table 5-6, p. 5-21). Therefore, a UF_A of 3 was applied. In regard to hepatic effects, the question above points out that: (1) uncertainty remains regarding the potential differences in sensitivity across species due to the involvement of both PPARα-dependent and-independent pathways; and (2) data are lacking to determine with confidence the relative contribution of each of these pathways. The available data are simply not adequate to determine the relative sensitivity of humans compared to laboratory animals with respect to the observed hepatic effects. Default UF values are intended to be applied in this very situation, where the data are inadequate to inform a more chemical-specific approach. In my experience, the application of a default UF_A of 3 for potential interspecies TD differences is standard EPA practice when interspecies TK adjustments have been performed and there is a lack of chemical-specific information on TD for a more data-informed approach, as is the case here. Accordingly, when TK adjustments have been performed (also, the current assessment considered associated uncertainties in selecting the approach) and data to support a more chemical-specific TD adjustment are lacking, which is frequently the case and the case here, standard practice dictates that the default value of 3 is both applicable and appropriate to account for this uncertainty. The method used by the EPA for interspecies TK adjustments accounted for associated uncertainties by limiting them (i.e., the TK extrapolation method associated with the least uncertainty was used by the EPA), and a more conservative animal-to-human TK adjustment approach (the ratio of clearance values) compared to default body weight scaling has already been utilized by the EPA. As such, a higher UF_A value is not justified either by standard practice or available data.[5 (see Haney footnote below)] **8c:** P. 4-2, lines 24-25 state that "no human studies were available to inform the potential for PFHxA exposure to affect sensitive subpopulations or lifestages." However, Section 4.2 (*Conclusions Regarding Susceptible Populations and Lifestages*) does discuss pertinent laboratory animal data. For example, potential intraspecies differences in terms of sex differences in TK are discussed for rats, where toxicological findings were either consistently observed at lower dose levels in males compared to females or the findings were observed only in males. The reason for this sex dependence is possibly due to sex-dependent PFHxA elimination caused by sex-specific differences in the expression (mRNA and protein) of the renal organic anion transporting polypeptide (Oatp) 1a1 (Kudo et al. 2001). Whether this sex-specific difference might also exist in humans is currently unclear. Additionally, given various developmental effects (i.e., perinatal mortality, reduced body weights, delays in time to eye opening), the prenatal

	and early postnatal window may represent a sensitive lifestage for PFHxA exposure. Consistent with standard practice and supporting scientific rationale, a full UF_H of 10 was applied for interindividual variability in humans in the absence of quantitative information on potential differences in TK and TD relating to NaPFHx/NH4+PFHxA/PFHxA exposure in humans (Table 5-6, p. 5-21). This UF_H value is also consistent with consideration of laboratory animal data, which show some intraspecies differences in sensitivity by sex (male rats being more sensitive to some effects) and identify the prenatal and early postnatal window as a potentially sensitive lifestage for PFHxA exposure. **8d:** The UF_S value of 1 that was used for developmental and hematopoietic effects, and a value > 1 used for hepatic effects (3 was proposed in this case), appear appropriate and scientifically justified. As clearly described in Table 5-6 (p. 5-21) for developmental effects… A UF_S of 1 is applied to developmental endpoints from the one-generation reproductive study by Loveless et al. (2009) and Iwai and Hoberman (2014). The developmental period is recognized as a susceptible lifestage and studies using exposure designs capturing sensitive developmental windows (i.e., gestation or lactation) are more relevant for induction of developmental effects than lifetime exposures (U.S. EPA, 1991). Although effects on body weights are not unique to development and studies evaluating the body weight effects of postnatal exposure are lacking, the current evidence for PFHxA suggests this is a sensitive lifestage for body weight effects of PFHxA exposure based on effects being measured at lower doses than adults. Put most simply, a $UF_S > 1$ is generally used to account for subchronic effects that may increase (e.g., incidence, severity, at lower doses) with longer chronic exposure and not applicable or used when a developmental effect is used as the critical effect for derivation of a chronic toxicity factor. A UF_S of 1 was also appropriately applied to hematopoietic endpoints in the Klaunig et al. (2015) study as the 51 wks of daily exposure represented more than 10% of a rodent life span and the incidence or severity of these outcomes is not anticipated by EPA to increase with increasing exposure duration (Table 5-6, p. 5-21). This reasoning and value are entirely appropriate. A UF_S of 3 was used for hepatocellular hypertrophy for the purpose of deriving a lifetime RfD. Although the endpoint was derived from a 90-d subchronic study (Loveless et al. 2009), the evidence supports a pathway where hepatocellular hypertrophy is the toxic effect altering homeostasis. The evidence suggests that hepatocellular hypertrophy is an adverse hepatic response to PFHxA exposure that worsens with longer exposure toxic effects such as necrosis (Table 5-6, p. 5-21). Accordingly, a UF_S value > 1 (3 was proposed in this case) is justified and appropriate. However, the EPA should consider (**Tier 2 Suggested Revision**) including a discussion of the specific study results justifying the specific UF_S value proposed for hepatocellular hypertrophy (i.e., 3 instead of 10). A UF_S of 1 was obviously appropriate for EPA's derivation of the subchronic RfD.

	It is entirely appropriate that a UF_L of 1 was applied for LOAEL-to-NOAEL extrapolation when the POD is a BMDL or a NOAEL, as this UF is essentially inapplicable in such cases. A UF_D of 3 is appropriate and justified, although there is a comment below concerning one of EPA's considerations. The EPA states (Table 5-6, p. 5-21)… A UF_D of 3 is applied because the evidence base for hepatic, hematopoietic, and developmental endpoints included two subchronic studies and one chronic study in Sprague-Dawley rats and developmental/reproductive studies in Sprague-Dawley rats and Crl:CD1 mice. Limitations, as described in U.S. EPA (2002c) were used as the basis for a $UF_D = 3$. These limitations included a lack of informative human studies for most outcomes, subchronic or chronic toxicity studies in more than one species, or a multigenerational study. For developmental outcomes, pups were indirectly exposed via the dam (i.e., via placental or lactational transfer); thus, the dose received by the pups is unclear and might be significantly less than that administered to the dams. I note that Table 5-6 contains the following as one justification for a UF_D of 3… "For developmental outcomes, pups were indirectly exposed via the dam (i.e., via placental or lactational transfer); thus, the dose received by the pups is unclear and might be significantly less than that administered to the dams." I do not see this as a factor significantly supporting a UF_D of 3. The RfD is derived to protect against developmental effects in offspring (i.e., F_1 PND 0 body weight decreases) based on the oral dose to the dams, not based on an estimated dose to the developing pups for which there could have been residual uncertainty. The actual dose to the developing pups is irrelevant for this type of derivation, as the RfD is not based on that dose (i.e., it is not expressed in terms of a direct developing pup intake protective of pup health) but rather a dam dose that protects developing pups from the given effect observed on PND 0 irrespective of the quantitative relationship between the dam and developing pup doses. Moreover, knowledge of maternal dose causing developmental toxicity is the most practical and therefore important data/basis for protection against developmental effects observed on PND 0, since even if developing pup doses causing toxicity were known those doses would still have to be related back to maternal dose for derivation of a useful intake estimate for protection against such effects (e.g., an RfD). Consequently, in terms of being able to protect against the critical developmental effect (F_1 PND 0 body weight decreases), knowledge of the maternal PFHxA doses producing such effects should be considered the critical data for assessing database uncertainty in this regard. In regard to uncertainty of pup dose due to lactational transfer, it is noted that the body weight effects are greater at PND 0 for the lower doses compared to PNDs 7, 14, and 21, so the comments above on PND 0 pups appear most relevant to this discussion. The table does cite, however, other sufficient considerations relevant to supporting a UF_D of 3… "These limitations include a lack of informative human studies for most outcomes, subchronic or chronic toxicity studies in more than one species, or a multigenerational study." The above comments also apply to Tables 5-8 (p. 5-24) and 5-12 (p. 5-31) in the context of confidence in the evidence base. It is recommended that "the dose received by the pups is unclear and might be significantly less than that administered to the dams" be considered by EPA (**Tier 2 Suggested Revision**) for removal as a cited factor that in a meaningful way diminishes confidence in the database relevant to deriving the RfD. Otherwise, since developing organism (e.g., pup) doses are commonly unknown, it is noted that by EPA's reasoning a UF_D of 3 might automatically be applied anytime the

	basis for an RfD or candidate RfD is developmental effects. Moreover, it is not needed as the EPA cites other considerations that are sufficient to support a UF_D of 3. **Haney Footnote 5:** Even if a validated PBPK model were available, there would be residual uncertainty in animal-to-human TK adjustments, yet an additional $UF_{A\text{-}TK}$ of 3 would not be used under standard EPA practice, only the remaining default $UF_{A\text{-}TD}$ of 3 would be applied. Residual uncertainty in animal-to-human dosimetric adjustments is typical, amongst other uncertainties associated with use of laboratory animal data for chemical dose-response assessment (e.g., potential for species-specific MOA(s) and interspecies differences in TD, potential high-to-low dose extrapolation issues such as dose-dependent transitions in MOA, selection of a dose-response/BMD model to estimate the POD). However, the process for toxicity factor derivation based on laboratory animal data is generally designed to be conservative in nature (e.g., use of the most sensitive species and oftentimes the most sensitive sex within that species in the absence of a completely elucidated MOA(s) to fully support human relevance) and includes standard practices for the application of UFs that overall, are intended to result in a tendency towards erring on the side of safety (i.e., conservatism). Given that the full UF_A of 10 is divided into two factors of 3.16 (the square root of 10), one each for TK and TD, and that TK adjustment has already been performed by the EPA in this case, to apply an additional $UF_{A\text{-}TK}$ (e.g., say a 3) would not only be contrary to standard EPA practice, but might also understandably be considered as double adjusting for interspecies TK differences in a process that is already inherently conservative overall. In this reviewer's opinion, the appropriate place for consideration of, and accounting for, uncertainty in animal-to-human TK adjustments is in selection of the method and inputs, which the EPA did in the *Uncertainty of animal-human extrapolation of PFHxA dosimetry* section (pp. 5-15 through 5-17) of the draft assessment.
Leung	This is not my area of expertise; I am unable to comment.
Ng	**8a:** The uncertainty associated with the evidence base (database uncertainty, UF_D, is assumed to be reference in this charge question) is given a value of 3 suggesting a medium level of uncertainty. This is appropriate given the number of available high quality animal studies and lack of informative human studies. **8b:** The interspecies differences are partially accounted for in derivation of the HED, as noted in discussion of pharmacokinetics. However there remain potential sources of variability, particularly as relates to pathways and expression of relative receptors, binding proteins, and transporters. Therefore, a UF_A of 3 is applied to account for this remaining uncertainty and is well justified. **Editorial Comment:** Table 5-6 on page 5-21 first row states "see text above" but appears to be referring to text below the table. **8c:** A somewhat high UF_H of 10 is applied to account for interindividual variability in humans given lack of specific PK data for PFHxA, but is consistent with observations of high variability, e.g. in human half lives of other PFHxS. **8d:** No modifications suggested.
Savitz	Lacking necessary expertise to comment

Zoeller	**8b:** The Agency devoted a great deal of effort to evaluate the animal, human and mechanistic data to develop scientifically justified conclusions about toxicokinetics and toxicodynamics to calculate RfDs. The Uf_A of 3, however, did not seem well described. **Tier 2 Suggested Revision:** The Agency should consider a more explicit description of the reasoning for choosing a UF_A of 3 instead of 1 or 10. **8c:** The Agency's UF_H of 10 for intraspecies variability is both justified and well described. No Recommendation. **8d:** The Agency tabulated the justifications for UF_I, UF_D and UF_S in Tables 5-6, 5-7 and 5-11 with narrative justification accompanying these tables. A UF_S of 1 does not seem to consider the data showing that PFHxA exposure causes a reduction in serum thyroid hormone, but there is little information beyond that. Moreover, there is data suggesting that eye-opening is delayed by PFHxA exposure, which is a potential thyroid endpoint, but this relationship is not evaluated empirically. Considering this, the UF_S of 1 does not appear to cover this level of uncertainty for development. **Tier 2 Suggested Revision:** Recommend revising the UF_S to 10.

3.9 The Toxicological Review concludes that there is inadequate information to assess carcinogenic potential for PFHxA and that this descriptor applies to oral and inhalation routes of human exposure. Please comment on whether the available animal and mechanistic studies and the analysis presented in the Toxicological Review are scientifically justified and clearly described.

Reviewer	Comments
Faustman	Yes, this reviewer agrees with the conclusion by US EPA to not go forward with a cancer estimate. Only one possible study (high confidence) is currently available and that did not have a positive outcome to model for human assessment. Also note the largely negative results from the in vitro measures of genotoxicity as with the other sections a read-across for the related PFAS compounds would be desirable.
Georgopoulos	Yes, the Toxicological Review for PFHxA reasonably concludes that information for assessing carcinogenic potential for PFHxA and related to oral and inhalation routes of human exposure is inadequate. The available animal and mechanistic studies are clearly described in the Toxicological Review and scientifically justified.
Haney	Yes, the available animal and mechanistic studies along with the analysis presented in the Toxicological Review support the conclusion that there is inadequate evidence to assess carcinogenic potential for PFHxA. As indicated in Section 3.3 of the document: (1) no studies of potential carcinogenicity in exposed humans were identified; (2) only one animal study (Klaunig et al. 2015) evaluated the potential carcinogenicity of oral PFHxA exposure (via histological evaluation of the lung, kidney, stomach, and liver of male

	rats) and did not observe significant treatment-related effects; and (3) the few studies examining markers of potential genotoxicity were largely null. This limited evidence amounts to inadequate information to confidently assess the carcinogenic potential of PFHxA for any route of exposure. Accordingly, consistent with EPA guidance (EPA 2005) to apply a standard descriptor as part of the hazard narrative and to express a conclusion regarding the weight of evidence for the carcinogenic hazard potential, a descriptor of *inadequate information to assess carcinogenic potential* was applied by the EPA for oral and inhalation exposure to PFHxA and is clearly scientifically justified.
Leung	The summary of in vitro and animal, and lack of human studies related to the potential carinonogenic effects of PFHxA exposure is clearly outlined, and the conclusions are reasonable as presented.
Ng	No studies were available for humans or human cells. Several animal studies were available, but only one was a high confidence in vivo cancer bioassay. This reported null findings for nonneoplastic and neoplastic lesions in lungs, kidney, stomach, and liver of exposed rats. Several mammalian and prokaryotic cell system in vitro studies were available, but again showed no significant effects of PFHxA exposure.
Savitz	No comments, agree with assessments and rationale.
Zoeller	The Agency identified that there is not adequate information to assess the carcinogenic potential for PFHxA. This is scientifically justified and well described. **No Recommendation**.

3.10 Given the conclusion there was inadequate information to assess carcinogenic potential for PFHxA (Charge Question 5), the Toxicological Review does not derive quantitative estimates for cancer effects for either oral or inhalation exposures. Is this decision scientifically justified and clearly described?

Reviewer	Comments
Faustman	Yes, this action is justifiable.
Georgopoulos	Yes, the decision to not derive quantitative estimates for cancer effects for either oral or inhalation exposure is the logical consequence of the conclusion that there was inadequate evidence to assess carcinogenic potential for PFHxA.
Haney	Yes, the decision to not derive quantitative estimates for cancer effects for oral or inhalation exposures is scientifically justified. The available animal and mechanistic studies along with the analysis presented in the Toxicological Review support the conclusion that there is inadequate evidence to assess carcinogenic potential for PFHxA. The limited data available provide no reliable basis (i.e., database) for confidently deriving quantitative estimates of excess cancer risk due to PFHxA exposure (oral or

	inhalation). That is, consistent with "the lack of adequate data on the potential carcinogenicity of PFHxA, quantitative estimates for either oral (oral slope factor, OSF) or inhalation (inhalation unit risk; IUR) exposure were not derived" (p. 5-33, lines 11-13). Furthermore, no robust scientific foundation has been laid, critically reviewed and broadly accepted by the scientific community for the use of any surrogate PFAS with carcinogenicity data (e.g., PFOA) for this purpose.
Leung	The conclusion that there is inadequate information to assess the carcinogenic potential of PFHxA exposure via oral or inhalation routes is scientifically justified and clearly described.
Ng	Due to lack of data to evaluate carcinogenic potential, the decision to derive neither an oral slope factor nor an inhalation unit risk is well justified.
Savitz	Comment: This seems like the only logical decision.
Zoeller	The Agency has justified and documented the decision not to derive quantitative estimates for cancer effects. **No Recommendation.**

ADDITIONAL COMMENTS

Reviewer	Comments
Faustman	**Perflorinated Compounds Lit Search - Tier 3 Future Considerations** **PubMed Search: "(PFHxa) AND (perfluorohexanoic acid)" (2020-present)** **Appel, Mareike**, Martin Forsthuber, Romualdo Ramos, Raimund Widhalm, Sebastian Granitzer, Maria Uhl, Markus Hengstschläger, Tanja Stamm, and Claudia Gundacker. 2022. "The Transplacental Transfer Efficiency of Per- and Polyfluoroalkyl Substances (PFAS): A First Meta-Analysis." Journal of Toxicology and Environmental Health. Part B, Critical Reviews 25 (1): 23–42. https://doi.org/10.1080/10937404.2021.2009946. Arinaitwe, Kenneth, Nils Keltsch, Anthony Taabu-Munyaho, Thorsten Reemtsma, and Urs Berger. 2021. "Perfluoroalkyl Substances (PFASs) in the Ugandan Waters of Lake Victoria: Spatial Distribution, Catchment Release and Public Exposure Risk via Municipal Water Consumption." The Science of the Total Environment 783 (August): 146970. https://doi.org/10.1016/j.scitotenv.2021.146970. Bai, Xuelian, and Yeongkwon Son. 2021. "Perfluoroalkyl Substances (PFAS) in Surface Water and Sediments from Two Urban Watersheds in Nevada, USA." The Science of the Total Environment 751 (January): 141622. https://doi.org/10.1016/j.scitotenv.2020.141622.

Caron-Beaudoin, Élyse, Pierre Ayotte, Caty Blanchette, Gina Muckle, Ellen Avard, Sylvie Ricard, and Mélanie Lemire. 2020. "Perfluoroalkyl Acids in Pregnant Women from Nunavik (Quebec, Canada): Trends in Exposure and Associations with Country Foods Consumption." Environment International 145 (December): 106169. https://doi.org/10.1016/j.envint.2020.106169.

Chen, Dongmei, Ying Zhao, Wei Xu, Yuanhu Pan, Qu Wei, and Shuyu Xie. 2020. "Biotransformation and Tissue Bioaccumulation of 8:2 Fluorotelomer Alcohol in Broiler by Oral Exposure." Environmental Pollution (Barking, Essex: 1987) 267 (December): 115611. https://doi.org/10.1016/j.envpol.2020.115611.

Chen, Huilun, Qianyu Wang, Yanping Cai, Rongfang Yuan, Fei Wang, and Beihai Zhou. 2020. "Investigation of the Interaction Mechanism of Perfluoroalkyl Carboxylic Acids with Human Serum Albumin by Spectroscopic Methods." International Journal of Environmental Research and Public Health 17 (4): E1319. https://doi.org/10.3390/ijerph17041319.

Claessens, Julien, Catherine Pirard, and Corinne Charlier. 2022. "Determination of Contamination Levels for Multiple Endocrine Disruptors in Hair from a Non-Occupationally Exposed Population Living in Liege (Belgium)." The Science of the Total Environment 815 (April): 152734. https://doi.org/10.1016/j.scitotenv.2021.152734.

DeLuca, Nicole M., Michelle Angrish, Amina Wilkins, Kris Thayer, and Elaine A. Cohen Hubal. 2021. "Human Exposure Pathways to Poly- and Perfluoroalkyl Substances (PFAS) from Indoor Media: A Systematic Review Protocol." Environment International 146 (January): 106308. https://doi.org/10.1016/j.envint.2020.106308.

Dennis, Nicole M., Farzana Hossain, Seenivasan Subbiah, Adcharee Karnjanapiboonwong, Michael L. Dennis, Chris McCarthy, Christopher G. Heron, et al. 2021. "Chronic Reproductive Toxicity Thresholds for Northern Bobwhite Quail (Colinus Virginianus) Exposed to Perfluorohexanoic Acid (PFHxA) and a Mixture of Perfluorooctane Sulfonic Acid (PFOS) and PFHxA." Environmental Toxicology and Chemistry 40 (9): 2601–14. https://doi.org/10.1002/etc.5135.

Dennis, Nicole M., Farzana Hossain, Seenivasan Subbiah, Adcharee Karnjanapiboonwong, Michael L. Dennis, Christopher McCarthy, W. Andrew Jackson, Jordan P. Crago, Christopher J. Salice, and Todd A. Anderson. 2022. "Species- and Tissue-Specific Chronic Toxicity Values for Northern Bobwhite Quail (Colinus Virginianus) Exposed to Perfluorohexane Sulfonic Acid and a Binary Mixture of Perfluorooctane Sulfonic Acid and Perfluorohexane Sulfonic Acid." Environmental Toxicology and Chemistry 41 (1): 219–29. https://doi.org/10.1002/etc.5238.

Dzierlenga, Anika L., Veronica G. Robinson, Suramya Waidyanatha, Michael J. DeVito, Max A. Eifrid, Seth T. Gibbs, Courtney A. Granville, and Chad R. Blystone. 2020. "Toxicokinetics of Perfluorohexanoic Acid (PFHxA), Perfluorooctanoic Acid (PFOA) and Perfluorodecanoic Acid (PFDA) in Male and Female Hsd:Sprague Dawley SD Rats Following Intravenous or Gavage Administration." Xenobiotica; the Fate of Foreign Compounds in Biological Systems 50 (6): 722–32. https://doi.org/10.1080/00498254.2019.1683776.

East, Andrew, Richard H. Anderson, and Christopher J. Salice. 2021. “Per- and Polyfluoroalkyl Substances (PFAS) in Surface Water Near US Air Force Bases: Prioritizing Individual Chemicals and Mixtures for Toxicity Testing and Risk Assessment.” Environmental Toxicology and Chemistry 40 (3): 859–70. https://doi.org/10.1002/etc.4893.

Fulong, Cressa Ria P., Mary Grace E. Guardian, Diana S. Aga, and Timothy R. Cook. 2020. “A Self-Assembled Iron(II) Metallacage as a Trap for Per- and Polyfluoroalkyl Substances in Water.” Inorganic Chemistry 59 (10): 6697–6708. https://doi.org/10.1021/acs.inorgchem.9b03405.

Gaballah, Shaza, Adam Swank, Jon R. Sobus, Xia Meng Howey, Judith Schmid, Tara Catron, James McCord, Erin Hines, Mark Strynar, and Tamara Tal. 2020. “Evaluation of Developmental Toxicity, Developmental Neurotoxicity, and Tissue Dose in Zebrafish Exposed to GenX and Other PFAS.” Environmental Health Perspectives 128 (4): 47005. https://doi.org/10.1289/EHP5843.

Garcia-Barrios, Joshua, Mallory Drysdale, Mylène Ratelle, Éric Gaudreau, Alain LeBlanc, Mary Gamberg, and Brian D. Laird. 2021. “Biomarkers of Poly- and Perfluoroalkyl Substances (PFAS) in Sub-Arctic and Arctic Communities in Canada.” International Journal of Hygiene and Environmental Health 235 (June): 113754. https://doi.org/10.1016/j.ijheh.2021.113754.

Guo, Xiaochun, Shengnan Zhang, Xiaohui Liu, Shaoyong Lu, Qin Wu, and Ping Xie. 2021. “Evaluation of the Acute Toxicity and Neurodevelopmental Inhibition of Perfluorohexanoic Acid (PFHxA) in Zebrafish Embryos.” Ecotoxicology and Environmental Safety 225 (December): 112733. https://doi.org/10.1016/j.ecoenv.2021.112733.

Hamid, Hanna, Loretta Y. Li, and John R. Grace. 2021. “Exploring Indirect Photolysis of 6:2 Fluorotelomer Sulfonate in Landfill Leachate under Simulated Sunlight: Effect of Humic Acid and Nitrate.” Environmental Science and Pollution Research International 28 (8): 9508–16. https://doi.org/10.1007/s11356-020-11417-4.

Han, Ji-Seok, Sumi Jang, Hwa-Young Son, Yong-Bum Kim, Younhee Kim, Jung-Ho Noh, Mi-Jeong Kim, and Byoung-Seok Lee. 2020. “Subacute Dermal Toxicity of Perfluoroalkyl Carboxylic Acids: Comparison with Different Carbon-Chain Lengths in Human Skin Equivalents and Systemic Effects of Perfluoroheptanoic Acid in Sprague Dawley Rats.” Archives of Toxicology 94 (2): 523–39. https://doi.org/10.1007/s00204-019-02634-z.

Helmer, Ross W., Donald M. Reeves, and Daniel P. Cassidy. 2022. “Per- and Polyfluorinated Alkyl Substances (PFAS) Cycling within Michigan: Contaminated Sites, Landfills and Wastewater Treatment Plants.” Water Research 210 (February): 117983. https://doi.org/10.1016/j.watres.2021.117983.

Holmquist, Hanna, Peter Fantke, Ian T. Cousins, Mikołaj Owsianiak, Ioannis Liagkouridis, and Gregory M. Peters. 2020. “An (Eco)Toxicity Life Cycle Impact Assessment Framework for Per- And Polyfluoroalkyl Substances.” Environmental Science & Technology 54 (10): 6224–34. https://doi.org/10.1021/acs.est.9b07774.

Hua, Zulin, Ying Lu, Kejian Chu, Yuanyuan Liu, Yixin Ma, Li Gu, Jianyi Wu, Wachirasak Leelawattananun, and Sereyvatanak Ky. 2022. “Shift in the Distribution and Fate of Perfluoroalkyl Acids by Sluice Gates in the Multi-Environment Media of Rivers.” Journal of Environmental Management 305 (March): 114436. https://doi.org/10.1016/j.jenvman.2022.114436.

Jiang, Lilong, Yanjun Hong, Guangshan Xie, Jinghui Zhang, Hongna Zhang, and Zongwei Cai. 2021. “Comprehensive Multi-Omics Approaches Reveal the Hepatotoxic Mechanism of Perfluorohexanoic Acid (PFHxA) in Mice.” The Science of the Total Environment 790 (October): 148160. https://doi.org/10.1016/j.scitotenv.2021.148160.

Kibambe, M. G., M. N. B. Momba, A. P. Daso, and M. a. A. Coetzee. 2020. “Evaluation of the Efficiency of Selected Wastewater Treatment Processes in Removing Selected Perfluoroalkyl Substances (PFASs).” Journal of Environmental Management 255 (February): 109945. https://doi.org/10.1016/j.jenvman.2019.109945.

Lasters, Robin, Thimo Groffen, Lieven Bervoets, and Marcel Eens. 2021. “Perfluoroalkyl Acid (PFAA) Profile and Concentrations in Two Co-Occurring Tit Species: Distinct Differences Indicate Non-Generalizable Results across Passerines.” The Science of the Total Environment 761 (March): 143301. https://doi.org/10.1016/j.scitotenv.2020.143301.

Lenka, Swadhina Priyadarshini, Melanie Kah, and Lokesh P. Padhye. 2022. “Occurrence and Fate of Poly- and Perfluoroalkyl Substances (PFAS) in Urban Waters of New Zealand.” Journal of Hazardous Materials 428 (April): 128257. https://doi.org/10.1016/j.jhazmat.2022.128257.

Li, Juan, Jiafan Li, Yuning Ma, Bo Chen, Xirui Wang, Xianting Jiao, Yihui Jin, Zhemin Shen, Tao Yuan, and Xiaodan Yu. 2021. “Urine Concentrations of Perfluoroalkyl Acids in Children and Contributions of Dietary Factors: A Cross-Sectional Study from Shanghai, China.” Environmental Science and Pollution Research International 28 (16): 20440–50. https://doi.org/10.1007/s11356-020-12293-8.

Li, Na, Guang-Guo Ying, Huachang Hong, and Wen-Jing Deng. 2021. “Perfluoroalkyl Substances in the Urine and Hair of Preschool Children, Airborne Particles in Kindergartens, and Drinking Water in Hong Kong.” Environmental Pollution (Barking, Essex: 1987) 270 (February): 116219. https://doi.org/10.1016/j.envpol.2020.116219.

Li, Yijing, Jennifer Bräunig, Phong K. Thai, Mario Rebosura, Jochen F. Mueller, and Zhiguo Yuan. 2022. “Formation and Fate of Perfluoroalkyl Acids (PFAAs) in a Laboratory-Scale Urban Wastewater System.” Water Research 216 (June): 118295. https://doi.org/10.1016/j.watres.2022.118295.

Lin, Huiju, Sachi Taniyasu, Nobuyoshi Yamashita, Muhammad Kamran Khan, Saiyada Shadiah Masood, Sumayya Saied, and Haider Abbas Khwaja. 2022. “Per- and Polyfluoroalkyl Substances in the Atmospheric Total Suspended Particles in Karachi, Pakistan: Profiles, Potential Sources, and Daily Intake Estimates.” Chemosphere 288 (Pt 2): 132432. https://doi.org/10.1016/j.chemosphere.2021.132432.

Liu, Baolin, Hong Zhang, Yong Yu, Liuwei Xie, Juying Li, Xinxuan Wang, and Weihua Dong. 2020. “Perfluorinated Compounds (PFCs) in Soil of the Pearl River Delta, China: Spatial Distribution, Sources, and Ecological Risk Assessment.” Archives of Environmental Contamination and Toxicology 78 (2): 182–89. https://doi.org/10.1007/s00244-019-00674-1.

Liu, Jiao-Jiao, Xin-Xin Cui, Ya-Wen Tan, Peng-Xin Dong, Yan-Qiu Ou, Qing-Qing Li, Chu Chu, et al. 2022. “Per- and Perfluoroalkyl Substances Alternatives, Mixtures and Liver Function in Adults: A Community-Based Population Study in China.” Environment International 163 (May): 107179. https://doi.org/10.1016/j.envint.2022.107179.

Lu, Yintao, Jianfa Gao, Hue T. Nguyen, Soumini Vijayasarathy, Peng Du, Xiqing Li, Hong Yao, Jochen F. Mueller, and Phong K. Thai. 2021. “Occurrence of Per- and Polyfluoroalkyl Substances (PFASs) in Wastewater of Major Cities across China in 2014 and 2016.” Chemosphere 279 (September): 130590. https://doi.org/10.1016/j.chemosphere.2021.130590.

Lv, Ming, Yangyang Xie, Hui Yu, Tao Sun, Liping Song, and Fenghua Wang. 2022. “Effects of Perfluoroalkyl Substances on Soil Respiration and Enzymatic Activity: Differences in Carbon Chain-Length Dependence.” Journal of Environmental Science and Health. Part. B, Pesticides, Food Contaminants, and Agricultural Wastes, March, 1–13. https://doi.org/10.1080/03601234.2022.2047563.

Makowska, Krystyna, Julia Martín, Andrzej Rychlik, Irene Aparicio, Juan Luis Santos, Esteban Alonso, and Slawomir Gonkowski. 2021. “Assessment of Exposure to Perfluoroalkyl Substances (PFASs) in Dogs by Fur Analysis.” Environmental Pollution (Barking, Essex: 1987) 286 (October): 117435. https://doi.org/10.1016/j.envpol.2021.117435.

Méndez, Valentina, Sophie Holland, Shefali Bhardwaj, James McDonald, Stuart Khan, Denis O’Carroll, Russell Pickford, et al. 2022. “Aerobic Biotransformation of 6:2 Fluorotelomer Sulfonate by Dietzia Aurantiaca J3 under Sulfur-Limiting Conditions.” The Science of the Total Environment 829 (March): 154587. https://doi.org/10.1016/j.scitotenv.2022.154587.

Miner, K. R., H. Clifford, T. Taruscio, M. Potocki, G. Solomon, M. Ritari, I. E. Napper, A. P. Gajurel, and P. A. Mayewski. 2021. “Deposition of PFAS ‘forever Chemicals’ on Mt. Everest.” The Science of the Total Environment 759 (March): 144421. https://doi.org/10.1016/j.scitotenv.2020.144421.

Palma, Davide, Dimitra Papagiannaki, Manuel Lai, Rita Binetti, Mohamad Sleiman, Marco Minella, and Claire Richard. 2021. “PFAS Degradation in Ultrapure and Groundwater Using Non-Thermal Plasma.” Molecules (Basel, Switzerland) 26 (4): 924. https://doi.org/10.3390/molecules26040924.

Rice, Penelope A., Jason Aungst, Jessica Cooper, Omari Bandele, and Shruti V. Kabadi. 2020a. “Comparative Analysis of the Toxicological Databases for 6:2 Fluorotelomer Alcohol (6:2 FTOH) and Perfluorohexanoic Acid (PFHxA).” Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association 138 (April): 111210. https://doi.org/10.1016/j.fct.2020.111210.

Rodríguez-Varela, Mario, Juan C. Durán-Álvarez, Blanca Jiménez-Cisneros, Olivia Zamora, and Blanca Prado. 2021. "Occurrence of Perfluorinated Carboxylic Acids in Mexico City's Wastewater: A Monitoring Study in the Sewerage and a Mega Wastewater Treatment Plant." The Science of the Total Environment 774 (June): 145060. https://doi.org/10.1016/j.scitotenv.2021.145060

Si, Yuanyuan, Jiankun Huang, Zhihong Liang, Guoqiang Liu, Da Chen, Ying Guo, and Fei Wang. 2021. "Occurrence and Ecological Risk Assessment of Perfluoroalkyl Substances (PFASs) in Water and Sediment from an Urban River in South China." Archives of Environmental Contamination and Toxicology 81 (1): 133–41. https://doi.org/10.1007/s00244-021-00855-x.

Sim, Wonjin, Sol Choi, Gyojin Choo, Mihee Yang, Ju-Hyun Park, and Jeong-Eun Oh. 2021. "Organophosphate Flame Retardants and Perfluoroalkyl Substances in Drinking Water Treatment Plants from Korea: Occurrence and Human Exposure." International Journal of Environmental Research and Public Health 18 (5): 2645. https://doi.org/10.3390/ijerph18052645.

Skaggs, Christopher S., and Brian A. Logue. 2021. "Ultratrace Analysis of Per- and Polyfluoroalkyl Substances in Drinking Water Using Ice Concentration Linked with Extractive Stirrer and High Performance Liquid Chromatography - Tandem Mass Spectrometry." Journal of Chromatography. A 1659 (December): 462493. https://doi.org/10.1016/j.chroma.2021.462493.

Sörengård, Mattias, Pablo Gago-Ferrero, Dan B Kleja, and Lutz Ahrens. 2021. "Laboratory-Scale and Pilot-Scale Stabilization and Solidification (S/S) Remediation of Soil Contaminated with per- and Polyfluoroalkyl Substances (PFASs)." Journal of Hazardous Materials 402 (January): 123453. https://doi.org/10.1016/j.jhazmat.2020.123453.

Sun, Mei, Jia'nan Cui, Junyu Guo, Zihan Zhai, Peng Zuo, and Jianbo Zhang. 2020. "Fluorochemicals Biodegradation as a Potential Source of Trifluoroacetic Acid (TFA) to the Environment." Chemosphere 254 (September): 126894. https://doi.org/10.1016/j.chemosphere.2020.126894.

Tang, Jiaxi, Yongle Zhu, Biao Xiang, Yu Li, Ting Tan, Ying Xu, and Mengxue Li. 2022. "Multiple Pollutants in Groundwater near an Abandoned Chinese Fluorine Chemical Park: Concentrations, Correlations and Health Risk Assessments." Scientific Reports 12 (1): 3370. https://doi.org/10.1038/s41598-022-07201-8.

Timshina, Alina, Juan J. Aristizabal-Henao, Bianca F. Da Silva, and John A. Bowden. 2021. "The Last Straw: Characterization of per- and Polyfluoroalkyl Substances in Commercially-Available Plant-Based Drinking Straws." Chemosphere 277 (August): 130238. https://doi.org/10.1016/j.chemosphere.2021.130238.

Wang, Siquan, Yizhi Cai, Liya Ma, Xiaoping Lin, Qin Li, Yongyu Li, and Xinhong Wang. 2022. "Perfluoroalkyl Substances in Water, Sediment, and Fish from a Subtropical River of China: Environmental Behaviors and Potential Risk." Chemosphere 288 (Pt 1): 132513. https://doi.org/10.1016/j.chemosphere.2021.132513.

Wasel, Ola, Kathryn M. Thompson, Yu Gao, Amy E. Godfrey, Jiejun Gao, Cecon T. Mahapatra, Linda S. Lee, Maria S. Sepúlveda, and Jennifer L. Freeman. 2021. "Comparison of Zebrafish in Vitro and in Vivo Developmental Toxicity Assessments of Perfluoroalkyl Acids (PFAAs)." Journal of Toxicology and Environmental Health. Part A 84 (3): 125–36. https://doi.org/10.1080/15287394.2020.1842272.

Wu, Jian-Yi, Li Gu, Zu-Lin Hua, Zhong-Yan Liang, Ke-Jian Chu, and Xin-Xin He. 2021. "Per-, Poly-Fluoroalkyl Substances (PFASs) Pollution in Benthic Riverine Ecosystem: Integrating Microbial Community Coalescence and Biogeochemistry with Sediment Distribution." Chemosphere 281 (October): 130977. https://doi.org/10.1016/j.chemosphere.2021.130977.

Wu, Jian-Yi, Zu-Lin Hua, and Li Gu. 2021. "Planktonic Microbial Responses to Perfluorinated Compound (PFC) Pollution: Integrating PFC Distributions with Community Coalescence and Metabolism." The Science of the Total Environment 788 (September): 147743. https://doi.org/10.1016/j.scitotenv.2021.147743.

Xiao, Shao-Ke, Qi Wu, Chang-Gui Pan, Chao Yin, Ying-Hui Wang, and Ke-Fu Yu. 2021. "Distribution, Partitioning Behavior and Potential Source of Legacy and Alternative per- and Polyfluoroalkyl Substances (PFASs) in Water and Sediments from a Subtropical Gulf, South China Sea." Environmental Research 201 (October): 111485. https://doi.org/10.1016/j.envres.2021.111485.

Yao, Xinyun, Ning-Ning Song, Jia Wang, Xian Zhao, Meng-Yuan Cheng, Jiaqi Zuo, and Kaipei Qiu. 2021. "Influence of Electrolyte Concentration on Single-Molecule Sensing of Perfluorocarboxylic Acids." Frontiers in Chemistry 9: 732378. https://doi.org/10.3389/fchem.2021.732378.

Yong, Zhi Yuan, Ki Yong Kim, and Jeong-Eun Oh. 2021. "The Occurrence and Distributions of Per- and Polyfluoroalkyl Substances (PFAS) in Groundwater after a PFAS Leakage Incident in 2018." Environmental Pollution (Barking, Essex: 1987) 268 (Pt B): 115395. https://doi.org/10.1016/j.envpol.2020.115395.

Zhan, Jiaxun, Ai Zhang, Paul Héroux, Ying Guo, Zhuyu Sun, Zhenyu Li, Jingyi Zhao, and Yanan Liu. 2020. "Remediation of Perfluorooctanoic Acid (PFOA) Polluted Soil Using Pulsed Corona Discharge Plasma." Journal of Hazardous Materials 387 (April): 121688.https://doi.org/10.1016/j.jhazmat.2019.121688.

Zhang, Shengnan, Xiaochun Guo, Shaoyong Lu, Jia He, Qin Wu, Xiaohui Liu, Zhenyang Han, and Ping Xie. 2022. "Perfluorohexanoic Acid Caused Disruption of the Hypothalamus-Pituitary-Thyroid Axis in Zebrafish Larvae." Ecotoxicology and Environmental Safety 232 (March): 113283. https://doi.org/10.1016/j.ecoenv.2022.113283.

Zhang, Weilan, and Yanna Liang. 2020. "Removal of Eight Perfluoroalkyl Acids from Aqueous Solutions by Aeration and Duckweed." The Science of the Total Environment 724 (July): 138357. https://doi.org/10.1016/j.scitotenv.2020.138357.

Zhang, Weilan, Quan Zhang, and Yanna Liang. 2022. "Ineffectiveness of Ultrasound at Low Frequency for Treating Per- and Polyfluoroalkyl Substances in Sewage Sludge."

Chemosphere 286 (Pt 2): 131748. https://doi.org/10.1016/j.chemosphere.2021.131748.

Zhang, Yueqing, Yunqiao Zhou, Aiguo Zhang, Juying Li, Jia Yu, Yezhi Dou, Jian He, and Deyang Kong. 2021. "Perfluoroalkyl Substances in Drinking Water Sources along the Yangtze River in Jiangsu Province, China: Human Health and Ecological Risk Assessment." Ecotoxicology and Environmental Safety 218 (April): 112289. https://doi.org/10.1016/j.ecoenv.2021.112289.

Zhao, Shuyan, Tianqi Liu, Lingyan Zhu, Liping Yang, Yvlu Zong, Huanting Zhao, Longhui Hu, and Jingjing Zhan. 2021. "Formation of Perfluorocarboxylic Acids (PFCAs) during the Exposure of Earthworms to 6:2 Fluorotelomer Sulfonic Acid (6:2 FTSA)." The Science of the Total Environment 760 (March): 143356. https://doi.org/10.1016/j.scitotenv.2020.143356.

PubMed Search: "(Sodium perfluorohexanoate)" (2020-present)

(Note: asterisked references are duplicates of the "(PFHxa) AND (perfluorohexanoic acid)" PubMed search)

Ahmadireskety, Atiye, Bianca Ferreira Da Silva, Timothy G. Townsend, Richard A. Yost, Helena M. Solo-Gabriele, and John A. Bowden. 2021. "Evaluation of Extraction Workflows for Quantitative Analysis of Per- and Polyfluoroalkyl Substances: A Case Study Using Soil Adjacent to a Landfill." The Science of the Total Environment 760 (March): 143944. https://doi.org/10.1016/j.scitotenv.2020.143944.

Anderson, Janet K., Anthony L. Luz, and Philip E. Goodrum. 2020. "Letter to the Editor Regarding Rice et al. (2020)." Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association 145 (November): 111756. https://doi.org/10.1016/j.fct.2020.111756.

*Appel, Mareike, Martin Forsthuber, Romualdo Ramos, Raimund Widhalm, Sebastian Granitzer, Maria Uhl, Markus Hengstschläger, Tanja Stamm, and Claudia Gundacker. 2022. "The Transplacental Transfer Efficiency of Per- and Polyfluoroalkyl Substances (PFAS): A First Meta-Analysis." Journal of Toxicology and Environmental Health. Part B, Critical Reviews 25 (1): 23–42. https://doi.org/10.1080/10937404.2021.2009946.

*Arinaitwe, Kenneth, Nils Keltsch, Anthony Taabu-Munyaho, Thorsten Reemtsma, and Urs Berger. 2021. "Perfluoroalkyl Substances (PFASs) in the Ugandan Waters of Lake Victoria: Spatial Distribution, Catchment Release and Public Exposure Risk via Municipal Water Consumption." The Science of the Total Environment 783 (August): 146970. https://doi.org/10.1016/j.scitotenv.2021.146970.

*Bai, Xuelian, and Yeongkwon Son. 2021. "Perfluoroalkyl Substances (PFAS) in Surface Water and Sediments from Two Urban Watersheds in Nevada, USA." The Science of the Total Environment 751 (January): 141622. https://doi.org/10.1016/j.scitotenv.2020.141622.

Bertanza, Giorgio, Giacomo Umberto Capoferri, Massimo Carmagnani, Francesco Icarelli, Sabrina Sorlini, and Roberta Pedrazzani. 2020. "Long-Term Investigation on the Removal of Perfluoroalkyl Substances in a Full-Scale Drinking Water Treatment Plant

in the Veneto Region, Italy." The Science of the Total Environment 734 (September): 139154. https://doi.org/10.1016/j.scitotenv.2020.139154.

Bil, Wieneke, Marco Zeilmaker, Styliani Fragki, Johannes Lijzen, Eric Verbruggen, and Bas Bokkers. 2021. "Risk Assessment of Per- and Polyfluoroalkyl Substance Mixtures: A Relative Potency Factor Approach." Environmental Toxicology and Chemistry 40 (3): 859–70. https://doi.org/10.1002/etc.4835.

*Caron-Beaudoin, Élyse, Pierre Ayotte, Caty Blanchette, Gina Muckle, Ellen Avard, Sylvie Ricard, and Mélanie Lemire. 2020. "Perfluoroalkyl Acids in Pregnant Women from Nunavik (Quebec, Canada): Trends in Exposure and Associations with Country Foods Consumption." Environment International 145 (December): 106169. https://doi.org/10.1016/j.envint.2020.106169.

*Chen, Dongmei, Ying Zhao, Wei Xu, Yuanhu Pan, Qu Wei, and Shuyu Xie. 2020. "Biotransformation and Tissue Bioaccumulation of 8:2 Fluorotelomer Alcohol in Broiler by Oral Exposure." Environmental Pollution (Barking, Essex: 1987) 267 (December): 115611. https://doi.org/10.1016/j.envpol.2020.115611.

*Chen, Huilun, Qianyu Wang, Yanping Cai, Rongfang Yuan, Fei Wang, and Beihai Zhou. 2020. "Investigation of the Interaction Mechanism of Perfluoroalkyl Carboxylic Acids with Human Serum Albumin by Spectroscopic Methods." International Journal of Environmental Research and Public Health 17 (4): E1319. https://doi.org/10.3390/ijerph17041319.

Chen, Ying, Lei Zhang, Fangxuan Li, Jindong Sheng, Changxiao Xu, Dan Li, Hu Yu, and Wenxin Liu. 2021. "Combination of Chemotherapy and Photodynamic Therapy with Oxygen Self-Supply in the Form of Mutual Assistance for Cancer Therapy." International Journal of Nanomedicine 16: 3679–94. https://doi.org/10.2147/IJN.S298146.

*Claessens, Julien, Catherine Pirard, and Corinne Charlier. 2022. "Determination of Contamination Levels for Multiple Endocrine Disruptors in Hair from a Non-Occupationally Exposed Population Living in Liege (Belgium)." The Science of the Total Environment 815 (April): 152734. https://doi.org/10.1016/j.scitotenv.2021.152734.

***DeLuca, Nicole M.,** Michelle Angrish, Amina Wilkins, Kris Thayer, and Elaine A. Cohen Hubal. 2021. "Human Exposure Pathways to Poly- and Perfluoroalkyl Substances (PFAS) from Indoor Media: A Systematic Review Protocol." Environment International 146 (January): 106308. https://doi.org/10.1016/j.envint.2020.106308.

*Dennis, Nicole M., Farzana Hossain, Seenivasan Subbiah, Adcharee Karnjanapiboonwong, Michael L. Dennis, Chris McCarthy, Christopher G. Heron, et al. 2021. "Chronic Reproductive Toxicity Thresholds for Northern Bobwhite Quail (Colinus Virginianus) Exposed to Perfluorohexanoic Acid (PFHxA) and a Mixture of Perfluorooctane Sulfonic Acid (PFOS) and PFHxA." Environmental Toxicology and Chemistry 40 (9): 2601–14. https://doi.org/10.1002/etc.5135.

*Dennis, Nicole M., Farzana Hossain, Seenivasan Subbiah, Adcharee Karnjanapiboonwong, Michael L. Dennis, Christopher McCarthy, W. Andrew Jackson,

Jordan P. Crago, Christopher J. Salice, and Todd A. Anderson. 2022. “Species- and Tissue-Specific Chronic Toxicity Values for Northern Bobwhite Quail (Colinus Virginianus) Exposed to Perfluorohexane Sulfonic Acid and a Binary Mixture of Perfluorooctane Sulfonic Acid and Perfluorohexane Sulfonic Acid.” Environmental Toxicology and Chemistry 41 (1): 219–29. https://doi.org/10.1002/etc.5238.

Duan, Yishuang, Hongwen Sun, Yiming Yao, Yue Meng, and Yongcheng Li. 2020. “Distribution of Novel and Legacy Per-/Polyfluoroalkyl Substances in Serum and Its Associations with Two Glycemic Biomarkers among Chinese Adult Men and Women with Normal Blood Glucose Levels.” Environment International 134 (January): 105295. https://doi.org/10.1016/j.envint.2019.105295.

*__Dzierlenga, Anika L__., Veronica G. Robinson, Suramya Waidyanatha, Michael J. DeVito, Max A. Eifrid, Seth T. Gibbs, Courtney A. Granville, and Chad R. Blystone. 2020. “Toxicokinetics of Perfluorohexanoic Acid (PFHxA), Perfluorooctanoic Acid (PFOA) and Perfluorodecanoic Acid (PFDA) in Male and Female Hsd:Sprague Dawley SD Rats Following Intravenous or Gavage Administration.” Xenobiotica; the Fate of Foreign Compounds in Biological Systems 50 (6): 722–32. https://doi.org/10.1080/00498254.2019.1683776.

*East, Andrew, Richard H. Anderson, and Christopher J. Salice. 2021. “Per- and Polyfluoroalkyl Substances (PFAS) in Surface Water Near US Air Force Bases: Prioritizing Individual Chemicals and Mixtures for Toxicity Testing and Risk Assessment.” Environmental Toxicology and Chemistry 40 (3): 859–70. https://doi.org/10.1002/etc.4893.

*Fulong, Cressa Ria P., Mary Grace E. Guardian, Diana S. Aga, and Timothy R. Cook. 2020. “A Self-Assembled Iron(II) Metallacage as a Trap for Per- and Polyfluoroalkyl Substances in Water.” Inorganic Chemistry 59 (10): 6697–6708. https://doi.org/10.1021/acs.inorgchem.9b03405.

*__Gaballah, Shaza,__ Adam Swank, Jon R. Sobus, Xia Meng Howey, Judith Schmid, Tara Catron, James McCord, Erin Hines, Mark Strynar, and Tamara Tal. 2020. “Evaluation of Developmental Toxicity, Developmental Neurotoxicity, and Tissue Dose in Zebrafish Exposed to GenX and Other PFAS.” Environmental Health Perspectives 128 (4): 47005. https://doi.org/10.1289/EHP5843.

*Gao, Chang, Zu-Lin Hua, and Xiao-Qing Li. 2022. “Distribution, Sources, and Dietetic-Related Health Risk Assessment of Perfluoroalkyl Acids (PFAAs) in the Agricultural Environment of an Industrial-Agricultural Interaction Region (IAIR), Changshu, East China.” The Science of the Total Environment 809 (February): 152159. https://doi.org/10.1016/j.scitotenv.2021.152159.

*Garcia-Barrios, Joshua, Mallory Drysdale, Mylène Ratelle, Éric Gaudreau, Alain LeBlanc, Mary Gamberg, and Brian D. Laird. 2021. “Biomarkers of Poly- and Perfluoroalkyl Substances (PFAS) in Sub-Arctic and Arctic Communities in Canada.” International Journal of Hygiene and Environmental Health 235 (June): 113754. https://doi.org/10.1016/j.ijheh.2021.113754.

*__Guo, Xiaochun__, Shengnan Zhang, Xiaohui Liu, Shaoyong Lu, Qin Wu, and Ping Xie. 2021. “Evaluation of the Acute Toxicity and Neurodevelopmental Inhibition of

Perfluorohexanoic Acid (PFHxA) in Zebrafish Embryos." Ecotoxicology and Environmental Safety 225 (December): 112733. https://doi.org/10.1016/j.ecoenv.2021.112733.

*Hamid, Hanna, Loretta Y. Li, and John R. Grace. 2021. "Exploring Indirect Photolysis of 6:2 Fluorotelomer Sulfonate in Landfill Leachate under Simulated Sunlight: Effect of Humic Acid and Nitrate." Environmental Science and Pollution Research International 28 (8): 9508–16. https://doi.org/10.1007/s11356-020-11417-4.

***Han, Ji-Seok**, Sumi Jang, Hwa-Young Son, Yong-Bum Kim, Younhee Kim, Jung-Ho Noh, Mi-Jeong Kim, and Byoung-Seok Lee. 2020. "Subacute Dermal Toxicity of Perfluoroalkyl Carboxylic Acids: Comparison with Different Carbon-Chain Lengths in Human Skin Equivalents and Systemic Effects of Perfluoroheptanoic Acid in Sprague Dawley Rats." Archives of Toxicology 94 (2): 523–39. https://doi.org/10.1007/s00204-019-02634-z

*Helmer, Ross W., Donald M. Reeves, and Daniel P. Cassidy. 2022. "Per- and Polyfluorinated Alkyl Substances (PFAS) Cycling within Michigan: Contaminated Sites, Landfills and Wastewater Treatment Plants." Water Research 210 (February): 117983. https://doi.org/10.1016/j.watres.2021.117983.

*Holmquist, Hanna, Peter Fantke, Ian T. Cousins, Mikołaj Owsianiak, Ioannis Liagkouridis, and Gregory M. Peters. 2020. "An (Eco)Toxicity Life Cycle Impact Assessment Framework for Per- And Polyfluoroalkyl Substances." Environmental Science & Technology 54 (10): 6224–34. https://doi.org/10.1021/acs.est.9b07774.

*Hua, Zulin, Ying Lu, Kejian Chu, Yuanyuan Liu, Yixin Ma, Li Gu, Jianyi Wu, Wachirasak Leelawattananun, and Sereyvatanak Ky. 2022. "Shift in the Distribution and Fate of Perfluoroalkyl Acids by Sluice Gates in the Multi-Environment Media of Rivers." Journal of Environmental Management 305 (March): 114436. https://doi.org/10.1016/j.jenvman.2022.114436.

***Jiang, Lilong**, Yanjun Hong, Guangshan Xie, Jinghui Zhang, Hongna Zhang, and Zongwei Cai. 2021. "Comprehensive Multi-Omics Approaches Reveal the Hepatotoxic Mechanism of Perfluorohexanoic Acid (PFHxA) in Mice." The Science of the Total Environment 790 (October): 148160. https://doi.org/10.1016/j.scitotenv.2021.148160.

*Kibambe, M. G., M. N. B. Momba, A. P. Daso, and M. a. A. Coetzee. 2020. "Evaluation of the Efficiency of Selected Wastewater Treatment Processes in Removing Selected Perfluoroalkyl Substances (PFASs)." Journal of Environmental Management 255 (February): 109945. https://doi.org/10.1016/j.jenvman.2019.109945.

*Lasters, Robin, Thimo Groffen, Lieven Bervoets, and Marcel Eens. 2021. "Perfluoroalkyl Acid (PFAA) Profile and Concentrations in Two Co-Occurring Tit Species: Distinct Differences Indicate Non-Generalizable Results across Passerines." The Science of the Total Environment 761 (March): 143301. https://doi.org/10.1016/j.scitotenv.2020.143301.

*Lenka, Swadhina Priyadarshini, Melanie Kah, and Lokesh P. Padhye. 2022. "Occurrence and Fate of Poly- and Perfluoroalkyl Substances (PFAS) in Urban Waters of New

Zealand." Journal of Hazardous Materials 428 (April): 128257. https://doi.org/10.1016/j.jhazmat.2022.128257.

***Li, Juan**, Jiafan Li, Yuning Ma, Bo Chen, Xirui Wang, Xianting Jiao, Yihui Jin, Zhemin Shen, Tao Yuan, and Xiaodan Yu. 2021. "Urine Concentrations of Perfluoroalkyl Acids in Children and Contributions of Dietary Factors: A Cross-Sectional Study from Shanghai, China." Environmental Science and Pollution Research International 28 (16): 20440–50. https://doi.org/10.1007/s11356-020-12293-8.

*Li, Na, Guang-Guo Ying, Huachang Hong, and Wen-Jing Deng. 2021. "Perfluoroalkyl Substances in the Urine and Hair of Preschool Children, Airborne Particles in Kindergartens, and Drinking Water in Hong Kong." Environmental Pollution (Barking, Essex: 1987) 270 (February): 116219. https://doi.org/10.1016/j.envpol.2020.116219.

Li, Xiao-Qing, Zu-Lin Hua, Jian-Yun Zhang, and Li Gu. 2022. "Interactions between Dissolved Organic Matter and Perfluoroalkyl Acids in Natural Rivers and Lakes: A Case Study of the Northwest of Taihu Lake Basin, China." Water Research 216 (June): 118324. https://doi.org/10.1016/j.watres.2022.118324.

*Li, Yijing, Jennifer Bräunig, Phong K. Thai, Mario Rebosura, Jochen F. Mueller, and Zhiguo Yuan. 2022. "Formation and Fate of Perfluoroalkyl Acids (PFAAs) in a Laboratory-Scale Urban Wastewater System." Water Research 216 (June): 118295. https://doi.org/10.1016/j.watres.2022.118295.

*Lin, Huiju, Sachi Taniyasu, Nobuyoshi Yamashita, Muhammad Kamran Khan, Saiyada Shadiah Masood, Sumayya Saied, and Haider Abbas Khwaja. 2022. "Per- and Polyfluoroalkyl Substances in the Atmospheric Total Suspended Particles in Karachi, Pakistan: Profiles, Potential Sources, and Daily Intake Estimates." Chemosphere 288 (Pt 2): 132432. https://doi.org/10.1016/j.chemosphere.2021.132432.

*Liu, Baolin, Hong Zhang, Yong Yu, Liuwei Xie, Juying Li, Xinxuan Wang, and Weihua Dong. 2020. "Perfluorinated Compounds (PFCs) in Soil of the Pearl River Delta, China: Spatial Distribution, Sources, and Ecological Risk Assessment." Archives of Environmental Contamination and Toxicology 78 (2): 182–89. https://doi.org/10.1007/s00244-019-00674-1.

*Liu, Jiao-Jiao, Xin-Xin Cui, Ya-Wen Tan, Peng-Xin Dong, Yan-Qiu Ou, Qing-Qing Li, Chu Chu, et al. 2022. "Per- and Perfluoroalkyl Substances Alternatives, Mixtures and Liver Function in Adults: A Community-Based Population Study in China." Environment International 163 (May): 107179. https://doi.org/10.1016/j.envint.2022.107179.

Liu, Shuyu, Renjun Yang, Nuoya Yin, and Francesco Faiola. 2020. "Effects of Per- and Poly-Fluorinated Alkyl Substances on Pancreatic and Endocrine Differentiation of Human Pluripotent Stem Cells." Chemosphere 254 (September): 126709. https://doi.org/10.1016/j.chemosphere.2020.126709.

Liu, YingYing, Carol J. Ptacek, Rachel J. Baldwin, Janice M. Cooper, and David W. Blowes. 2020. "Application of Zero-Valent Iron Coupled with Biochar for Removal of Perfluoroalkyl Carboxylic and Sulfonic Acids from Water under Ambient

Environmental Conditions." The Science of the Total Environment 719 (June): 137372. https://doi.org/10.1016/j.scitotenv.2020.137372.

*Lu, Yintao, Jianfa Gao, Hue T. Nguyen, Soumini Vijayasarathy, Peng Du, Xiqing Li, Hong Yao, Jochen F. Mueller, and Phong K. Thai. 2021. "Occurrence of Per- and Polyfluoroalkyl Substances (PFASs) in Wastewater of Major Cities across China in 2014 and 2016." Chemosphere 279 (September): 130590. https://doi.org/10.1016/j.chemosphere.2021.130590.

*Lv, Ming, Yangyang Xie, Hui Yu, Tao Sun, Liping Song, and Fenghua Wang. 2022. "Effects of Perfluoroalkyl Substances on Soil Respiration and Enzymatic Activity: Differences in Carbon Chain-Length Dependence." Journal of Environmental Science and Health. Part. B, Pesticides, Food Contaminants, and Agricultural Wastes, March, 1–13. https://doi.org/10.1080/03601234.2022.2047563.

*Makowska, Krystyna, Julia Martín, Andrzej Rychlik, Irene Aparicio, Juan Luis Santos, Esteban Alonso, and Slawomir Gonkowski. 2021. "Assessment of Exposure to Perfluoroalkyl Substances (PFASs) in Dogs by Fur Analysis." Environmental Pollution (Barking, Essex: 1987) 286 (October): 117435. https://doi.org/10.1016/j.envpol.2021.117435.

*Méndez, Valentina, Sophie Holland, Shefali Bhardwaj, James McDonald, Stuart Khan, Denis O'Carroll, Russell Pickford, et al. 2022. "Aerobic Biotransformation of 6:2 Fluorotelomer Sulfonate by Dietzia Aurantiaca J3 under Sulfur-Limiting Conditions." The Science of the Total Environment 829 (March): 154587. https://doi.org/10.1016/j.scitotenv.2022.154587.

*Miner, K. R., H. Clifford, T. Taruscio, M. Potocki, G. Solomon, M. Ritari, I. E. Napper, A. P. Gajurel, and P. A. Mayewski. 2021. "Deposition of PFAS 'forever Chemicals' on Mt. Everest." The Science of the Total Environment 759 (March): 144421. https://doi.org/10.1016/j.scitotenv.2020.144421.

Morales-McDevitt, Maya E., Matthew Dunn, Ahsan Habib, Simon Vojta, Jitka Becanova, and Rainer Lohmann. 2022. "Poly- and Perfluorinated Alkyl Substances in Air and Water from Dhaka, Bangladesh." Environmental Toxicology and Chemistry 41 (2): 334–42. https://doi.org/10.1002/etc.5255.

Ojo, Atinuke F., Qing Xia, Cheng Peng, and Jack C. Ng. 2021. "Evaluation of the Individual and Combined Toxicity of Perfluoroalkyl Substances to Human Liver Cells Using Biomarkers of Oxidative Stress." Chemosphere 281 (October): 130808. https://doi.org/10.1016/j.chemosphere.2021.130808.

*Palma, Davide, Dimitra Papagiannaki, Manuel Lai, Rita Binetti, Mohamad Sleiman, Marco Minella, and Claire Richard. 2021. "PFAS Degradation in Ultrapure and Groundwater Using Non-Thermal Plasma." Molecules (Basel, Switzerland) 26 (4): 924. https://doi.org/10.3390/molecules26040924.

Raine, J. C., S. Su, E. Lin, Z. L. Yang, J. P. Giesy, and P. D. Jones. 2021. "Prefertilization Exposure of Rainbow Trout Eggs to Per- and Polyfluoroalkyl Substances to Simulate

Accumulation During Oogenesis." Environmental Toxicology and Chemistry 40 (11): 3159–65. https://doi.org/10.1002/etc.5200.

*Rice, Penelope A., Jason Aungst, Jessica Cooper, Omari Bandele, and Shruti V. Kabadi. 2020a. "Comparative Analysis of the Toxicological Databases for 6:2 Fluorotelomer Alcohol (6:2 FTOH) and Perfluorohexanoic Acid (PFHxA)." Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association 138 (April): 111210. https://doi.org/10.1016/j.fct.2020.111210.

*Rodríguez-Varela, Mario, Juan C. Durán-Álvarez, Blanca Jiménez-Cisneros, Olivia Zamora, and Blanca Prado. 2021. "Occurrence of Perfluorinated Carboxylic Acids in Mexico City's Wastewater: A Monitoring Study in the Sewerage and a Mega Wastewater Treatment Plant." The Science of the Total Environment 774 (June): 145060. https://doi.org/10.1016/j.scitotenv.2021.145060.

*Si, Yuanyuan, Jiankun Huang, Zhihong Liang, Guoqiang Liu, Da Chen, Ying Guo, and Fei Wang. 2021. "Occurrence and Ecological Risk Assessment of Perfluoroalkyl Substances (PFASs) in Water and Sediment from an Urban River in South China." Archives of Environmental Contamination and Toxicology 81 (1): 133–41. https://doi.org/10.1007/s00244-021-00855-x.

*Sim, Wonjin, Sol Choi, Gyojin Choo, Mihee Yang, Ju-Hyun Park, and Jeong-Eun Oh. 2021. "Organophosphate Flame Retardants and Perfluoroalkyl Substances in Drinking Water Treatment Plants from Korea: Occurrence and Human Exposure." International Journal of Environmental Research and Public Health 18 (5): 2645. https://doi.org/10.3390/ijerph18052645.

*Skaggs, Christopher S., and Brian A. Logue. 2021. "Ultratrace Analysis of Per- and Polyfluoroalkyl Substances in Drinking Water Using Ice Concentration Linked with Extractive Stirrer and High Performance Liquid Chromatography - Tandem Mass Spectrometry." Journal of Chromatography. A 1659 (December): 462493. https://doi.org/10.1016/j.chroma.2021.462493.

Sörengård, Mattias, Pablo Gago-Ferrero, Dan B Kleja, and Lutz Ahrens. 2021. "Laboratory-Scale and Pilot-Scale Stabilization and Solidification (S/S) Remediation of Soil Contaminated with per- and Polyfluoroalkyl Substances (PFASs)." Journal of Hazardous Materials 402 (January): 123453. https://doi.org/10.1016/j.jhazmat.2020.123453.

*Sun, Mei, Jia'nan Cui, Junyu Guo, Zihan Zhai, Peng Zuo, and Jianbo Zhang. 2020. "Fluorochemicals Biodegradation as a Potential Source of Trifluoroacetic Acid (TFA) to the Environment." Chemosphere 254 (September): 126894. https://doi.org/10.1016/j.chemosphere.2020.126894.

*Tang, Jiaxi, Yongle Zhu, Biao Xiang, Yu Li, Ting Tan, Ying Xu, and Mengxue Li. 2022. "Multiple Pollutants in Groundwater near an Abandoned Chinese Fluorine Chemical Park: Concentrations, Correlations and Health Risk Assessments." Scientific Reports 12 (1): 3370. https://doi.org/10.1038/s41598-022-07201-8.

*Timshina, Alina, Juan J. Aristizabal-Henao, Bianca F. Da Silva, and John A. Bowden. 2021. "The Last Straw: Characterization of per- and Polyfluoroalkyl Substances in

Commercially-Available Plant-Based Drinking Straws." Chemosphere 277 (August): 130238. https://doi.org/10.1016/j.chemosphere.2021.130238.

Vela-Soria, Fernando, Laura Serrano-López, Javier García-Villanova, Tomás de Haro, Nicolas Olea, and Carmen Freire. 2020. "HPLC-MS/MS Method for the Determination of Perfluoroalkyl Substances in Breast Milk by Combining Salt-Assisted and Dispersive Liquid-Liquid Microextraction." Analytical and Bioanalytical Chemistry 412 (28): 7913–23. https://doi.org/10.1007/s00216-020-02924-x.

*Wang, Siquan, Yizhi Cai, Liya Ma, Xiaoping Lin, Qin Li, Yongyu Li, and Xinhong Wang. 2022. "Perfluoroalkyl Substances in Water, Sediment, and Fish from a Subtropical River of China: Environmental Behaviors and Potential Risk." Chemosphere 288 (Pt 1): 132513. https://doi.org/10.1016/j.chemosphere.2021.132513.

***Wasel, Ola**, Kathryn M. Thompson, Yu Gao, Amy E. Godfrey, Jiejun Gao, Cecon T. Mahapatra, Linda S. Lee, Maria S. Sepúlveda, and Jennifer L. Freeman. 2021. "Comparison of Zebrafish in Vitro and in Vivo Developmental Toxicity Assessments of Perfluoroalkyl Acids (PFAAs)." Journal of Toxicology and Environmental Health. Part A 84 (3): 125–36. https://doi.org/10.1080/15287394.2020.1842272.

Wilson, Timothy B., Gavin Stevenson, Robert Crough, Jesuina de Araujo, Nilhan Fernando, Arif Anwar, Tyrone Scott, José A. Quinteros, Peter C. Scott, and Michael J. G. Archer. 2021. "Evaluation of Residues in Hen Eggs After Exposure of Laying Hens to Water Containing Per- and Polyfluoroalkyl Substances." Environmental Toxicology and Chemistry 40 (3): 735–43. https://doi.org/10.1002/etc.4723.

*Wu, Jian-Yi, Li Gu, Zu-Lin Hua, Zhong-Yan Liang, Ke-Jian Chu, and Xin-Xin He. 2021. "Per-, Poly-Fluoroalkyl Substances (PFASs) Pollution in Benthic Riverine Ecosystem: Integrating Microbial Community Coalescence and Biogeochemistry with Sediment Distribution." Chemosphere 281 (October): 130977. https://doi.org/10.1016/j.chemosphere.2021.130977.

*Wu, Jian-Yi, Zu-Lin Hua, and Li Gu. 2021. "Planktonic Microbial Responses to Perfluorinated Compound (PFC) Pollution: Integrating PFC Distributions with Community Coalescence and Metabolism." The Science of the Total Environment 788 (September): 147743. https://doi.org/10.1016/j.scitotenv.2021.147743.

*Xiao, Shao-Ke, Qi Wu, Chang-Gui Pan, Chao Yin, Ying-Hui Wang, and Ke-Fu Yu. 2021. "Distribution, Partitioning Behavior and Potential Source of Legacy and Alternative per- and Polyfluoroalkyl Substances (PFASs) in Water and Sediments from a Subtropical Gulf, South China Sea." Environmental Research 201 (October): 111485. https://doi.org/10.1016/j.envres.2021.111485.

*Yao, Xinyun, Ning-Ning Song, Jia Wang, Xian Zhao, Meng-Yuan Cheng, Jiaqi Zuo, and Kaipei Qiu. 2021. "Influence of Electrolyte Concentration on Single-Molecule Sensing of Perfluorocarboxylic Acids." Frontiers in Chemistry 9: 732378. https://doi.org/10.3389/fchem.2021.732378.

*Yong, Zhi Yuan, Ki Yong Kim, and Jeong-Eun Oh. 2021. "The Occurrence and Distributions of Per- and Polyfluoroalkyl Substances (PFAS) in Groundwater after a

PFAS Leakage Incident in 2018." Environmental Pollution (Barking, Essex: 1987) 268 (Pt B): 115395. https://doi.org/10.1016/j.envpol.2020.115395.

Yu, Liang, Xiaodong Liu, Zulin Hua, Yuan Zhang, and Hongqin Xue. 2022. "Spatial and Temporal Trends of Perfluoroalkyl Acids in Water Bodies: A Case Study in Taihu Lake, China (2009-2021)." Environmental Pollution (Barking, Essex: 1987) 293 (January): 118575. https://doi.org/10.1016/j.envpol.2021.118575.

*Zhan, Jiaxun, Ai Zhang, Paul Héroux, Ying Guo, Zhuyu Sun, Zhenyu Li, Jingyi Zhao, and Yanan Liu. 2020. "Remediation of Perfluorooctanoic Acid (PFOA) Polluted Soil Using Pulsed Corona Discharge Plasma." Journal of Hazardous Materials 387 (April): 121688. https://doi.org/10.1016/j.jhazmat.2019.121688.

***Zhang, Shengnan,** Xiaochun Guo, Shaoyong Lu, Jia He, Qin Wu, Xiaohui Liu, Zhenyang Han, and Ping Xie. 2022. "Perfluorohexanoic Acid Caused Disruption of the Hypothalamus-Pituitary-Thyroid Axis in Zebrafish Larvae." Ecotoxicology and Environmental Safety 232 (March): 113283. https://doi.org/10.1016/j.ecoenv.2022.113283.

*Zhang, Weilan, and Yanna Liang. 2020. "Removal of Eight Perfluoroalkyl Acids from Aqueous Solutions by Aeration and Duckweed." The Science of the Total Environment 724 (July): 138357. https://doi.org/10.1016/j.scitotenv.2020.138357.

*Zhang, Weilan, Quan Zhang, and Yanna Liang. 2022. "Ineffectiveness of Ultrasound at Low Frequency for Treating Per- and Polyfluoroalkyl Substances in Sewage Sludge." Chemosphere 286 (Pt 2): 131748. https://doi.org/10.1016/j.chemosphere.2021.131748.

Zhang, Yanyan, Jinxia Liu, Audrey Moores, and Subhasis Ghoshal. 2020. "Transformation of 6:2 Fluorotelomer Sulfonate by Cobalt(II)-Activated Peroxymonosulfate." Environmental Science & Technology 54 (7): 4631–40. https://doi.org/10.1021/acs.est.9b07113.

*Zhang, Yueqing, Yunqiao Zhou, Aiguo Zhang, Juying Li, Jia Yu, Yezhi Dou, Jian He, and Deyang Kong. 2021. "Perfluoroalkyl Substances in Drinking Water Sources along the Yangtze River in Jiangsu Province, China: Human Health and Ecological Risk Assessment." Ecotoxicology and Environmental Safety 218 (April): 112289. https://doi.org/10.1016/j.ecoenv.2021.112289.

*Zhao, Shuyan, Tianqi Liu, Lingyan Zhu, Liping Yang, Yvlu Zong, Huanting Zhao, Longhui Hu, and Jingjing Zhan. 2021. "Formation of Perfluorocarboxylic Acids (PFCAs) during the Exposure of Earthworms to 6:2 Fluorotelomer Sulfonic Acid (6:2 FTSA)." The Science of the Total Environment 760 (March): 143356. https://doi.org/10.1016/j.scitotenv.2020.143356.

Zhou, Jiaqi, Karsten Baumann, Naomi Chang, Glenn Morrison, Wanda Bodnar, Zhenfa Zhang, Joanna M. Atkin, Jason D. Surratt, and Barbara J. Turpin. 2022. "Per- and Polyfluoroalkyl Substances (PFASs) in Airborne Particulate Matter (PM2.0) Emitted During Floor Waxing: A Pilot Study." Atmospheric Environment (Oxford, England: 1994) 268 (January): 118845. https://doi.org/10.1016/j.atmosenv.2021.118845.

	Zhu, Yumin, Yibo Jia, Menglin Liu, Liping Yang, Shujun Yi, Xuemin Feng, and Lingyan Zhu. 2021. "Mechanisms for Tissue-Specific Accumulation and Phase I/II Transformation of 6:2 Fluorotelomer Phosphate Diester in Earthworm (M. Guillelmi)." Environment International 151 (June): 106451. https://doi.org/10.1016/j.envint.2021.106451. Zodrow, Jeanmarie M., Meredith Frenchmeyer, Kaitlyn Dally, Erin Osborn, Paul Anderson, and Craig Divine. 2021. "Development of Per and Polyfluoroalkyl Substances Ecological Risk-Based Screening Levels." Environmental Toxicology and Chemistry 40 (3): 921–36. https://doi.org/10.1002/etc.4975.
Georgopoulos	**GENERAL COMMENTS** **Tier 3 Future Consideration**: Future efforts and revisions of the assessment for PFHxA (and other PFAS) must consider cumulative risks It is reasonable to expect that individuals and subpopulations who will experience high PFHxA exposures will also generally have above average (and above median) exposures to other PFAS, including the major legacy PFAS. Furthermore, it is evident that PFHXA shares multiple common Adverse Outcome Pathways with other PFAS. It is therefore very important for EPA to develop (or to continue developing) a consistent, integrative, framework for cumulative risk assessments of PFAS mixtures, that include PFHxA. **Tier 3 Future Consideration:** Future efforts and revisions of the assessment for PFHxA (and other PFAS) must consider reasonable population exposure (and potential exposure) distributions Though, of course, the IRIS Program does not develop the exposure assessment component of risk assessment, it is still essential to have a reasonable understanding and characterization of the potential range of real-world exposures. As assessment of environmental measurements can be considered outside the scope of the IRIS program, the focus should probably be on available biomarker data, starting with NHANES (Calafat et al., 2019) and with on-going CDC biomonitoring studies across the US. The compilation and evaluation of human (PFHxA) biomarker studies should be a priority (ideally in conjunction with the development of a database for data available from these studies). This may require coordination with agencies/organizations worldwide (e.g., European Union, China). For example, it is known that various components of HBM4EU, the human biomonitoring initiative in Europe, includes collection of PFHxA blood data (see, e.g., the EU HBM Dashboard at https://www.hbm4eu.eu/what-we-do/european-hbm-platform/eu-hbm-dashboard/) and, although statistical summaries can be downloaded, the process for accessing the full data sets needs to be clarified. Finally, it should be recognized that the authors of the draft PFHxA Toxicological Review have developed a thorough, readable and balanced document and they deserve our thanks. **REFERENCES** **Note:** The list below only includes references not already cited in the draft US EPA Toxicological Review for PFHxA.

	Tier 3 Future Consideration: The new peer-reviewed studies included in this reference list should be considered by EPA for potential inclusion in the Supplemental material for the PFHxA Toxicological Review. Abraham, K., El-Khatib, A. H., Schwerdtle, T., & Monien, B. H. (2021). Perfluorobutanoic acid (PFBA): No high-level accumulation in human lung and kidney tissue. International Journal of Hygiene and Environmental Health, 237, 113830. Allendorf, F., Berger, U., Goss, K. U., & Ulrich, N. (2019). Partition coefficients of four perfluoroalkyl acid alternatives between bovine serum albumin (BSA) and water in comparison to ten classical perfluoroalkyl acids. Environmental Science: Processes & Impacts, 21(11), 1852-1863 Allendorf, F. (2021). Equilibrium sorption of perfluoroalkyl acids (PFAAs) and four of their alternatives in mammals (Doctoral dissertation, Dissertation, Halle (Saale), Martin-Luther-Universität Halle-Wittenberg, 2021). Amstutz, V. H., Cengo, A., Sijm, D. T. H. M., & Vrolijk, M. F. (2022). The impact of legacy and novel perfluoroalkyl substances on human cytochrome P450: An in vitro study on the inhibitory potential and underlying mechanisms. Toxicology, 468, 153116. Appel, M., Forsthuber, M., Ramos, R., Widhalm, R., Granitzer, S., Uhl, M., ... & Gundacker, C. (2022). The transplacental transfer efficiency of per-and polyfluoroalkyl substances (PFAS): a first meta-analysis. Journal of Toxicology and Environmental Health, Part B, 25(1), 23-42. Babayev, M., Capozzi, S. L., Miller, P., McLaughlin, K. R., Medina, S. S., Byrne, S., ... & Salamova, A. (2022). PFAS in drinking water and serum of the people of a southeast Alaska community: A pilot study. Environmental Pollution, 119246. Bao, J., Shao, L. X., Liu, Y., Cui, S. W., Wang, X., Lu, G. L., ... & Jin, Y. H. Target Analysis and Suspect Screening of Per-and Poly-Fluoroalkyl Substances in Paired Samples of Maternal Serum, Umbilical Cord Serum, and Placenta Near Fluorochemical Plants in Fuxin, China. SSRN http://dx.doi.org/10.2139/ssrn.4091456 Calafat, A. M., Kato, K., Hubbard, K., Jia, T., Botelho, J. C., & Wong, L. Y. (2019). Legacy and alternative per-and polyfluoroalkyl substances in the US general population: paired serum-urine data from the 2013–2014 National Health and Nutrition Examination Survey. Environment international, 131, 105048. Cao, L., Guo, Y., Chen, Y., Hong, J., Wu, J., & Hangbiao, J. (2022). Per-/polyfluoroalkyl substance concentrations in human serum and their associations with liver cancer. Chemosphere, 296, 134083. Chen, X., Feng, X., Sun, X., Li, Y., Yang, Y., Shan, G., & Zhu, L. (2022). Quantifying Indirect Contribution from Precursors to Human Body Burden of Legacy PFASs Based on Paired Blood and One-Week Duplicate Diet. Environmental Science & Technology. Chou, W. C., & Lin, Z. (2020). Probabilistic human health risk assessment of perfluorooctane sulfonate (PFOS) by integrating in vitro, in vivo toxicity, and human epidemiological studies using a Bayesian-based dose-response assessment coupled

with physiologically based pharmacokinetic (PBPK) modeling approach. Environment International, 137, 105581.

Claessens, J., Pirard, C., & Charlier, C. (2022). Determination of contamination levels for multiple endocrine disruptors in hair from a non-occupationally exposed population living in Liege (Belgium). Science of The Total Environment, 815, 152734.

Coperchini, F., De Marco, G., Croce, L., Denegri, M., Greco, A., Magri, F., ... & Chiovato, L. (2022) The Per-and Poly-Fluoroalkyl Substances, Pfoa, Pfhxa and C6o4 Differently Modulate the Expression of the Pro-Tumorigenic Chemokine Cxcl8 in Normal Thyroid Cells and in Thyroid Cancer Cell Lines. SSRN PREPRINT http://dx.doi.org/10.2139/ssrn.4060867

Cousins, I. T., DeWitt, J. C., Glüge, J., Goldenman, G., Herzke, D., Lohmann, R., ... & Wang, Z. (2020). Strategies for grouping per-and polyfluoroalkyl substances (PFAS) to protect human and environmental health. Environmental Science: Processes & Impacts, 22(7), 1444-1460.

DeLuca, N. M., Angrish, M., Wilkins, A., Thayer, K., & Hubal, E. A. C. (2021). Human exposure pathways to poly-and perfluoroalkyl substances (PFAS) from indoor media: A systematic review protocol. Environment international, 146, 106308.

Duan, Y., Sun, H., Yao, Y., Li, Y., Meng, Y., Lu, Y., ... & Chen, L. (2021). Serum concentrations of per-/polyfluoroalkyl substances and risk of type 2 diabetes: A case-control study. Science of The Total Environment, 787, 147476.

ECHA (European Chemicals Agency). (2019). Annex XV Restriction Report. Proposal for a Restriction. Substance Names: Undecafluorohexanoic acid (PFHxA), its salts and related substances. BAuA Federal Institute for Occupational Safety and Health Division 5 - Federal Office for Chemicals Friedrich-Henkel-Weg 1-25. D-44149 Dortmund, Germany.

EFSA Panel on Contaminants in the Food Chain (EFSA CONTAM Panel), Schrenk, D., Bignami, M., Bodin, L., Chipman, J. K., del Mazo, J., ... & Schwerdtle, T. (2020). Risk to human health related to the presence of perfluoroalkyl substances in food. EFSA Journal, 18(9), e06223.

Fiedler, H., Sadia, M., Krauss, T., Baabish, A., & Yeung, L. W. (2022). Perfluoroalkane acids in human milk under the global monitoring plan of the Stockholm Convention on Persistent Organic Pollutants (2008–2019). Frontiers of Environmental Science & Engineering, 16(10), 1-14.

Ishibashi, H., Hirano, M., Kim, E. Y., & Iwata, H. (2019). In vitro and in silico evaluations of binding affinities of perfluoroalkyl substances to Baikal seal and human peroxisome proliferator-activated receptor α. Environmental Science & Technology, 53(4), 2181-2188.

Jia, Y., Zhu, Y., Xu, D., Feng, X., Yu, X., Shan, G., & Zhu, L. (2022). Insights into the Competitive Mechanisms of Per-and Polyfluoroalkyl Substances Partition in Liver and Blood. Environmental Science & Technology.

Jiang, L., Hong, Y., Xie, G., Zhang, J., Zhang, H., & Cai, Z. (2021). Comprehensive multi-omics approaches reveal the hepatotoxic mechanism of perfluorohexanoic acid (PFHxA) in mice. Science of The Total Environment, 790, 148160.

Jin, H., Mao, L., Xie, J., Zhao, M., Bai, X., Wen, J., ... & Wu, P. (2020). Poly-and perfluoroalkyl substance concentrations in human breast milk and their associations with postnatal infant growth. Science of the Total Environment, 713, 136417.

Khazaee, M., Christie, E., Cheng, W., Michalsen, M., Field, J., & Ng, C. (2021). Perfluoroalkyl acid binding with peroxisome proliferator-activated receptors α, γ, and δ, and fatty acid binding proteins by equilibrium dialysis with a comparison of methods. Toxics, 9(3), 45.

Li, B. J., Chen, J. Y., Liu, Z. Z., Wang, J., & He, S. C. (2022). Pollution Characteristics and Health Risk Assessment of Perfluorinated Compounds in PM 2.5 in Zhejiang Province. Huan jing ke xue= Huanjing kexue, 43(2), 639-648. ARTICLE IN CHINESE

Li, N., Ying, G. G., Hong, H., & Deng, W. J. (2021). Perfluoroalkyl substances in the urine and hair of preschool children, airborne particles in kindergartens, and drinking water in Hong Kong. Environmental Pollution, 270, 116219.

Lin, H., Taniyasu, S., Yamashita, N., Khan, M. K., Masood, S. S., Saied, S., & Khwaja, H. A. (2022). Per-and polyfluoroalkyl substances in the atmospheric total suspended particles in Karachi, Pakistan: Profiles, potential sources, and daily intake estimates. Chemosphere, 288, 132432.

Liu, J. J., Cui, X. X., Tan, Y. W., Dong, P. X., Ou, Y. Q., Li, Q. Q., ... & Zhao, X. M. (2022). Per-and perfluoroalkyl substances alternatives, mixtures and liver function in adults: A community-based population study in China. Environment International, 163, 107179.

López, M. E., Göen, T., Mol, H., Nübler, S., Haji-Abbas-Zarrabi, K., Koch, H. M., ... & Castaño, A. (2021). The European human biomonitoring platform-Design and implementation of a laboratory quality assurance/quality control (QA/QC) programme for selected priority chemicals. International Journal of Hygiene and Environmental Health, 234, 113740.

Macheka, L. R., Abafe, O. A., Mugivhisa, L. L., & Olowoyo, J. O. (2022). Occurrence and infant exposure assessment of per and polyfluoroalkyl substances in breast milk from South Africa. Chemosphere, 288, 132601.

Maekawa, R., Ito, R., Iwasaki, Y., Saito, K., Akutsu, K., Takatori, S., ... & Sugino, N. (2017). Evidence of exposure to chemicals and heavy metals during pregnancy in Japanese women. Reproductive Medicine and Biology, 16(4), 337-348.

Modaresi, S. M. S., Wei, W., Emily, M., DaSilva, N. A., & Slitt, A. L. (2022). Per-and polyfluoroalkyl substances (PFAS) augment adipogenesis and shift the proteome in murine 3T3-L1 adipocytes. Toxicology, 465, 153044.

Neagu, M., Constantin, C., Bardi, G., & Duraes, L. (2021). Adverse outcome pathway in immunotoxicity of perfluoroalkyls. Current Opinion in Toxicology, 25, 23-29.

Nian, M., Zhou, W., Feng, Y., Wang, Y., Chen, Q., & Zhang, J. (2022). Emerging And Legacy PFAS And Cytokine Homeostasis In Women of Childbearing Age. (In Review, Scientific Reports) https://doi.org/10.21203/rs.3.rs-1217618/v1)

Pearce, R. G., Setzer, R. W., Strope, C. L., Wambaugh, J. F., & Sipes, N. S. (2017). Httk: R package for high-throughput toxicokinetics. Journal of statistical software, 79(4), 1.

Peng, L., Xu, W., Zeng, Q., Sun, F., Guo, Y., Zhong, S., ... & Chen, D. (2022). Exposure to perfluoroalkyl substances in waste recycling workers: Distributions in paired human serum and urine. Environment international, 158, 106963.

Pierozan, P., Cattani, D., & Karlsson, O. (2022). Tumorigenic activity of alternative per- and polyfluoroalkyl substances (PFAS): Mechanistic in vitro studies. Science of The Total Environment, 808, 151945.

Rawn, D. F., Dufresne, G., Clément, G., Fraser, W. D., & Arbuckle, T. E. (2022). Perfluorinated alkyl substances in Canadian human milk as part of the Maternal-Infant Research on Environmental Chemicals (MIREC) study. Science of The Total Environment, 154888.

Sonego, E., Simonetti, G., Di Filippo, P., Riccardi, C., Buiarelli, F., Fresta, A., ... & Pomata, D. (2022). Characterization of organophosphate esters (OPEs) and polyfluoralkyl substances (PFASs) in settled dust in specific workplaces. Environmental Science and Pollution Research, 1-15.

Tang, J., Zhu, Y., Li, Y., Xiang, B., Tan, T., Lv, L., & Luo, Q. (2022). Occurrence characteristics and health risk assessment of per-and polyfluoroalkyl substances from water in residential areas around fluorine chemical industrial areas, China. Environmental Science and Pollution Research, 1-11.

Wang, F., Wang, W., Zhao, D., Liu, J., Lu, P., Rose, N. L., & Zhang, G. (2022). Source apportionment and wet deposition of atmospheric poly-and per-fluoroalkyl substances in a metropolitan city centre of southwest China. Atmospheric Environment, 273, 118983.

Velarde, M. C., Chan, A. F. O., Sajo, M. E. J. V., Zakharevich, I., Melamed, J., Uy, G. L. B., ... & Gerona, R. R. (2022). Elevated levels of perfluoroalkyl substances in breast cancer patients within the Greater Manila Area. Chemosphere, 286, 131545.

Wang, Y., Gao, X., Liu, J., Lyu, B., Li, J., Zhao, Y., & Wu, Y. (2022). Exposure to Emerging and Legacy Polyfluoroalkyl Substances in the Sixth Total Diet Study—China, 2016–2019. China CDC Weekly, 4(9), 168.

Wasel, O., Thompson, K. M., Gao, Y., Godfrey, A. E., Gao, J., Mahapatra, C. T., ... & Freeman, J. L. (2021). Comparison of zebrafish in vitro and in vivo developmental toxicity assessments of perfluoroalkyl acids (PFAAs). Journal of Toxicology and Environmental Health, Part A, 84(3), 125-136.

Xie, M. Y., Lin, Z. Y., Liu, L. Y., Wu, C. C., Liu, Y. W., Huang, G. L., & Zeng, E. Y. (2022). Use of glioma to assess the distribution patterns of perfluoroalkyl and polyfluoroalkyl substances in human brain. Environmental Research, 204, 112011.

	Xu, Y., Fletcher, T., Pineda, D., Lindh, C. H., Nilsson, C., Glynn, A., ... & Li, Y. (2020). Serum half-lives for short-and long-chain perfluoroalkyl acids after ceasing exposure from drinking water contaminated by firefighting foam. Environmental health perspectives, 128(7), 077004. Xu, Y., Nielsen, C., Li, Y., Hammarstrand, S., Andersson, E. M., Li, H., ... & Jakobsson, K. (2021). Serum perfluoroalkyl substances in residents following long-term drinking water contamination from firefighting foam in Ronneby, Sweden. Environment international, 147, 106333. Yu, S., Ren, J., Lv, Z., Li, R., Zhong, Y., Yao, W., & Yuan, J. (2022). Prediction of the endocrine-disrupting ability of 49 per-and polyfluoroalkyl substances: In silico and epidemiological evidence. Chemosphere, 290, 133366. Zhang, S., Guo, X., Lu, S., He, J., Wu, Q., Liu, X., ... & Xie, P. (2022). Perfluorohexanoic acid caused disruption of the hypothalamus-pituitary-thyroid axis in zebrafish larvae. Ecotoxicology and Environmental Safety, 232, 113283. Zhang, Y. T., Zeeshan, M., Su, F., Qian, Z. M., Geiger, S. D., McMillin, S. E., ... & Dong, G. H. (2022). Associations between both legacy and alternative per-and polyfluoroalkyl substances and glucose-homeostasis: The Isomers of C8 health project in China. Environment international, 158, 106913. Zheng, G., Schreder, E., Dempsey, J. C., Uding, N., Chu, V., Andres, G., ... & Salamova, A. (2021). Per-and polyfluoroalkyl substances (PFAS) in breast milk: Concerning trends for current-use PFAS. Environmental Science & Technology, 55(11), 7510-7520. Zhou, J., Baumann, K., Chang, N., Morrison, G., Bodnar, W., Zhang, Z., ... & Turpin, B. J. (2022). Per-and polyfluoroalkyl substances (PFASs) in airborne particulate matter (PM2. 0) emitted during floor waxing: A pilot study. Atmospheric Environment, 268, 118845. Zhu, Q., Li, H., Wen, Z., Wang, Y., Li, X., Huang, T., ... & Ge, R. S. (2020). Perfluoroalkyl substances cause Leydig cell dysfunction as endocrine disruptors. Chemosphere, 253, 126764.
Haney	In conclusion, I support the draft assessment overall and believe the conclusions and RfD values therein are scientifically defensible given currently available data. It is obvious that the EPA has put a great deal of time and work into the draft PFHxA assessment. Similarly, the external scientific peer review panel has no doubt spent significant time and effort in reviewing and providing thoughtful comments on the draft assessment. At the same time, a great deal of scientific expertise and resources (e.g., subject area experts, total review time available) exist outside of any peer review panel or environmental regulatory agency. Additionally, the public is obviously an important stakeholder in chemical dose-response assessments such as this that will ultimately be utilized in the protection of public health. Consequently, in addition to careful review of comments from the peer review panel, the EPA should duly consider all public comments so that the draft assessment is the beneficiary of staff having considered the most

	diverse set of viewpoints and scientific perspectives possible. Thank you for the opportunity to have peer reviewed this important draft assessment.
Ng	**Summary Review Comments** Based on my independent review of the materials provided as well as the useful and enlightening discussions during the two days of the meeting, these are my overall impressions and recommendations for the review document: The authors have done a tremendous job in pulling together and evaluating available data for perflurohexanoic acid (PFHxA). That said, given the paucity of available high quality human studies, there is the opportunity to benefit from use of ancillary data and comparison to supporting evidence from other compounds. **Tier 2 Suggested Revision:** A general recommendation would therefore be to carefully consider how data from other PFAS either support or differ from PFHxA observations and how those could be explained by structure-activity relationships (e.g. chain length vs. half-live observations) as well as how data from other model systems (e.g. zebrafish) could help to fill data gaps. **Tier 2 Suggested Revision:** Finally, to harmonize the discussion of this supporting evidence across the different endpoints considered. My specific responses to the charge questions follow. It should be noted that in almost all cases the Tier 1 revisions suggested are meant to improve the clarity of the review, and do not materially change the conclusions drawn by the authors. **Clarification:** This is again a Tier 2 suggested revision. In terms of "harmonization" I meant to include under the different charge questions the same types of evidence. If structure-activity relationship information is available for example for hepatic effects and comments are made about what should be expected for PFHxA based on observations for other PFAS, then under developmental effects it should be stated whether similar structure-activity relationships could be considered or if such information is not available. Or observations for other models (e.g. zebrafish). ***Material Outside of the Charge Questions*** **EXECUTIVE SUMMARY** **Editorial Comments:** In the executive summary (p xiii, lines 12-13) and in section 1 (p. 1-2, lines 21-23) the review states that concerns about PFHxA and other PFAS "stem from the resistance of these compounds [to degradation], which leads to their persistence in the environment." While PFAS persistence is the most "uniform" of their hazards, in that most are or will transform into extremely persistent compounds, the main purpose of this review is to understand the toxicity of PFHxA—both the toxicity and, for longer-chain PFAS, the bioaccumulation of these substances are also important concerns, and probably are more responsible for initial interest into their properties and impacts than their persistence. I would recommend rephrasing this section in both locations in the document to reflect the multiple hazard dimensions driving concerns about PFAS. On p. xiv line 3 it states "Animal studies of PFHxA exposure exclusively examined the oral exposure route" – Suggest adding a clarification that other studies cited throughout the

	document that include IV dosing (and in a few cases IP injection?) were used as supplemental data to the PECO-included oral studies. Table ES-1, p. xv: lowercase “a” in “Na” for column 5 Line 4 on p. xvii, define “$BMDL_{5RD}$” on first use. **OVERVIEW OF BACKGROUND** **Tier 2 Suggested Revisions**: Table 1-1 provides available physicochemical properties of PFHxA. While these use available sources that include both experimental and estimated data, some context needs to be provided on reliability. For example, water solubility varies five orders of magnitude for the water solubility of the ammonium vs. sodium salts. Clearly one of these values is wrong as once dissociated these should behave similarly. The same is true for the bioconcentration factor. **PHARMACOKINETICS (section 3.1)** **Tier 1 Necessary Revision**: p. 3-4 lines 20-38: As noted in comments above, analysis of data from Perez et al. 2013 requires a caveat about potential issues in analysis/quantification of short-chain PFAS. **Tier 2 Suggested Revisions**: p. 3-5, lines 6-7: How is “substantial binding” to serum proteins defined? PFHxA has been shown in in vitro studies to bind less strongly than long-chain PFAS. How relevant is this? p. 3-9, lines 34-38: As noted in comments above regarding extrapolation of HED, interpretation of dose-dependent rodent PK should take into account that there are not only reabsorption-mediating kidney transporters (e.g. Oatp1a1) but also elimination-mediating ones (e.g. Oat1 and Oat3). **Editorial Comments**: p. 3-8, lines 19-20: Should be “perfluorobutanoic acid” (not benzoic)

APPENDIX A

LIST OF REVIEWERS

Reviewers for the External Peer Review of the EPA Draft "IRIS Toxicological Review of Perfluorohexanoic Acid (PFHxA) and Related Salts"

Elaine M. Faustman, Ph.D., DABT
(Panel Chair)
Professor, Environmental and Occupational Health Sciences, and Director, Institute for Risk Analysis and Risk Communication
School of Public Health
University of Washington
Seattle, WA

Panagiotis G. Georgopoulos, Ph.D.
Professor, Rutgers Biomedical and Health Sciences
Department of Environmental and Occupational Health and Justice
School of Public Health
Rutgers University
Piscataway, NJ

Joseph T. Haney, Jr., M.S.
Distinguished Toxicologist
Toxicology Division, Office of the Executive Director
Texas Commission on Environmental Quality
Austin, TX

Angela M. Leung, M.D., M.Sc.
Associate Professor of Medicine
David Geffen School of Medicine
University of California Los Angeles
Los Angeles, CA

Carla A. Ng, Ph.D.
Assistant Professor
Civil and Environmental Engineering
University of Pittsburgh
Pittsburgh, PA

David A. Savitz, Ph.D.
Professor, Epidemiology
School of Public Health
Brown University
Providence, RI

R. Thomas Zoeller, Ph.D.
Professor Emeritus
Department of Biology
University of Massachusetts Amherst
Amherst, MA

APPENDIX B

CHARGE TO REVIEWERS

Technical Charge to External Peer Reviewers

Contract No. EP-C-17-017
Task Order 68HERH20F0407 (ERG Task 44)
March 2022

External Peer Review of EPA's Draft IRIS Toxicological Review of Perfluorohexanoic Acid (PFHxA) and Related Salts

INTRODUCTION

The U.S. Environmental Protection Agency (EPA) is seeking a scientific peer review of the draft *IRIS Toxicological Review of Perfluorohexanoic Acid and Related Salts* developed in support of the Agency's online database, the Integrated Risk Information System (IRIS). IRIS is prepared and maintained by EPA's Center for Public Health and Environmental Assessment within the Office of Research and Development. IRIS assessments contain information about chemicals that can be used to support hazard identification and dose-response assessment, two of the four steps in the human health risk assessment process. When used by risk managers in combination with information on human exposure and other considerations, IRIS assessments support the Agency's regulatory activities and decisions to protect public health.

There is no existing IRIS assessment for perfluorohexanoic acid (PFHxA). The draft Toxicological Review of PFHxA is based on a comprehensive review of the available scientific literature on the noncancer and cancer health effects in humans and experimental animals exposed to PFHxA or related salts. The systematic review protocol for PFHxA and other appendices for toxicokinetic information, dose-response modeling, and other supporting materials are provided as *Supplemental Information* (see Appendices A to E) to the draft Toxicological Review.

REVIEW MATERIALS PROVIDED

- Draft PFHXA Toxicological Assessment
- Supplemental Material (PFHxA Appendices)

CHARGE QUESTIONS

When responding to the charge questions below, categorize any recommendations for EPA as part of this peer review into one of three categories (Tier 1, 2, or 3). The categories are useful for prioritizing the relative importance of comments, as follows:

- **Tier 1: *Necessary Revisions*** – Use this category for any revisions you believe are necessary to adequately support and substantiate the analyses or scientific basis for the assessment conclusions, or to improve the clarity of the presentation in the PFHxA Toxicological Review.
- **Tier 2: *Suggested Revisions*** – Use this category for any revisions you encourage EPA to implement to strengthen the analyses or scientific basis for the assessment conclusions, or to improve the clarity of the presentation in the PFHxA Toxicological Review.

- **Tier 3: *Future Considerations*** – Use this category for any advice you have for scientific exploration that might inform future work. While these recommendations are generally outside the immediate scope or needs of the PFHxA Toxicological Review, they could inform future reviews or research efforts.

Systematic Review Methods and Documentation

1. The Toxicological Review for PFHxA describes and applies a systematic review protocol for identifying and screening pertinent studies. The protocol is described in brief detail in Section 1.2.1 (*Literature Searching and Screening*) and in full detail in Appendix A (*Systematic Review Protocol for the PFAS IRIS Assessments*). Please comment on whether the search strategy and screening criteria for PFHxA literature are clearly described. If applicable, please identify additional peer-reviewed studies of PFHxA that the assessment should incorporate[1].

2. The Toxicological Review provides an overview of individual study evaluations and the results of those evaluations are made available in the Health Assessment Workplace Collaborative linked here HAWC. Note that a "HAWC FAQ for assessment readers" document, linked here (scroll to the bottom of the page, and the document is available for download under "attachments"), is intended to help the reviewer navigate this on-line resource. Data from studies considered informative to the assessment are synthesized in the relevant health effect-specific sections, and study data are available in HAWC.

    a. Please comment on whether the study confidence conclusions for the PFHxA studies are scientifically justified and clearly described, considering the important methodological features of the assessed outcomes. Please indicate any study confidence conclusions that are not justified and explain any alternative study evaluation decisions.

    b. Results from individual PFHxA studies are presented and synthesized in the health system-specific sections. Please comment on whether the presentation and analysis of study results are clear, appropriate, and effective to allow for scientifically supported syntheses of the findings across sets of studies.

Noncancer Hazard Identification

3. For each health effect considered in the assessment and outlined below, please comment on whether the available data have been clearly and appropriately synthesized to describe the strengths and limitations. For each, please also comment on whether the weight-of-evidence decisions for hazard identification are scientifically justified and clearly described.

    a. For hepatic effects, the Toxicological Review concludes the available ***evidence indicates*** PFHxA likely causes hepatic effects in humans under relevant exposure circumstances. This conclusion is based on studies of rats showing increased liver weight, hepatocellular hypertrophy, increased serum enzymes, and decreased serum globulins. The hepatic

[1] Newly identified studies (i.e., studies identified by EPA or the public that meet PECO criteria but were not addressed in the external review draft, for example due to recent publication) will be characterized by EPA and presented to the peer review panel. This characterization will focus on EPA's judgment of whether the studies would have a material impact on the conclusions (i.e., identified hazards or toxicity values) in the external review draft. The peer review panel is asked to review EPA's characterization and provide tiered recommendations to EPA regarding which studies, if any, to incorporate into the assessment before finalizing.

findings for PFHxA were similar for other PFAS and determined to be adverse and relevant to humans.

i. Additional considerations influenced the hepatic effects hazard identification decisions. Appendix A (*Systematic Review Protocol for the PFAS IRIS Assessments*) outlines the human relevance of hepatic effects in animals that involve PPARα receptors as a key science issue. To the extent supported by the PFHxA literature (and to a lesser extent, literature for other PFAS), the Toxicological Review evaluates the evidence relevant to the potential involvement of PPARα and non-PPARα pathways with respect to the reported hepatic effects. The Toxicological Review ultimately concludes evidence from *in vivo* (including genetic mouse models) and *in vitro* studies support a potential role for multiple pathways operant in the induction of hepatic effects from PFHxA exposure but those pathways cannot be specifically determined. Please comment on whether the conclusions regarding the available animal and mechanistic studies are scientifically justified and clearly described. The hepatic findings for PFHxA were similar for other PFAS and determined to be adverse and relevant to humans.

b. For developmental effects, the Toxicological Review concludes the available ***evidence indicates*** PFHxA likely causes developmental effects in humans under relevant exposure circumstances. This judgment is based primarily on gestational exposure experiments in mice, with supportive findings in rats exposed throughout gestation and lactation, showing increased perinatal mortality, decreased offspring body weight, and delayed eye opening. These effects are similar to those observed for other PFAS following developmental exposure and were determined to be adverse and relevant to humans.

c. For hematopoietic effects, the Toxicological Review concludes the available ***evidence indicates*** PFHxA likely causes hematopoietic effects in humans under relevant exposure circumstances. This judgment is based on consistent findings, including decreased red blood cells [RBCs], hematocrit, and hemoglobin, across study designs that, when interpreted together, signifies PFHxA-related hematological effects such as anemia. These findings were determined to be adverse and relevant to humans.

d. For endocrine effects, the Toxicological Review concludes the available evidence suggests, but is not sufficient to infer, that PFHxA may cause endocrine effects in humans under relevant exposure circumstances. This conclusion is based on some evidence of thyroid effects based on hormone and histopathological changes in two rat studies; however, the data is limited, lacking consistency across studies, and histopathological changes may be explained by non-thyroid related effects.

e. For all other potential health effects (i.e., renal, male and female reproductive, immune, and nervous system), the Toxicological Review concluded the available evidence is inadequate to assess whether PFHxA may cause effects in humans under relevant exposure circumstances. In general, these conclusions were driven by sparse evidence bases or data that were largely null.

Noncancer Toxicity Value Data Selection

4. For PFHxA, no RfC was derived. The study chosen for use in deriving the RfD is the Loveless et al. (2009) one-generation reproductive toxicity study based on decreased offspring body weight in rats exposed continuously throughout gestation and lactation to PFHxA sodium salt via the dam. Is the

selection of this study and these effects for use in deriving the RfD for PFHxA scientifically justified and clearly described?

   a. If yes, please provide an explanation.

   b. If no, please provide an alternative study(ies) or effect(s) that should be used to support the derivation of the RfD and detail the rationale for use of such an alternative.

   c. As part of the responses in "a" or "b" above, please comment on whether the effects selected are appropriate for use in deriving the RfD, including considerations regarding adversity (or appropriateness in representing an adverse change) and the scientific support for their selection.

   d. Given the lack of studies on inhalation exposure to PFHxA, no reference concentration (RfC) is derived. Please comment on this decision.

5. In addition, for PFHxA, an RfD for less-than-lifetime ("subchronic") exposures is derived. No "subchronic" RfC was derived. The same study and outcome were chosen for use in deriving the RfD. Is the selection of this study and these effects for the derivation of the subchronic RfD for PFHxA scientifically justified and clearly described?

   a. If yes, please provide an explanation.

   b. If no, please provide an alternative study(ies) and/or effect(s) that should be used to support the derivation of the subchronic RfD and detail the rationale for use of such an alternative.

   c. As part of the responses in "a" or "b" above, please comment on whether the effects selected are appropriate for use in deriving the RfD, including considerations regarding adversity (or appropriateness in representing an adverse change) and the scientific support for their selection.

   d. Given the lack of studies on inhalation exposure to PFHxA, no "subchronic" RfC is derived. Please comment on this decision.

Noncancer Toxicity Value Derivation

6. EPA used benchmark dose modeling (USEPA, 2012) to identify points-of-departure (PODs) for oral exposure to PFHxA. Are the modeling approaches used, selection and justification of benchmark response levels, and the selected models used to identify each POD for toxicity value derivation scientifically justified and clearly described?

7. Appendix A identifies the potential for pharmacokinetic differences across species and sexes as a key science issue and lays out a hierarchy for using relevant pharmacokinetic data in extrapolating oral doses between laboratory animals and humans. Section 5.2.1 describes the various approaches considered and the rationale for the selected approach. Given what is known and not known about the potential interspecies differences in PFHxA pharmacokinetics, EPA used the ratio of human-to-animal serum clearance values assuming the volume of distribution (V_d) in humans is equivalent to that in monkeys to adjust the POD to estimate a human equivalent dose (HED) in the derivation of the respective RfDs.

   a. Is applying the ratio of human-to-animal serum clearance values for PFHxA scientifically justified and clearly described? If not, please provide an explanation and detail the preferred alternative approach.

b. Does the Toxicological Review clearly describe the uncertainties in evaluating the pharmacokinetic differences between the experimental animal data and humans?

8. EPA has evaluated and applied uncertainty factors to account for intraspecies variability (UFH), interspecies differences (UF_A), database limitations (UF_D), exposure duration (UF_S), and LOAEL-to-NOAEL extrapolation (UF_L) for PFHxA.

    a. Is uncertainty in the derivation of the toxicity values scientifically justified and clearly described? Please describe and provide comments, if needed.

    b. For uncertainty in interspecies differences (UF_A), a value of 3 is applied to account for remaining uncertainty in characterizing the pharmacokinetic and pharmacodynamic differences between laboratory animals and humans after calculation of the HED. For developmental and hematopoietic outcomes, the evidence base lacked chemical-and species-specific information that would have been useful for informing the UFA; for hepatic outcomes, however, available mechanistic and supplemental information was useful for further evaluating the interspecies uncertainty factor. Some data indicate a PPARα-dependent pathway that might support a UF_A of 1. Evidence for non-PPARα modes of action, however, is available in the PFHxA (and larger PFAS) database. Thus, uncertainty remains regarding the potential differences in sensitivity across species due to the involvement of both PPARα-dependent and-independent pathways. Further, data are lacking to determine with confidence the relative contribution of each of these pathways. As such, the Toxicological Review concludes the available data are not adequate to determine if humans are likely to be equally or less sensitive than laboratory animals with respect to the observed hepatic effects and that a value of UF_A=3 is warranted to account for the residual uncertainty in pharmacodynamic differences across species. Please comment on whether the available animal and mechanistic studies support this conclusion and whether the analysis presented in the Toxicological Review is scientifically justified and clearly described.

    c. To inform uncertainty in intraspecies variability (UF_H), the assessment evaluates and considers the available evidence on potential susceptibility to PFHxA within different populations or lifestages, including any potential human health impacts from early life exposure. Are the available information and data appropriately considered and the resultant UF_H values scientifically justified and clearly described?

    d. Are the provided rationales for the remaining uncertainty factors (UF_L, UF_D, UF_S) scientifically justified and clearly described? If not, please explain.

Carcinogenicity Hazard Identification and Toxicity Value Derivation

9. The Toxicological Review concludes that there is *inadequate information to assess carcinogenic potential* for PFHxA and that this descriptor applies to oral and inhalation routes of human exposure. Please comment on whether the available animal and mechanistic studies and the analysis presented in the Toxicological Review are scientifically justified and clearly described.

10. Given the conclusion there was *inadequate information to assess carcinogenic potential* for PFHxA (Charge Question 5), the Toxicological Review does not derive quantitative estimates for cancer effects for either oral or inhalation exposures. Is this decision scientifically justified and clearly described?

APPENDIX C

MEETING AGENDA

External Peer Review of the EPA Draft "IRIS Toxicological Review of Perfluorohexanoic Acid (PFHxA) and Related Salts"

Monday, May 16, 2022: 10:00 AM - 5:30 PM EDT
Tuesday, May 17, 2022: 12:00 PM - 3:00 PM EDT
Virtual Meeting via Zoom.gov

Note: Daily meeting start times are fixed; discussion and break times may be adjusted by reviewers.

Final Agenda

DAY 1: Monday, May 16

10:00 AM **Meeting Purpose, Peer Review Process & Reviewer Intros** *Jan Connery, ERG (facilitator)*

10:20 AM **U.S. EPA Office of Research and Development (ORD) Background Presentation**

11:05 AM **Reviewer Discussion Agenda and Process** ..... *Jan Connery, ERG*

11:10 AM **Chair Opening Remarks to Panel** ..... *Peer Review Chair*

11:15 AM **Reviewer Discussions** ..... *Peer Review Panel*

Systematic Review Methods and Documentation

Charge Question 1 (~45 minutes): The Toxicological Review for PFHxA describes and applies a systematic review protocol for identifying and screening pertinent studies. The protocol is described in brief detail in Section 1.2.1 (Literature Searching and Screening) and in full detail in Appendix A (Systematic Review Protocol for the PFAS IRIS Assessments). Please comment on whether the search strategy and screening criteria for PFHxA literature are clearly described. If applicable, please identify additional peer-reviewed studies of PFHxA that the assessment should incorporate.

12:00 PM BREAK

12:15 PM **Reviewer Discussions (cont.)** ..... *Peer Review Panel*

Charge Question 2 (~30 minutes): The Toxicological Review provides an overview of individual study evaluations and the results of those evaluations are made available in the Health Assessment Workplace Collaborative (HAWC). Data from studies considered informative to the assessment are synthesized in the relevant health effect-specific sections, and study data are available in HAWC.

a) Please comment on whether the study confidence conclusions for the PFHxA studies are scientifically justified and clearly described, considering the important methodological features of the assessed outcomes. Please indicate any study confidence conclusions that are not justified and explain any alternative study evaluation decisions.

b) Results from individual PFHxA studies are presented and synthesized in the health system-specific sections. Please comment on whether the presentation and analysis of study results are clear, appropriate, and effective to allow for scientifically supported syntheses of the findings across sets of studies.

Agenda (cont.)

DAY 1: Monday, May 16 (cont.)

12:45 PM **Reviewer Discussions (cont.)** .....*Peer Review Panel*

Non-Cancer Hazard Identification

Charge Question 3: For each health effect considered in the assessment and outlined below, please comment on whether the available data have been clearly and appropriately synthesized to describe the strengths and limitations. For each, please also comment on whether the weight-of-evidence decisions for hazard identification are scientifically justified and clearly described.

a) ***For hepatic effects***, the Toxicological Review concludes the available evidence indicates PFHxA likely causes hepatic effects in humans under relevant exposure circumstances. This conclusion is based on studies of rats showing increased liver weight, hepatocellular hypertrophy, increased serum enzymes, and decreased serum globulins. The hepatic findings for PFHxA were similar for other PFAS and determined to be adverse and relevant to humans. *(~35 minutes)*

   i) Additional considerations influenced the hepatic effects hazard identification decisions. Appendix A (Systematic Review Protocol for the PFAS IRIS Assessments) outlines the human relevance of hepatic effects in animals that involve PPARα receptors as a key science issue. To the extent supported by the PFHxA literature (and to a lesser extent, literature for other PFAS), the Toxicological Review evaluates the evidence relevant to the potential involvement of PPARα and non-PPARα pathways with respect to the reported hepatic effects. The Toxicological Review ultimately concludes evidence from in vivo (including genetic mouse models) and in vitro studies support a potential role for multiple pathways operant in the induction of hepatic effects from PFHxA exposure but those pathways cannot be specifically determined. Please comment on whether the conclusions regarding the available animal and mechanistic studies are scientifically justified and clearly described. The hepatic findings for PFHxA were similar for other PFAS and determined to be adverse and relevant to humans. *(~15 of 35 minutes)*

1:00 PM *Carcinogenicity Hazard Identification and Toxicity Value Derivation*

Charge Question 9 (~15 minutes): The Toxicological Review concludes that there is *inadequate information to assess carcinogenic potential* for PFHxA and that this descriptor applies to oral and inhalation routes of human exposure. Please comment on whether the available animal and mechanistic studies and the analysis presented in the Toxicological Review are scientifically justified and clearly described.

Charge Question 10 (~10 minutes): Given the conclusion there *was inadequate information to assess carcinogenic potential* for PFHxA (Charge Question 5), the Toxicological Review does not derive quantitative estimates for cancer effects for either oral or inhalation exposures. Is this decision scientifically justified and clearly described?

1:25 PM **Reviewer Discussions, Charge Question 3 (cont.)** .....*Peer Review Panel*

Non-Cancer Hazard Identification (cont.)

c) ***For hematopoietic effects***, the Toxicological Review concludes the available evidence indicates PFHxA likely causes hematopoietic effects in humans under relevant exposure circumstances. This judgment is based on consistent findings, including decreased red blood cells [RBCs], hematocrit, and hemoglobin, across study designs that, when interpreted together, signifies PFHxA-related hematological effects such as anemia. These findings were determined to be adverse and relevant to humans. *(~15 minutes)*

1:40 PM BREAK

Agenda (cont.)

DAY 1: Monday, May 16 (cont.)

2:00 PM **Reviewer Discussions, Charge Question 3 (cont.)** ..... *Peer Review Panel*

Non-Cancer Hazard Identification (cont.)

a) (Continued) ***For hepatic effects***, the Toxicological Review concludes the available evidence indicates PFHxA likely causes hepatic effects in humans under relevant exposure circumstances. This conclusion is based on studies of rats showing increased liver weight, hepatocellular hypertrophy, increased serum enzymes, and decreased serum globulins. The hepatic findings for PFHxA were similar for other PFAS and determined to be adverse and relevant to humans. *(~20 of 35 minutes)*

b) ***For developmental effects***, the Toxicological Review concludes the available evidence indicates PFHxA likely causes developmental effects in humans under relevant exposure circumstances. This judgment is based primarily on gestational exposure experiments in mice, with supportive findings in rats exposed throughout gestation and lactation, showing increased perinatal mortality, decreased offspring body weight, and delayed eye opening. These effects are similar to those observed for other PFAS following developmental exposure and were determined to be adverse and relevant to humans. *(~15 minutes)*

d) ***For endocrine effects***, the Toxicological Review concludes the available evidence suggests, but is not sufficient to infer, that PFHxA may cause endocrine effects in humans under relevant exposure circumstances. This conclusion is based on some evidence of thyroid effects based on hormone and histopathological changes in two rat studies; however, the data is limited, lacking consistency across studies, and histopathological changes may be explained by non-thyroid related effects. *(~20 minutes)*

e) ***For all other potential health effects (i.e., renal, male and female reproductive, immune, and nervous system),*** the Toxicological Review concluded the available evidence is inadequate to assess whether PFHxA may cause effects in humans under relevant exposure circumstances. In general, these conclusions were driven by sparse evidence bases or data that were largely null. *(~10 minutes)*

3:05 PM *Noncancer Toxicity Value Data Selection*

Charge Question 4 (~45 minutes): For PFHxA, no RfC was derived. The study chosen for use in deriving the RfD is the Loveless et al. (2009) one-generation reproductive toxicity study based on decreased offspring body weight in rats exposed continuously throughout gestation and lactation to PFHxA sodium salt via the dam. Is the selection of this study and these effects for use in deriving the RfD for PFHxA scientifically justified and clearly described?

a) If yes, please provide an explanation.

b) If no, please provide an alternative study(ies) or effect(s) that should be used to support the derivation of the RfD and detail the rationale for use of such an alternative.

c) As part of the responses in "a" or "b" above, please comment on whether the effects selected are appropriate for use in deriving the RfD, including considerations regarding adversity (or appropriateness in representing an adverse change) and the scientific support for their selection.

d) Given the lack of studies on inhalation exposure to PFHxA, no reference concentration (RfC) is derived. Please comment on this decision.

3:50 PM BREAK

Agenda (cont.)

DAY 1: Monday, May 16 (cont.)

4:10 PM **Reviewer Discussions (cont.)** ..... *Peer Review Panel*

Noncancer Toxicity Value Data Selection (cont.)

Charge Question 5 (~35 minutes): In addition, for PFHxA, an RfD for less-than-lifetime ("subchronic") exposures is derived. No "subchronic" RfC was derived. The same study and outcome were chosen for use in deriving the RfD. Is the selection of this study and these effects for the derivation of the subchronic RfD for PFHxA scientifically justified and clearly described?

a) If yes, please provide an explanation.

b) If no, please provide an alternative study(ies) and/or effect(s) that should be used to support the derivation of the subchronic RfD and detail the rationale for use of such an alternative.

c) As part of the responses in "a" or "b" above, please comment on whether the effects selected are appropriate for use in deriving the RfD, including considerations regarding adversity (or appropriateness in representing an adverse change) and the scientific support for their selection.

d) Given the lack of studies on inhalation exposure to PFHxA, no "subchronic" RfC is derived. Please comment on this decision.

4:45 PM *Noncancer Toxicity Value Derivation*

Charge Question 6 (~20 minutes): EPA used benchmark dose modeling (USEPA, 2012) to identify points-of-departure (PODs) for oral exposure to PFHxA. Are the modeling approaches used, selection and justification of benchmark response levels, and the selected models used to identify each POD for toxicity value derivation scientifically justified and clearly described?

Charge Question 7 (~30 minutes): Appendix A identifies the potential for pharmacokinetic differences across species and sexes as a key science issue and lays out a hierarchy for using relevant pharmacokinetic data in extrapolating oral doses between laboratory animals and humans. Section 5.2.1 describes the various approaches considered and the rationale for the selected approach. Given what is known and not known about the potential interspecies differences in PFHxA pharmacokinetics, EPA used the ratio of human-to-animal serum clearance values assuming the volume of distribution (Vd) in humans is equivalent to that in monkeys to adjust the POD to estimate a human equivalent dose (HED) in the derivation of the respective RfDs.

a) Is applying the ratio of human-to-animal serum clearance values for PFHxA scientifically justified and clearly described? If not, please provide an explanation and detail the preferred alternative approach.

b) Does the Toxicological Review clearly describe the uncertainties in evaluating the pharmacokinetic differences between the experimental animal data and humans?

5:30 PM **ADJOURN Day 1**

Agenda (cont.)

DAY 2: Tuesday, May 17

Noon **Day 1 Recap**, **Day 2 Agenda and Process** ..... *Jan Connery, ERG*

12:05 PM **Reviewer Discussions** ..... *Peer Review Panel*

Noncancer Toxicity Value Derivation (cont.)

Charge Question 7 (continued if needed): Appendix A identifies the potential for pharmacokinetic differences across species and sexes as a key science issue and lays out a hierarchy for using relevant pharmacokinetic data in extrapolating oral doses between laboratory animals and humans. Section 5.2.1 describes the various approaches considered and the rationale for the selected approach. Given what is known and not known about the potential interspecies differences in PFHxA pharmacokinetics, EPA used the ratio of human-to-animal serum clearance values assuming the volume of distribution (Vd) in humans is equivalent to that in monkeys to adjust the POD to estimate a human equivalent dose (HED) in the derivation of the respective RfDs.

Charge Question 8 (~40 minutes): EPA has evaluated and applied uncertainty factors to account for intraspecies variability (UFH), interspecies differences (UFA), database limitations (UFD), exposure duration (UFS), and LOAEL-to-NOAEL extrapolation (UFL) for PFHxA.

a) Is uncertainty in the derivation of the toxicity values scientifically justified and clearly described? Please describe and provide comments, if needed.

b) For uncertainty in interspecies differences (UFA), a value of 3 is applied to account for remaining uncertainty in characterizing the pharmacokinetic and pharmacodynamic differences between laboratory animals and humans after calculation of the HED. For developmental and hematopoietic outcomes, the evidence base lacked chemical-and species-specific information that would have been useful for informing the UFA; for hepatic outcomes, however, available mechanistic and supplemental information was useful for further evaluating the interspecies uncertainty factor. Some data indicate a PPARα-dependent pathway that might support a UFA of 1. Evidence for non-PPARα modes of action, however, is available in the PFHxA (and larger PFAS) database. Thus, uncertainty remains regarding the potential differences in sensitivity across species due to the involvement of both PPARα-dependent and-independent pathways. Further, data are lacking to determine with confidence the relative contribution of each of these pathways. As such, the Toxicological Review concludes the available data are not adequate to determine if humans are likely to be equally or less sensitive than laboratory animals with respect to the observed hepatic effects and that a value of UFA=3 is warranted to account for the residual uncertainty in pharmacodynamic differences across species. Please comment on whether the available animal and mechanistic studies support this conclusion and whether the analysis presented in the Toxicological Review is scientifically justified and clearly described.

c) To inform uncertainty in intraspecies variability (UFH), the assessment evaluates and considers the available evidence on potential susceptibility to PFHxA within different populations or lifestages, including any potential human health impacts from early life exposure. Are the available information and data appropriately considered and the resultant UFH values scientifically justified and clearly described?

d) Are the provided rationales for the remaining uncertainty factors (UFL, UFD, UFS) scientifically justified and clearly described? If not, please explain.

1:00 PM **Reviewer Integrative Comments and Discussion** ..... *Peer Review Panel*

1:30 PM BREAK

Agenda (cont.)

DAY 2: Tuesday, May 17 (cont.)

Reviewer Discussions (cont.) ..... *Peer Review Panel*

1:40 PM **Individual Reviewer Recommendations**

2:50 PM **Closing Remarks** ..... *EPA, ERG*

3:00 PM **ADJOURN DAY 2**

ehp Environmental Health Perspectives

Vol. 128, No. 4 | Research

Evaluation of Developmental Toxicity, Developmental Neurotoxicity, and Tissue Dose in Zebrafish Exposed to GenX and Other PFAS

Shaza Gaballah, Adam Swank, Jon R. Sobus, Xia Meng Howey, Judith Schmid, Tara Catron, James McCord, Erin Hines, Mark Strynar, and Tamara Tal

Published: 9 April 2020 | CID: 047005 | https://doi.org/10.1289/EHP5843 | **Cited by:** 3

Abstract

Background: Per- and polyfluoroalkyl substances (PFAS) are a diverse class of industrial chemicals with widespread environmental occurrence. Exposure to long-chain PFAS is associated with developmental toxicity, prompting their replacement with short-chain and fluoroether compounds. There is growing public concern over the safety of replacement PFAS.

Objective: We aimed to group PFAS based on shared toxicity phenotypes.

Methods: Zebrafish were developmentally exposed to 4,8-dioxa-3H-perfluorononanoate (ADONA), perfluoro-2-propoxypropanoic acid (GenX Free Acid), perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid (PFESA1), perfluorohexanesulfonic acid (PFHxS), perfluorohexanoic acid (PFHxA), perfluoro-*n*-octanoic acid (PFOA), perfluorooctanesulfonic acid (PFOS), or 0.4% dimethyl sulfoxide (DMSO) daily from 0–5 d post fertilization (dpf). At 6 dpf, developmental toxicity and developmental neurotoxicity assays were performed, and targeted analytical chemistry was used to measure media and tissue doses. To test whether aliphatic sulfonic acid PFAS cause the same toxicity phenotypes, perfluorobutanesulfonic acid

(PFPeS; 5-carbon), PFHxS (6-carbon), perfluoroheptanesulfonic acid (PFHpS; 7-carbon), and PFOS (8-carbon) were evaluated.

Results: PFHxS or PFOS exposure caused failed swim bladder inflation, abnormal ventroflexion of the tail, and hyperactivity at nonteratogenic concentrations. Exposure to PFHxA resulted in a unique hyperactivity signature. ADONA, PFESA1, or PFOA exposure resulted in detectable levels of parent compound in larval tissue but yielded negative toxicity results. GenX was unstable in DMSO, but stable and negative for toxicity when diluted in deionized water. Exposure to PFPeS, PFHxS, PFHpS, or PFOS resulted in a shared toxicity phenotype characterized by body axis and swim bladder defects and hyperactivity.

Conclusions: All emerging fluoroether PFAS tested were negative for evaluated outcomes. Two unique toxicity signatures were identified arising from structurally dissimilar PFAS. Among sulfonic acid aliphatic PFAS, chemical potencies were correlated with increasing carbon chain length for developmental neurotoxicity, but not developmental toxicity. This study identified relationships between chemical structures and *in vivo* phenotypes that may arise from shared mechanisms of PFAS toxicity. These data suggest that developmental neurotoxicity is an important end point to consider for this class of widely occurring environmental chemicals. https://doi.org/10.1289/EHP5843

Introduction

Per- and polyfluoroalkyl substances (PFAS) are a structurally diverse class of industrial chemicals that contain aliphatic chains with all or some of the carbons bonded to fluorines ($-C_nF_{2n}-$) and carboxylic acid or sulfonic acid terminal moieties (OECD 2018). There are 4,370 unique PFAS structures (OECD 2018) with 602 compounds currently in commercial use in the United States (U.S. EPA 2019). PFAS have flame-retardant, water-resistant, and surfactant-like properties (Banks et al. 1994; Kissa 2001). This class of compounds is therefore widely used as protectants in paper and packaging products, water- and grease-repellent textiles, nonstick cookware coatings, and firefighting foams (Lindstrom et al. 2011). PFAS are extremely stable due to the carbon–fluorine bond strength (Banks et al. 1994; Kissa 2001). Based on their structurally inherent thermal and chemical stability, PFAS persist in the environment where they are generally resistant to biodegradation, photooxidation, direct photolysis, and hydrolysis (Schultz et al. 2003). As a result, they are widely detected in the environment (Dauchy et al. 2019; Pan et al. 2018), wildlife (Cui et al. 2018; Escoruela et al. 2018; Route et al. 2014), drinking water (Guelfo and Adamson 2018; Guelfo et al. 2018), and humans (Daly et al. 2018; Hurley et al. 2018; Jain 2018).

Since the voluntary phaseout of perfluoro-*n*-octanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) in the early 2000s, time trends of National Health and Nutrition Examination Survey (NHANES) PFOS and PFOA serum levels are generally indicative of reduced human exposures (Jain 2018). Despite reductions, exposures are still widespread, with PFAS detectable in 95% of NHANES subjects (2013–2014) (CDC 2019) and in pregnant women, maternal

serum levels for PFOS ($35.3\ \mathrm{ng/mL}$) and PFOA ($5.6\ \mathrm{ng/mL}$) have been reported (Fei et al. 2007). Of additional concern, an examination of these compounds in U.S. children 3–11 years of age, most of whom were born after PFOS and PFOA were phased out of use, revealed detectable levels of 14 PFAS, including PFOS and PFOA, in more than 60% of study subjects (Ye et al. 2018). A longitudinal study in Finnish children and adolescents showed that although serum levels of PFOS, PFOA, perfluorohexanesulfonic acid (PFHxS), and perfluorohexanoic acid (PFHxA) decreased over the study period, calculated body burdens generally remained constant and, in some cases, increased (Koponen et al. 2018). In humans, PFAS exposure has been associated with reduced birth weight (Apelberg et al. 2007; Fei et al. 2007), although weak associations with low birth weight or conflicting data have also been reported (Manzano-Salgado et al. 2017; Shoaff et al. 2018; Whitworth et al. 2012). In animal studies, early life stage exposure to PFOS or PFOA have been linked to developmental toxicity in chickens and mice (Jiang et al. 2012; Tucker et al. 2015), immunotoxicity in mice (reviewed by DeWitt et al. 2009), and developmental (Huang et al. 2010; Padilla et al. 2012; Truong et al. 2014) and reproductive toxicity in zebrafish (Jantzen et al. 2017).

To address toxicity concerns, longer alkyl chain PFAS like PFOS and PFOA have been replaced with shorter alkyl chain compounds such as perfluorobutanesulfonic acid (PFBS) or large fluoroether PFAS such as perfluoro-2-propoxypropanoic acid (GenX) and 4,8-dioxa-3H-perfluorononanoate (ADONA). Alternative chemistries that retain the long-chain character, such as ADONA, were engineered with ether linkages and sites of hydrogenation in efforts to reduce biological half-lives (Fromme et al. 2017). Replacement PFAS are therefore increasingly detected in the environment, including in surface water (De Silva et al. 2011; McCord et al. 2018; Pan et al. 2018; Strynar et al. 2015; Wang et al. 2016) and drinking water (Kaboré et al. 2018; McCord et al. 2018). Environmental screening efforts have also identified relevant exposures to PFAS by-products, such as sulfonated fluorovinyl ethers (i.e., PFESA compounds), that are not strictly chemicals of commerce (McCord et al. 2018; Strynar et al. 2015). Growing concern over the safety of GenX and other replacement PFAS has unsurprisingly led to a greater demand for toxicity data (Blum et al. 2015; Borg et al. 2017; Scheringer et al. 2014). However, traditional mammalian toxicity assays can be costly and time consuming, and it is challenging to test multiple chemicals and concentrations of chemicals in parallel. Because PFAS exposures have been historically linked to complex toxicity outcomes involving whole organisms (e.g., developmental toxicity) or specific organ systems (e.g., immunotoxicity), the use of a rapid *in vivo* animal screening system is justified.

The zebrafish is a widely used *in vivo* model for toxicity testing (Hamm et al. 2019; Padilla et al. 2012). Development is rapid, with organogenesis complete by 3 d post fertilization (3 dpf). The zebrafish genome contains orthologs for $\sim 70\%$ of human genes (Howe et al. 2013) and $\sim 86\%$ of the genes that are known human drug targets (Gunnarsson et al. 2008). Zebrafish developmental toxicity testing can be completed in a matter of days by directly exposing the developing organism to xenobiotics. Post-hatch, automated locomotor behavior tests can be used to assess swimming behavior in response to a variety of stimuli as a functional neurodevelopmental outcome. One major limitation of the zebrafish

model for toxicity testing relates to chemical dosimetry. Zebrafish embryos are exposed to xenobiotics via immersion. In most studies, nominal waterborne concentrations are generally reported when making determinations on compound toxicity (i.e., positive or negative for toxicity). However, based on physicochemical properties like LogP and differences in exposure parameters (e.g., static vs. semi-static exposures), both of which can affect the uptake, distribution, metabolism, and elimination of test chemicals, the internal tissue dose does not generally reflect nominal exposure media concentrations (Brox et al. 2014, 2016; Kirla et al. 2016; Souder and Gorelick 2017).

The developmental toxicity and developmental neurotoxicity of a subset of PFAS, such as PFOS and PFOA, have been previously evaluated in zebrafish (Hagenaars et al. 2011; Huang et al. 2010; Jantzen et al. 2016; Khezri et al. 2017; Spulber et al. 2014; Ulhaq et al. 2013a, 2013b). PFOS exposure results in failed swim bladder inflation, abnormal ventroflexion of the tail (Hagenaars et al. 2011; Huang et al. 2010; Jantzen et al. 2016; Ulhaq et al. 2013a), and hyperactivity (Hurley et al. 2018; Khezri et al. 2017; Spulber et al. 2014), whereas results for PFOA exposures are quite mixed for both developmental toxicity and behavior (Hagenaars et al. 2011; Huang et al. 2010; Jantzen et al. 2016; Khezri et al. 2017; Padilla et al. 2012; Truong et al. 2014; Ulhaq et al. 2013a, 2013b). However, because replacement PFAS such as GenX and ADONA are detected in the environment yet lack adequate data on their potential toxicity, the goal of this study was to assess the developmental toxicity, developmental neurotoxicity, and tissue doses of multiple aliphatic PFAS (e.g., PFOS, PFOA, PFHxS, and PFHxA), several emerging replacement PFAS (e.g., GenX and ADONA), and a polymer production by-product [e.g., perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid (PFESA1)] in parallel, using zebrafish as a test organism. In addition, the potential of sulfonic acid PFAS with varying alkyl chain lengths to elicit similar toxicity phenotypes was assessed.

Methods

Zebrafish Husbandry

All procedures involving zebrafish were approved by the U.S. Environmental Protection Agency (EPA) National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee and carried out in accordance with the relevant guidelines and regulations. Embryos were obtained from a mixed wild-type (WT) adult zebrafish line (*Danio rerio*) that was generated and maintained as previously described (Phelps et al. 2017). Briefly, to maintain genetic diversity, a minimum of one WT line (AB and/or Tupfel long fin WT strains) was added one time per year. Zebrafish adults were housed in 6-L tanks at an approximate density of $8\ \text{fish/L}$. Adults were fed Gemma Micro 300 (Skretting) once daily and shell free E-Z Egg (Brine Shrimp Direct) twice daily Mondays through Fridays. Both food sources were fed once daily on weekends. U.S. EPA WT zebrafish were maintained on a 14 h:10 h light cycle at 28.5°C and bred every 2–3 weeks. For embryo collection, 60–100 adults were placed in 10- or 20-L angled static breeding tanks overnight. The following morning, adults were transferred to new angled bottom tanks containing fish facility water, and embryos were collected 30–40 min later.

Chemical Preparation

ADONA [Chemical Abstracts Service Registry No. (CASRN): 958445-44-8; Catalog No. NaDONA] was purchased from Wellington Laboratories (Table 1). GenX Free Acid (CASRN: 13252-13-6; Catalog No. 2121-3-13), PFHxA (CASRN: 307-24-4; Catalog No. 2121-3-39), PFHxS (CASRN: 3871-99-6; Catalog No. 6164-3-X4), PFOA (CASRN: 335-67-1; Catalog No. 2121-3-18), PFOS (CASRN: 1763-23-1; Catalog No. 6164-3-08), perfluorobutanesulfonic acid (PFBS; CASRN: 375-73-5; Catalog No. 6164-3-09), and perfluoroheptanesulfonic acid (PFHpS; CASRN: 375-92-8; Catalog No. 6164-3-2S) were purchased from Synquest. Perfluoropentanesulfonic acid (PFPeS; CASRN 2706-91-4; Catalog No. 6164-3-2U) was synthesized for the study by Synquest Laboratories and chlorpyrifos (CASRN: 2921-88-2; Catalog No. 45395) was purchased from Sigma-Aldrich. PFESA1 (CASRN: 29311-67-9) was obtained from Chemours (Table 1). Stock solutions ($20\ \mathrm{mM}$ or $25\ \mathrm{mM}$) were prepared either by mixing liquid chemical or dissolving neat chemical into molecular-grade dimethyl sulfoxide (DMSO) ($>99.9\%$) or deionized (DI) water, and aliquots were stored at $-80\ ^{\circ}\mathrm{C}$. For each experiment, $250\times$ working solutions were prepared by thawing single-use stock solution aliquots and performing semi- or quarter-log serial dilutions in DMSO or DI water in a 96-well polycarbonate microtiter plate. Stock plates containing $250\times$ working solutions were sealed (Biorad; Catalog No. MSB1001) and stored at room temperature in the dark and used for the duration of each study (maximum storage time of 5 weeks).

Table 1 Test chemicals.

Chemical	Name	CASRN	MW (g/mol)	LogP[a] (OPERA[b])
4,8-Dioxa-3H-perfluorononanoate	ADONA	958445-44-8	400.05	3.96
Perfluoro-2-propoxypropanoic acid	GenX Free Acid	13252-13-6	330.05	3.21
Perfluorobutanesulfonic acid	PFBS	375-73-5	300.1	3.10
Perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid	PFESA1	29311-67-9	444.12	6.02
Perfluoroheptanesulfonic acid	PFHpS	375-92-8	450.12	2.83
Perfluorohexanoic acid	PFHxA	307-24-4	314.05	2.78
Perfluorohexanesulfonic		3871-		

acid	PFHxS	99-6	438.21	3.87
Perfluoro-*n*-octanoic acid	PFOA	335-67-1	414.07	3.79
Perfluorooctanesulfonic acid	PFOS	1763-23-1	500.13	2.77
Perfluoropentanesulfonic acid	PFPeS	2706-91-4	350.11	3.18

Note: CASRN, Chemical Abstracts Service Registration Number; MW, molecular weight.

[a]Partition coefficient.

[b]OPEn structure-activity/property Relationship App (OPERA) (https://comptox.epa.gov/dashboard).

Study Design

In Study 1 (Figure 1), the developmental toxicity and developmental neurotoxicity and the media and internal tissue doses of ADONA, GenX Free Acid, PFESA1, PFHxA, PFHxS, PFOA, and PFOS were determined, using DMSO as a vehicle. All chemicals except PFESA1 were tested in parallel and shared the same DMSO control samples for all three assays. PFESA1 was obtained subsequently from Chemours and therefore had unique, experiment-specific control data. In Study 1, GenX Free Acid diluted in DMSO was determined to be unstable, resulting in a null data set that was therefore excluded. In Study 2 (Figure 1), zebrafish were exposed to GenX Free Acid diluted in DI water and evaluated in the developmental toxicity (DevTox) and developmental neurotoxicity (DNT) assays. Measured media and tissue doses were also obtained. Last, in a sulfonic acid PFAS follow-up study (Study 3) (Figure 1), the ability of PFBS (4-carbon), PFPeS (5-carbon), PFHxS (6-carbon), PFHpS (7-carbon), or PFOS (8-carbon) exposure to cause developmental toxicity or developmental neurotoxicity was assessed. All chemicals tested in Study 3, except PFPeS, were exposed in parallel and have shared DMSO control data. PFPeS was synthesized for this study and tested separately, with an experiment-specific DMSO control.

Figure 1. Study design. Zebrafish were semi-statically exposed to test PFAS daily, from 0 5 dpf. At 6 dpf. developmental toxicity, developmental neurotoxicity,

and PFAS tissue concentrations were assessed. Test PFAS included in Study 1, solubilized in DMSO (final concentration 0.4% DMSO), are highlighted in light blue. Because GenX Free Acid was not stable in DMSO, the compound was retested in all three assays using DI water as a diluent in Study 2 (highlighted in blue). In Study 3, a set of sulfonic acid aliphatic PFAS solubilized in DMSO were tested in the DevTox and DNT assays (shown in green). Note: ADONA, 4,8-dioxa-3H-perfluorononanoate; DevTox, developmental toxicity; DI, deionized; DMSO, dimethyl sulfoxide; DNT, developmental neurotoxicity; dpf, days post fertilization; GenX Free Acid, perfluoro-2-propoxypropanoic acid; PFAS, per- and polyfluoroalkyl substances; PFBS, perfluorobutanesulfonic acid; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHpS, perfluoroheptanesulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid; PFPeS, perfluoropentanesulfonic acid.

Chemical Exposures

At 0 dpf, zebrafish embryos were bleached as previously described (Tal et al. 2017). A single embryo at the dome-to-epiboly stages (Kimmel et al. 1995) was placed into each individual well of a 96-well plate containing a $40\text{-}\mu\mathrm{m}$ nylon mesh filter (Millipore, Catalog No. MANMN4010) with $400\,\mu\mathrm{L}$ of 10% Hanks' balanced salt solution (HBSS) per well. Filter inserts containing zebrafish embryos were transferred to 96-well culture trays (Millipore, Catalog No. MAMCS9610) containing $250\,\mu\mathrm{L}$ of 10% HBSS (Westerfield 2007) and $1\,\mu\mathrm{L}$ of $250\times$ working solutions per well. A final concentration of 0.4% DMSO was used for all exposure groups and as a vehicle control. In the case of GenX Free Acid in Study 2 (Figure 1), DI water was used as a vehicle control. Daily, from 1 5 dpf, plates underwent 100% media changes to refresh chemical dosing solutions by blotting (Brandel; Catalog No. FPXLR-196) and transferring mesh inserts containing zebrafish to new bottom plates (Millipore; Catalog No. MAMCS9610). To minimize evaporation, plates were sealed (Biorad; Catalog No. MSA5001) and wrapped with parafilm. Plates were maintained on a 14 h:10 h light cycle at 26.0°C and scored daily for death, malformations, hatching, and swim bladder inflation. At 6 dpf, plates were evaluated by two independent observers and DevTox or DNT assays were performed or media and tissue were collected for analytical chemistry analyses as described below.

Developmental Toxicity Assay

In Study 1 (Figure 1), zebrafish were exposed, as described in the "Chemical Exposures" section, to 0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, or $80.0\,\mu\mathrm{M}$ PFOS, PFOA, PFHxS, PFHxA, or ADONA, or 0.4% DMSO. Six 96-well plates were

tested with a single chemical concentration included on each microtiter plate. Subsequently, as part of Study 1, zebrafish were exposed to 0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, or $80.0\mu M$ PFESA1, or 0.4% DMSO. The number of biological replicates per study and additional experimental details are shown in Table 2. In Study 2, GenX Free Acid diluted in DI water was tested by exposing zebrafish to 0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, or $80.0\mu M$ of the compound or DI water. In a follow-up study to assess the toxicity of aliphatic sulfonic acid PFAS (Study 3), a higher starting concentration was used to increase the likelihood of observing both malformations with shorter-chain compounds and malformations at multiple test concentrations. Zebrafish were exposed to 1.7, 3.1, 5.5, 9.8, 17.6, 31.4, 56.0, or $100.0\mu M$ of PFBS, PFHxS, PFHpS, or PFOS or 0.4% DMSO. Subsequently, zebrafish were exposed to 1.7, 3.1, 5.5, 9.8, 17.6, 31.4, 56.0, or $100.0\mu M$ PFPeS, or 0.4% DMSO. Chlorpyrifos was used as positive control for malformations ($8.0\mu M$) and lethality ($80.0\mu M$) (Padilla et al. 2012; Tal et al. 2017). To conduct DevTox assay assessments, at 6 dpf, two independent observers evaluated zebrafish larvae for survival, hatching, swim bladder inflation, and malformations, including curved body axis, shortened trunk, pericardial edema, yolk sac edema, necrotic yolk sac, pectoral fin abnormalities and head/jaw abnormalities. Directly after assessments, data were reviewed and, in the case of discrepancies, consensus calls were reached. Toxicity values were assigned to descriptive data (i.e., $\text{normal}=0$, $\text{abnormal}=20$, severely $\text{abnormal}=50$, and $\text{dead}=100$), modified from a previously described approach (Padilla et al. 2012). Briefly, animals with a single malformation were scored as abnormal, whereas animals with ≥ 2 malformations were scored as severely abnormal. A study inclusion criterion based on a previously published study (Padilla et al. 2012) was applied where microtiter plates with $>15\%$ abnormal or dead DMSO or DI water control larvae were excluded (one plate from Study 3 was excluded).

Table 2 Study-specific metrics.

Study	Name	Diluent and/or vehicle	Assay	Concentrations tested (μM)	Expos replica (*n*)
1	ADONA	DMSO	DevTox	0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, 80.0	6
			DNT	4.4, 7.9, 14.0, 25.1, 44.8, 80.0	24
			Chemistry	25.1, 44.8, 80.0	5
1	GenX Free	DMSO	DevTox, DNT,	Not stable in DMSO; res	

	Acid		Chemistry		
1	PFESA1	DMSO	DevTox	0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, 80.0	6
			DNT	4.4, 7.9, 14.0, 25.1, 44.8, 80.0	24
			Chemistry	25.1, 44.8, 80.0	4
1	PFHxA	DMSO	DevTox	0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, 80.0	6
			DNT	4.4, 7.9, 14.0, 25.1, 44.8, 80.0	24
			Chemistry	25.1, 44.8, 80.0	4
1	PFHxS	DMSO	DevTox	0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, 80.0	6
			DNT	4.4, 7.9, 14.0, 25.1, 44.8, 80.0	24
			Chemistry	14.0, 25.1, 44.8	4
1	PFOA	DMSO	DevTox	0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, 80.0	6
			DNT	4.4, 7.9, 14.0, 25.1, 44.8, 80.0	24
			Chemistry	25.1, 44.8, 80.0	4
1	PFOS	DMSO	DevTox	0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, 80.0	6
			DNT	0.2, 0.3, 0.6, 1.0, 1.8, 3.1	24
			Chemistry	1.0, 1.8, 3.1	4
2	GenX Free Acid	DI water	DevTox	0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, 80.0	6
			DNT	4.4, 7.9, 14.0, 25.1, 44.8, 80.0	24
			Chemistry	25.1, 44.8, 80.0	4

3	PFBS	DMSO	DevTox	1.7, 3.1, 5.5, 9.8, 17.6, 31.4, 56.0, 100.0	10
			DNT	5.5, 9.8, 17.6, 31.4, 56.0, 100.0	25
3	PFPeS	DMSO	DevTox	1.7, 3.1, 5.5, 9.8, 17.6, 31.4, 56.0, 100.0	6
			DNT	3.1, 5.5, 9.8, 17.6, 31.4, 56.0	24
3	PFHxS	DMSO	DevTox	1.7, 3.1, 5.5, 9.8, 17.6, 31.4, 56.0, 100.0	10
			DNT	3.1, 5.5, 9.8, 17.6, 31.4, 56.0	25
3	PFHpS	DMSO	DevTox	1.7, 3.1, 5.5, 9.8, 17.6, 31.4, 56.0, 100.0	10
			DNT	1.7, 3.1, 5.5, 9.8, 17.6, 31.4	25
3	PFOS	DMSO	DevTox	1.7, 3.1, 5.5, 9.8, 17.6, 31.4, 56.0, 100.0	10
			DNT	0.5, 1.0, 1.7, 3.1, 5.5, 9.8	25

Note: ADONA, 4,8-dioxa-3H-perfluorononanoate; DevTox, developmental toxicity; DI, deionized; DMSO, dimethyl sulfoxide; DNT, developmental neurotoxicity; GenX Free Acid, perfluoro-2-propoxypropanoic acid; PFBS, perfluorobutanesulfonic acid; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHpS, perfluoroheptanesulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid; PFPeS, perfluoropentanesulfonic acid.

[a]Replicate numbers indicate single animals except for chemistry samples comprising pools of 10 larvae.

[b]Indicates total number of 96-well microtiter plates assessed for each study and/or assay. For Study 1 DevTox and DNT assays, ADONA, GenX Free Acid, PFESA1, PFHxA, PFHxS, PFOA, and PFOS were tested in parallel and shared the same 0.4% DMSO control samples. PFESA1 was obtained subsequently and had unique, experiment-specific control data. In Study 1, GenX Free Acid diluted in 0.4% DMSO was determined to be unstable,

resulting in a null data set. In Study 2 (Figure 1), zebrafish were exposed to GenX Free Acid diluted in DI water and evaluated in the DevTox and DNT assays. Measured media and tissue doses were also obtained. Study 3 (Figure 1) examined the ability of PFBS (4-carbon), PFPeS (5-carbon), PFHxS (6-carbon), PFHpS (7-carbon), or PFOS (8-carbon) exposure to cause developmental toxicity or developmental neurotoxicity. All chemicals tested in Study 3, except PFPeS, were exposed in parallel and have shared DMSO control data. PFPeS was synthesized for this study and tested separately, with an experiment-specific DMSO control.

Developmental Neurotoxicity Assay

To increase the likelihood of observing behavioral effects in morphologically normal larvae, the highest concentration evaluated in the DNT assay was the lowest observed effect concentration (LOEC) determined in DevTox assay. Zebrafish were exposed in parallel to 4.4, 7.9, 14.0, 25.1, 44.8, or $80.0\mu M$ PFOA, PFHxS, PFHxA, or ADONA or 0.2, 0.3, 0.6, 1.0, 1.8, or $3.1\mu M$ PFOS or 0.4% DMSO. Subsequently, as part of Study 1, exposure to 4.4, 7.9, 14.0, 25.1, 44.8, or $80.0\mu M$ PFESA1, or 0.4% DMSO was evaluated. In Study 2, zebrafish were exposed to 4.4, 7.9, 14.0, 25.1, 44.8, or $80.0\mu M$ GenX Free Acid, or DI water. In Study 3, to increase the likelihood of observing malformations with shorter-chain compounds at multiple test concentrations, the highest concentration evaluated was $100.0\mu M$. Zebrafish were exposed to 5.5, 9.8, 17.6, 31.4, 56.0, or $100.0\mu M$ PFBS; 3.1, 5.5, 9.8, 17.6, 31.4, or $56.0\mu M$ PFHxS; 1.7, 3.1, 5.5, 9.8, 17.6, or $31.4\mu M$ PFHpS; or 0.5, 1.0, 1.7, 3.1, 5.5, or $9.8\mu M$ PFOS, or 0.4% DMSO. Subsequently, as part of Study 3, zebrafish were exposed to 3.1, 5.5, 9.8, 17.6, 31.4, or $56.0\mu M$ PFPeS, or 0.4% DMSO. Chemical exposures and assessments were performed daily, as described above. To evaluate swimming behavior in a light/dark behavior test, microtiter plates were placed in a dark, temperature-controlled behavior testing room set to 26.0°C for at least 2 h prior to testing. At the time of testing, microtiter plates were placed on a Noldus tracking apparatus. Locomotor activity was recorded (30 frames/s) for a total of 60 min consisting of a 20-min dark acclimation period (0 lux) that was not analyzed followed by a 40-min testing period consisting of a 20-min light period (5.0 lux) and 20-min dark period (0 lux). Videos were analyzed using Ethovision software (version 3.1; Noldus Information Technology) as previously described (Jarema et al. 2015). Locomotor activity was collected for each individual fish for each 2-min period (minimum distance moved, set to 0.135cm). Thus, for a 40-min test, 20 data points were collected per larvae. Based on microtiter plate inclusion criterion (i.e., $<15\%$ abnormal or dead control larvae), two plates from Study 1 were excluded. Four additional criteria for inclusion of individual larvae were applied. One, all larvae that were identified as abnormal, severely abnormal, or dead were excluded. Two, larvae with uninflated swim bladders were removed from analyses. Three, individual larvae that moved $<2\text{cm}$ in either 10-min dark period were removed. Four, concentrations of test PFAS with fewer than 13 animals remaining (i.e.,

$>50\%$ death or malformations within the test group) were excluded from behavior analyses. Also as part of the DNT assay, media samples were collected. At 6 dpf, $150\text{-}\mu\mathrm{L}$ media samples ($\mathrm{n}=3/\text{chemical concentration}$) were collected and stored at $-80\,^{\circ}\mathrm{C}$ until further analysis.

Tissue Sample Preparation for Analytical Chemistry

Zebrafish were exposed to 25.1, 44.8, or $80.0\,\mu\mathrm{M}$ ADONA, PFOA, PFESA1, or PFHxA, 14.0, 25.1, or $44.8\,\mu\mathrm{M}$ PFHxS, or 1.0, 1.8, or $3.1\,\mu\mathrm{M}$ PFOS, or 0.4% DMSO. The highest concentration evaluated was the no observed effect concentration (NOEC) determined in Study 1. In Study 2, zebrafish were exposed to 25.1, 44.8, or $80.0\,\mu\mathrm{M}$ GenX Free Acid or 0.4% DMSO. According to the previously described microtiter plate inclusion criterion, 15/15 plates were included in the study. Larvae were anesthetized by rapid cooling in chilled 10% HBSS. Groups of 10 anesthetized larvae were pooled to comprise one biological replicate ($\mathrm{n}=4$) in $500\,\mu\mathrm{L}$ of 10% HBSS, flash frozen in liquid nitrogen, and stored at $-80\,^{\circ}\mathrm{C}$. In the case of ADONA, $\mathrm{n}=5$.

Analytical Chemistry

PFAS native standards were obtained from SynQuest and Sigma-Aldrich. $^{13}\mathrm{C}$-labeled PFAS standards were obtained from Wellington Laboratories. Stock solutions of the PFAS were prepared in 95% methanol with 5% aqueous $2.5\mathrm{M}$ sodium hydroxide and stored at room temperature in plastic. Intermediate standards were prepared daily in methanol or acetonitrile.

Exposure Media Analysis

Exposure media samples (10% HBSS) and quality control (QC) samples (10% HBSS) at microgram-per-milliliter concentrations were diluted and fortified with a surrogate [i.e., perfluorononanoic acid (PFNA)] and internal standards. Calibration standards were prepared at nanogram-per-milliliter concentrations in aqueous $2.5\ \mathrm{mM}$ ammonium acetate with 20% methanol. Standards and samples were analyzed with ACQUITY ultra-high-performance liquid chromatography (UPLC) system and Quattro Premier XE triple quadrupole mass spectrometer (Waters Corporation) operated in negative electrospray ionization (ESI) mode. ESI source conditions were optimized for the $[\mathrm{M\text{-}H}]^-$ ion of PFAS as follows: capillary voltage $-1.97\ \mathrm{kV}$, source temperature 150°C, desolvation temperature 350°C, cone gas flow $2\mathrm{L/h}$, and desolvation gas flow $350\mathrm{L/h}$. Compound-specific tandem mass spectrometry (MSMS) parameters were used to collect two multiple reaction monitoring (MRM) transitions for the $[\mathrm{M\text{-}H}]^-$ ion of each target analyte (Table 3). UPLC separation was achieved using ACQUITY UPLC BEH C18 Column, 130 , $1.7\,\mu\mathrm{M}$, $2.1\mathrm{mm}\times 50\mathrm{mm}$ (Waters Corp P/N 186,002,350) at 50°C with the gradient elution at a flow rate of $500\,\mu\mathrm{L/min}$ using $2.5\ \mathrm{mM}$ ammonium acetate in methanol and water with a $50\text{-}\mu\mathrm{L}$ injection. Data collection,

integration, calibration, and quantitation were performed using MassLynx software (version 4.1; Waters Corporation). Concentration for each PFAS analyte was determined by internal standard technique using isotopically labeled internal standards and calibration standards prepared in solvent. Qualitative identification was based on relative retention time and peak abundance ratio of two MRM transitions. Exposure media analysis was verified and evaluated using blanks, calibration standards, and QC standards prepared at three concentrations across the methods range. Batch results for exposure media were evaluated based on the following criteria: the calibration curve used a minimum of seven standards with a correlation coefficient of >0.99, standards accuracy tolerance $<20\%$ (30% at LLOQ), QC standard accuracy tolerance $<20\%$ (30% PFOS), QC standard precision expressed as percent relative standard deviation $\%\mathrm{RSD}\ <20\%$, $>75\%$ of QC standards satisfied accuracy criteria. Exposure media analysis method performance characteristics are listed in Excel Tables S1 and S2.

Table 3 Compound-specific Quattro Premier XE MSMS parameters.

Compound name	Parent (m/z)	Daughter (m/z)	Cone (V)	Collision (V)
GenX 1°	329.07	284.06	10	5
GenX 2°	329.07	184.72	10	23
$C_3 13$ GenX IS	332.00	287.06	10	5
PFHxA 1°	312.91	268.81	15	9
PFHxA 2°	312.91	118.64	15	25
$C_2 13$ PFHxA IS	315.00	269.81	15	9
PFHxS 1°	398.85	98.57	55	37
PFHxS 2°	398.85	79.62	55	41
$C_3 13$ PFHxS IS	401.85	79.62	55	41
PFOA 1°	412.93	368.84	15	11
PFOA 2°	412.93	168.63	15	21
$C_4 13$ PFOA IS	417.00	372.00	15	11
PFOS 1°	499.00	98.57	60	41
PFOS 2°	499.00	79.62	60	45
$C_4 13$ PFOS IS	503.00	98.57	60	41
ADONA 1°	377.02	250.83	15	13

ADONA 2°	377.02	84.70	15	29
PFESA1 (Nafion, BP 1) 1°	442.98	146.69	35	29
PFESA1 (Nafion, BP 1) 2°	442.98	262.79	35	19
PFNA 1°	463.00	418.90	15	13
PFNA 2°	463.00	218.84	15	17
C_513 PFNA IS	468.00	423.00	15	13

Note: 1° denotes the primary multiple reaction monitoring (MRM) transition for a compound used for quantitative analysis and 2° denotes the secondary MRM transition for a compound used for qualitative identification confirmation. ADONA, 4,8-dioxa-3H-perfluorononanoate; GenX Free Acid, perfluoro-2-propoxypropanoic acid; MSMS, tandem mass spectrometry; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid; PFPeS, perfluoropentanesulfonic acid; Quattro Premier XE, triple quadrupole mass spectrometer.

Tissue Analysis

Tissue samples were prepared by protein precipitation. Flash frozen samples, consisting of pools of ten 6-dpf zebrafish larvae, were homogenized using $\sim 100\text{mg}$ of 1.0-mm diameter zirconia/silica beads and a Fast-Prep-24™ homogenizer (MP Biomedicals) in $100\mu\text{L}$ 0.1M formic acid fortified with the surrogate (i.e., PFNA). Protein was precipitated from the homogenate with $400\mu\text{L}$ of acetonitrile containing internal standards and separated by centrifugation at $14{,}000\ \text{rpm}$ for 15 min at 4°C. Fifty microliters of the extract was diluted with $200\mu\text{L}$ aqueous $0.4\ \text{mM}$ ammonium formate in the LC vial for analysis.

Standards and samples were analyzed with Vanquish UPLC and Orbitrap Fusion mass spectrometer (Thermo Electron) operated in negative ESI mode. ESI source conditions were optimized for the $[\text{M-H}]^-$ ion of PFAS as follows: spray voltage $-3.5\ \text{kV}$, sheath gas 25 au (arbitrary units), aux gas 6 au, sweep gas 0 au, ion transfer tube temperature 300°C, and vaporizer temperature 30°C. High-resolution accurate mass (HRAM) MS1 scans were collected with the following parameters: detector type orbitrap, orbitrap resolution 30,000 full width at half maximum (FWHM), normal mass range, Use $\text{quadrapole isolation} = \text{true}$, scan range $70\ \ 700\text{m/z}$, radio frequency (RF) lens 60%, automatic gain control (AGC) target 4.00×10^5, max injection time of $50\ \text{ms}$, 1 microscan, data type set to profile, negative polarity, and source fragmentation disabled. Data-dependent orbitrap MSMS (ddMS2-OT) scans were collected for the $[\text{M-H}]^-$ ion [

$M\text{-}H\text{-}CO_2$ for GenX] using a target mass list for the eight PFAS and PFNA surrogate for identity confirmation. The apex detection was set to an expected peak width of 2 s at FWHM and desired apex peak window of 30%. The dynamic exclusion parameters were set to exclude after a one-time, 60-s exclusion duration, low and high mass tolerances of $10\,\mathrm{ppm}$, and exclude isotope was set to true. The intensity threshold for collecting an MSMS scan was set to $2.5\mathrm{E}+04$. Fragmentation was done by high-energy collisional dissociation (HCD). The ddMS2-OT scans were collected with the following parameters: orbitrap isolation mode, isolation window of $1.6\mathrm{m/z}$, isolation offset off, stepped HCD collision energy of $30\% \pm 10\%$, scan range of auto mass-to-charge ratio normal, orbitrap resolution of 30,000 FWHM, first mass $75\mathrm{m/z}$, max injection time of 54 s, AGC target 5.00×10^{4}, inject ions from available parallelizable time set to true, max injection time of 54 ms, 1 microscan, and data type set to centroid. UPLC separation was achieved using ACQUITY UPLC BEH C18 Column, 130 , $1.7\mu\mathrm{M}$, $2.1\mathrm{mm}\times50\mathrm{mm}$ (Waters Corp P/N 186,002,350) at 50°C with gradient elution at a flow rate of $300\mu\mathrm{L/min}$ using $0.4\ \mathrm{mM}$ ammonium formate in acetonitrile and water with a $10\text{-}\mu\mathrm{L}$ injection. Data collection, integration, calibration, and quantitation were performed using Xcalibur software (version 4.1; Thermo Electron). Concentration for each PFAS analyte was determined by internal standard technique using isotopically labeled internal standards and matrix-matched calibration standards. Quantitative analysis was performed using high-resolution MS1-extracted ion chromatograms. Qualitative identification was based on relative retention time, MS1 peak abundance ratio of $[\mathrm{M\text{-}H}]^-$ to a source decomposition product, and ddMS2-OT spectra. Tissue analysis was verified and evaluated using blanks, matrix-matched calibration standards, and matrix-matched QC standards prepared at three concentrations across the method's range. Batch results for exposure media were evaluated based on the following criteria: the calibration curve used a minimum of seven standards with a correlation coefficient of >0.99, standards accuracy tolerance $<20\%$ (30% at LLOQ), QC standard accuracy tolerance $<20\%$, QC standard precision expressed as $\%\mathrm{RSD}<20\%$, $>75\%$ of QC standards satisfy accuracy criteria. The tissue analysis method performance characteristics are listed in Excel Tables S3 and S4.

Statistics

For the DevTox assay, a Kruskal-Wallace nonparametric one-way analysis of variance (ANOVA) with a Dunn's multiple comparison test was used to detect differences between exposure groups ($^{*}p<0.05$, $^{**}p<0.0001$) and LOEC values were determined. If a test for linear trend was significant ($p<0.05$), with developmental toxicity observed at the highest concentration tested, nonlinear regression was performed with a Hill slope curve fitting for half maximal effective concentration (EC_{50}) value determinations.

For DNT assay results shown in Figures 3, 6, and 8, a repeated measures ANOVA analysis was used to detect differences in swimming behavior between

exposed and control larvae (Catron et al. 2019b; Irons et al. 2013; Phelps et al. 2017; Stevens et al. 2018). These analyses were performed using SAS (version 9.4; SAS Institute Inc.). Group means and standard errors were calculated by SAS Proc Means for each 10-min period included in the 40-min testing period [i.e., light 1 (L1), light 2 (L2), dark 1 (D1), and dark 2 (D2)]. Given that individual activity values were collected for each larva for every 2-min period, there were five data points for each 10-min light or dark period. First, means were calculated by individual larva across the five time points, then concentration group means, and standard errors were calculated using the larval means. Parametric analysis of locomotor data was conducted using SAS Proc Mixed. For each test chemical, a mixed-effects repeated measures model was run separately for each light or dark period (i.e., L1, L2, D1, D2). Each larva was considered a subject and an autoregressive covariance matrix was estimated across the five time points within each 10-min light or dark period. The fixed effects included in the model were as follows: experiment, plate nested within experiment, concentration, time, and the two-way interaction concentration by time. If the concentration effect within each 10-min light or dark period was significant ($p < 0.0125$) (i.e., because the same larvae were tested for four 10-min time periods), then pairwise *t*-tests were computed, comparing each concentration group to the control group ($p < 0.05$). Dunnett's test was used to adjust for multiple comparisons ($p < 0.05$). The parametric mixed model analysis included the ability to model design variables (e.g., plate or day of test), the correlated structure of the repeated distance measurements, and relationships among fixed effects (e.g., concentration, time). These statistics are displayed in Figures 3, 6, and 8 and Excel Tables S5–S7. To conclude that a test chemical was positive for developmental neurotoxicity, significance must have been detected at either more than one concentration within a time period or at the same concentration across multiple time periods.

In addition to the evaluation of mean movement measures described above, linear mixed-effects models (SAS Proc Mixed) were used to examine individual movement measures from individual zebrafish as a function of time, concentration, and $\text{time} \times \text{concentration}$. Data from light and dark periods were evaluated separately, given the clear differences in time trends during each period. Nearly half of the light period data were at or below the limit of detection (LOD) (0.135cm), whereas $<5\%$ of the dark period data were $<\text{LOD}$. The LOD was based on the minimum distance moved value, set to 0.135cm in Ethovision. This means that individual larva must move, from one frame to the next, a minimum of 0.135cm, to be considered in motion. All values $<\text{LOD}$ were initially assigned a value of LOD divided by the square root of 2. Inspection of QQ-plots (see Figure S1A,D) indicated that both light and dark period data were not normally distributed (Pleil 2016b). A square root transformation was therefore performed to improve the shape of the upper end of each measurement distribution (see Figure S1B,E). Because square root transformation did not sufficiently alter the lower end of each distribution, a multiple value imputation strategy was used (Pleil 2016a). This strategy is analogous to robust regression on order statistics (ROS), a widely used imputation strategy for censored data. Briefly, for the light period data, $\sim 50\%$ of measurements were $<\text{LOD}$ and values were therefore imputed for this $\sim 50\%$ (see Figure S1C). Zebrafish increased locomotor activity in the light period over time. An inverse trend between time and

the percentage of behavior measurements $<\mathrm{LOD}$ was observed (see Figure S2A). For chemicals that caused light-phase hyperactivity (i.e., PFOS and PFHxS), a modest inverse relationship between chemical concentration and the percentage of measurements $<\mathrm{LOD}$ was also observed (see Figure S2B,C). No clear trend between chemical concentration and percentage of values $<\mathrm{LOD}$ emerged for compounds that were negative for light period hyperactivity (see Figure S2C–G). For the dark period data, $\sim 5\%$ of measurements were $<\mathrm{LOD}$. Given the shape of the distribution (see Figure S1E), values were ultimately imputed for the lowest 30% of the data (see Figure S1F). This ROS imputation allowed the order of the raw data to be preserved in the final corrected distribution.

To carry out the ROS technique, for both light and dark period data sets, all measurements were ordered from smallest to largest. Values $<\mathrm{LOD}$ were all equivalent, and were therefore extracted, randomly sorted, and placed back into the main data sets. Next, the relative position ($\mathrm{p_r}$) of each measurement was determined according to the equation: $\mathrm{p_r} = (\mathrm{n} - 0.5)/\mathrm{N}$, where n is the rank for a given measurement and *N* is the total number of measurements. A *z*-score was then assigned to each measurement using the probit function in SAS (version 9.4). Here, the assigned *z*-score represents the quantile for a specific measurement, assuming it is from a standard normal distribution. In both data sets, the square root–adjusted measurements were regressed on *z*-scores where the regression was restricted to the upper 50% of the light period distribution and to the upper 70% of the dark period distribution. Regression equations were used to predict square root–adjusted measurements for the lower portions of the measurement distributions (see Figure S1C,F). The combined use of square root transformation and multiple value imputation allowed key regression assumptions (i.e., homoskedasticity and normality of residuals) to be met.

In all mixed models, the square root–transformed movement data were regressed on time, concentration, and the interaction of $\mathrm{time} \times \mathrm{concentration}$. For the light period, measurements were considered between $\mathrm{T} = 04$ and $\mathrm{T} = 20\ \mathrm{min}$. For the dark period, measurements were considered between $\mathrm{T} = 24$ and $\mathrm{T} = 40\ \mathrm{min}$. Data at $\mathrm{T} = 02$ (light period) and $\mathrm{T} = 22$ (dark period) minutes were considered to reflect transition periods and were, therefore, excluded from the analysis. All mixed models included a random effect for zebrafish, thus allowing partitioning of measurement variance into that which was observed between and within (over time) individual organisms. A compound symmetry covariance matrix was used, which assumes constant correlated errors between time points within organisms. Observed *p*-values for time indicate whether the linear effect of time on movement is significantly different than 0. The *p*-values for concentration indicate whether the intercept for movement (at $\mathrm{T} = 04\ \mathrm{min}$ for the light period, and $\mathrm{T} = 24\ \mathrm{min}$ for the dark period) differs across concentration groups (with DMSO set as the reference group). Finally, the *p*-values for $\mathrm{time} \times \mathrm{concentration}$ indicate whether the linear relationship between time and movement changes as a function of concentration (with DMSO set as the reference). Mixed model results were used to estimate zebrafish movement at specific time points in the light and

dark periods. Specifically, regression equations were used to estimate movement at $T = 10$ and $T = 20\ \text{min}$ in the light period, and at $T = 30$ and $T = 40\ \text{min}$ in the dark period [note: any estimates that were $< \text{LOD}$ (occurring in the light period only) were assigned a value of LOD divided by the square root of 2]. The difference in estimated movement during each period was ultimately used to gauge the magnitude of concentration-related effects on movement across all study chemicals.

For targeted analytical chemistry, PFAS concentrations were measured across media ($n = 3$ replicates) and tissue samples ($n = 4$ replicates, each comprising 10 pooled larvae). Measured media samples that were below the method detection limit ($< \text{MDL}$) (see Excel Table S8) were replaced with the value MDL divided by the square root of 2. To control for heteroskedasticity, log-transformation was performed followed by linear regression of log(measured media concentration) on log(nominal media concentration). Linear regression did not consider $\text{concentration} = 0$ samples (i.e., $< \text{MDL}$). Linear regression therefore considered measurements $> \text{MDL}$ and, further, met assumptions of normality and homoscedasticity. Significance indicates that a linear increase in measured concentration was observed in accordance with rising nominal concentrations ($p < 0.05$). Measured tissue samples $< \text{MDL}$ (see Excel Table S9) were replaced with the value MDL divided by the square root of 2. Welch's ANOVA was performed followed by a Dunnett T3 test (Dunnet 1980) ($p < 0.05$). If a single $\text{concentration} = 0$ sample was $> \text{MDL}$, all four exposure groups were considered for multiple comparison testing (this occurred for ADONA and PFESA1). If $\text{concentration} = 0$ samples were at or below the MDL, only the top three exposure groups were considered in the Dunnett T3 test (this occurred for PFHxA, PFHxS, PFOA, and PFOS). Additional one-sample Student's *t*-tests were then performed to compare measured concentrations to the MDL divided by the square root of 2 or to the lowest measured value above the MDL for $\text{concentration} = 0$ ($p < 0.05$).

Data Availability

The data sets generated during the current study are available in Science Hub by searching for the manuscript title at https://sciencehub.epa.gov/sciencehub/.

Results

Developmental Toxicity Phenotypes in Larval Zebrafish Exposed to PFAS

To determine whether exposure to PFAS caused developmental toxicity in larval zebrafish, embryos were exposed to $0.04\ \ 80.0\,\mu\text{M}$ PFOS, PFHxS, PFHxA, PFOA, ADONA, or PFESA1 or 0.4% DMSO daily from $0\ \ 5$ dpf (Study 1) (Figures 1 and 2). At 6 dpf, morphological assessments revealed developmental exposure to PFOS caused failed swim bladder inflation and ventroflexion of the tail, relative to DMSO control larvae (Figure 2A) and the EC_{50} value for developmental toxicity was calculated to be $7.5\,\mu\text{M}$ (Figure 2C). Exposure to PFHxS resulted in

the same morphological phenotypes as PFOS (Figure 2B,D) but was less potent ($EC_{50} = 92.7 \mu M$), although this value was derived from a concentration–response curve with just a single positive concentration and, therefore, may not be entirely reliable. In comparison, exposure to PFHxA, PFOA, ADONA, or PFESA1 did not cause concentration-dependent effects on survival or development (Figure 2E–H).

Figure 2. Measures of developmental toxicity in zebrafish exposed to PFAS. Zebrafish were semi-statically exposed to $0.04 \; 80.0 \mu M$ ADONA, GenX Free Acid, PFESA1, PFHxA, PFHxS, PFOA, or PFOS daily, from $0 \; 5$ dpf. At 6 dpf, larvae were assessed for developmental toxicity. Representative images for (A) PFOS and (B) PFHxS are shown. DevTox assay scores for (C) PFOS, (D) PFHxS, (E) PFHxA, (F) PFOA, (G) ADONA, or (H) PFESA1 are shown. Significance relative to the 0.4% DMSO control was determined by a Kruskal-Wallis ANOVA with a Dunn's multiple comparison test ($^{*}p < 0.05$, $^{**}p < 0.0001$). If a test for linear trend was significant ($p < 0.05$), with developmental toxicity observed at the highest concentration tested, nonlinear regression was performed with Hill slope curve fitting for half-maximal EC_{50} value determinations. $n = 6$ larvae per concentration per chemical tested. Note: ADONA, 4,8-dioxa-3H-perfluorononanoate; DevTox, developmental toxicity; DMSO, dimethyl sulfoxide; dpf, days post fertilization; EC_{50}, half maximal effective concentration; GenX Free Acid, perfluoro-2-propoxypropanoic acid; PFAS, per- and polyfluoroalkyl substances; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid.

Developmental Neurotoxicity Phenotypes in Larval Zebrafish Exposed to PFAS

To determine whether exposure to PFAS affect neurobehavioral development, zebrafish were exposed to $4.4 \; 80.0 \mu M$ PFHxS, PFHxA, PFOA, ADONA, or

PFESA1, $0.2\ \ 3.1\mu M$ PFOS or 0.4% DMSO daily, from $0\ \ 5$ dpf and locomotor activity was assessed at 6 dpf. Relative to DMSO, exposure to $1.0\mu M$ PFOS caused hyperactivity in the L1 period and exposure to $0.6\ \ 1.8\mu M$ PFOS caused hyperactivity in the L2 and D1 periods (Figure 3A,B). Like PFOS, developmental exposure to PFHxS caused hyperactivity in the L1 ($14.0\ \ 25.1\mu M$), L2 ($14.0\mu M$), and D1 (4.4, $14.0\ \ 25.1\mu M$) periods (Figure 3C,D). Finally, exposure to PFHxA resulted in hyperactivity relative to control in the L2 ($25.1\mu M$) and D1 ($14.0\ \ 25.1\mu M$) periods and, uniquely, in the D2 period ($14.0\ \ 25.1\mu M$) (Figure 3E,F). Zebrafish developmentally exposed to PFOA (Figure 3G,H), ADONA (Figure 3I,J), or PFESA1 (Figure 3K,L) did not exhibit differences in locomotor activity at 6 dpf. The effect of PFAS exposures on the slope of the response to light or dark stimuli was also determined (see Figures S3 and S4). Significant differences in estimated movement over the testing period were detected for all test chemicals (see Figure S3). However, the difference in estimated movement during each period, used to gauge the magnitude of concentration-related effects on movement across all study chemicals, only revealed qualitatively pronounced changes in larvae exposed to PFHxS or PFOS (see Figure S4).

Figure 3. Locomotor activity in zebrafish developmentally exposed to PFAS. Zebrafish were semi-statically exposed to $4.4\ \ 80.0\mu M$ ADONA, PFESA1, PFHxA, PFHxS, or PFOA, $0.2\ \ 3.1\mu M$ PFOS, or 0.4% DMSO as a vehicle control daily from $0\ \ 5$ dpf. At 6 dpf, larvae were assessed for developmental toxicity. Morphologically normal larvae with inflated swim bladders were subjected to behavioral testing. (A, C, E, G, I, K) Distance moved (cm) each 2-min period over the entire 40-min testing period are shown. (B, D, F, H, J, L) To make statistical comparisons, the mean distance moved during each 10-min light 1 (L1), 10-min light 2 (L2), 10-min dark 1 (D1), or 10-min dark 2 (D2) periods are shown. For all chemicals except PFESA1, 14–23 larvae were tested per chemical concentration and the same DMSO control larvae ($n=394$) were used. PFESA1 was tested separately ($n=35\ \ 40$ per chemical per concentration; 339 DMSO control larvae were evaluated). Repeated measures ANOVA models were run separately by period (L1, L2, D1, or D2). If a significant effect of concentration was detected ($p<0.0125$), within-period pairwise comparisons to control were computed using *t*-tests with a Dunnett adjustment for multiple comparisons (*$p<0.05$, **$p<0.001$).

Significance relative to period-specific DMSO controls are shown. Note: ADONA, 4,8-dioxa-3H-perfluorononanoate; ANOVA, analysis of variance; D, dark period; DMSO, dimethyl sulfoxide; dpf, days post fertilization; L, light period; PFAS, per- and polyfluoroalkyl substances; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid.

Bioaccumulation of PFAS in Larval Zebrafish

To measure media concentrations of PFAS, zebrafish were exposed to $4.4\ \ 80.0\mu M$ PFHxS, PFHxA, PFOA, ADONA, or PFESA1 or $0.2\ \ 3.1\mu M$ PFOS or DMSO daily, from $0\ \ 5$ dpf and at 6 dpf, exposure media was collected from wells. For all test chemicals, a linear increase in measured media concentration was observed (Figure 4). To quantitate tissue concentrations of test PFAS, zebrafish were exposed to $25.1\ \ 80.0\mu M$ PFHxA, PFOA, ADONA, or PFESA1, $14.0\ \ 44.8\mu M$ PFHxS, or $1.0\ \ 3.1\mu M$ PFOS or DMSO daily, from $0\ \ 5$ dpf and parent PFAS were measured in pools of 10 larvae $(n=4)$ (Figure 5; see also Excel Table S9). PFOS was the most bioaccumulative compound with calculated bioconcentration factor (BCF) values ranging from 684 to 1,375, depending on test concentration (Table 4). Fluoroether PFAS (i.e., ADONA and PFESA1) and PFHxA were the least bioaccumulative chemicals assessed (Table 4).

Figure 4. Media concentrations of test PFAS at 6 dpf. Zebrafish were semi-statically exposed to $4.4\ \ 80.0\mu M$ PFHxS, PFHxA, PFOA, ADONA, or PFESA1 or $0.2\ \ 3.1\mu M$ PFOS daily from $0\ \ 5$ dpf. At 6 dpf, media was collected for targeted analytical chemistry $(n=3)$. Measured media concentrations for (A) PFOS, (B) PFHxS, (C) PFHxA, (D) PFOA, (E) ADONA, and (F) PFESA1 are shown. One observation for PFOA nominal media concentration $14.1\mu M$ was $<MDL$ and therefore not shown on the plot. However, it was included in the regression analysis using the value MDL/sqrt(2). Note: ADONA, 4,8-dioxa-3H-perfluorononanoate; dpf, days post fertilization; MDL, method detection limit; PFAS, per- and polyfluoroalkyl substances; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS,

perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid; sqrt, square-root.

Figure 5. Internal tissue doses of test PFAS at 6 dpf. Zebrafish were semi-statically exposed to $25.1\ \ 80.0\mu M$ ADONA, PFOA, PFESA1, or PFHxA, or $14.0\ \ 44.8\mu M$ PFHxS, or $1.0\ \ 3.1\mu M$ PFOS. At 6 dpf, larvae were pooled and flash frozen ($n=4$ biological replicates with 10 pooled larvae per replicate) for targeted analytical chemistry. Measured internal tissue doses for (A) PFOS, (B) PFHxS, (C) PFHxA, (D) PFOA, (E) ADONA, and (F) PFESA1 are shown. Significance was determined by a Welch's ANOVA followed by a Dunnett T3 test ($p<0.05$). Additional one-sample Student's *t*-tests were performed for PFHxA, PFHxS, PFOA, and PFOS ($p<0.05$). Note: ADONA, 4,8-dioxa-3H-perfluorononanoate; ANOVA, analysis of variance; Dil, dilution; dpf, days post fertilization; PFAS, per- and polyfluoroalkyl substances; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid.

Table 4 Calculated bioconcentration factors (BCFs).

Compound name	Nominal concentration tested (μM)	Measured tissue dose (mg/kg)	Measured media dose (mg/L)	BCF_{dry} (L/kg)
ADONA	25.1	9.49 ± 3.1	9.98 ± 1.77	0.95
	44.8	16.0 ± 6.29	17.04 ± 2.24	0.94
	80.0	14.86 ± 3.11	26.52 ± 5.98	0.56
GenX Free	25.1	2.48 ± 0.66	5.46 ± 1.15	0.45
	44.8	2.02 ± 0.27	7.06 ± 0.73	0.29

Acid				
	80.0	5.11 ± 1.49	10.44 ± 1.57	0.49
	25.1	23.67 ± 17.47	7.67 ± 1.46	3.09
PFESA1	44.8	36.98 ± 17.44	14.11 ± 1.91	2.62
	80.0	43.16 ± 4.84	29.89 ± 2.87	1.44
	25.1	4.97 ± 4.40	10.76 ± 0.98	0.46
PFHxA	44.8	3.28 ± 0.56	18.22 ± 1.19	0.18
	80.0	8.77 ± 4.31	37.16 ± 7.53	0.24
	25.1	115.75 ± 25.19	9.91 ± 0.33	11.68
PFOA	44.8	124.13 ± 29.93	19.0 ± 2.45	6.52
	80.0	170.51 ± 41.12	33.01 ± 8.41	5.17
	14.0	107.21 ± 50.09	9.07 ± 0.36	11.82
PFHxS	25.1	128.55 ± 31.46	16.06 ± 1.97	8.01
	44.8	260.93 ± 57.16	28.04 ± 4.72	9.30
	1.0	220.10 ± 109.45	0.16 ± 0.007	1,374.8
PFOS	1.8	422.07 ± 182.86	0.31 ± 0.038	1,348.4
	3.1	677.86 ± 53.49	0.99 ± 0.194	684.03

Note: BCFs for ADONA, GenX Free Acid, PFESA1, PFHxA, PFHxS, PFOA, and PFOS in a 6-d zebrafish toxicity assay based on measured media and tissue concentrations reported in Excel Tables S8 and S9. BCF tissue doses (mg/kg) were calculated using a dry weight for 6 dpf larvae of 44 μg (Massei et al. 2015). ADONA, 4,8-dioxa-3H-perfluorononanoate; dpf, days post fertilization; GenX Free Acid, perfluoro-2-propoxypropanoic acid; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid.

Developmental Toxicity and Developmental Neurotoxicity Results in Larval Zebrafish Exposed to GenX Free Acid

Because GenX Free Acid was undetectable in media and zebrafish tissue at 6 dpf (data not shown) and, therefore, unstable in DMSO (see Figure S5), we retested the compound using DI water as a diluent. Daily exposure (0 5 dpf) to 0.0 80.0 μM GenX Free Acid was negative in the DevTox (Figure 6A) and DNT

(Figure 6B,C; see also Figures S6 and S7) assays, relative to the DI water control. GenX Free Acid diluted in DI water resulted in detectable levels of the parent compound in media (Figure 6D; see also Excel Table S8) and larval zebrafish tissue (Figure 6E; see also Excel Table S9). Similar to other fluoroether PFAS assessed in the current study (i.e., ADONA and PFESA1) (Table 3), GenX Free Acid exposure yielded extremely low BCF values ranging from 0.29 to 0.49, depending on test concentration (Table 4).

Figure 6. Developmental and behavioral assays and media and tissue concentrations in zebrafish exposed to GenX Free Acid diluted in DI water. (A) Developmental toxicity scores at 6 dpf obtained from zebrafish developmentally exposed to $0.04\ \ 80.0\mu M$ GenX Free Acid diluted in DI water daily, from $0\ \ 5$ dpf. Significance was determined by one-way ANOVA with a Tukey's multiple comparison test ($p < 0.05$). $n = 6$ larvae per concentration tested. For the DNT assay, zebrafish were exposed to $4.4\ \ 80.0\mu M$ GenX Free Acid daily, from $0\ \ 5$ dpf. At 6 dpf, locomotor activity was assessed. (B) Distance moved (cm) each 2-min period or (C) mean distance moved during the light 1 (L1), light 2 (L2), dark 1 (D1), or dark 2 (D2) 10-min periods are shown. Repeated measures ANOVA models were run separately by period (L1, L2, D1, or D2). If a significant effect of concentration was detected ($p < 0.0125$), within-period pairwise comparisons to control were computed using *t*-tests with a Dunnett adjustment for multiple comparisons (*$p < 0.05$). $n = 17\ \ 21$ zebrafish per concentration and 161 DI water control larvae were assessed. (D) Media concentrations and (E) internal tissue dose at 6 dpf following daily exposure to $25.1\ \ 80.0\mu M$ GenX Free Acid. $n = 3$ media replicates and $n = 4$ biological replicates each comprising 10 pooled larvae. Significance was determined by a Welch's ANOVA followed by a Dunnett T3 test ($p < 0.05$). Additional one-sample Student's *t*-tests were performed ($p < 0.05$). Note: ANOVA, analysis of variance; D, dark phase; DevTox, developmental toxicity; DI, deionized; Dil, dilution; DNT, developmental neurotoxicity; dpf, days post fertilization; GenX Free Acid, perfluoro-2-propoxypropanoic acid; L, Light period.

Developmental Toxicity and Developmental Neurotoxicity Phenotypes in Larval Zebrafish Exposed to Alkyl Sulfonic Acid PFAS

Because exposure to structurally similar aliphatic sulfonic acid PFAS PFOS (8-carbon) or PFHxS (6-carbon) resulted in consistent morphological (Figure 2A–D) and behavioral (Figure 3A–D) phenotypes relative to the DMSO control, we hypothesized that sulfonic acid PFAS with perfluorinated alkyl chains would elicit the same toxicity outcomes. To test this hypothesis, zebrafish were exposed to $0.0\ \ 100.0\mu M$ PFBS (4-carbon), PFPeS (5-carbon), PFHxS (6-carbon), PFHpS (7-carbon), or PFOS (8-carbon) (Figure 1) daily, from $0\ \ 5$ dpf and assessed for developmental toxicity at 6 dpf. PFBS was negative for developmental toxicity (Figure 7A). All other sulfonic acid PFAS resulted in significant developmental toxicity characterized by failed swim bladder inflation and ventroflexion of the tail (Figure 7B–E). Interestingly, PFPeS was quite potent for developmental toxicity with a calculated EC_{50} value of $48.8\mu M$ (Figure 7B). The same five alkyl sulfonic acid PFAS shown in Figure 7 were also tested in the DNT assay. Exposure to PFBS did not result in significant locomotor effects (Figure 8A,B; see also Figures S8 and S9), whereas relative to DMSO alone, exposure to PFPeS resulted in hyperactivity in the L1 ($5.5\mu M$) and L2 ($3.1\ \ 5.5\mu M$) periods but had no effect in the dark periods (Figure 8C,D; see also Figures S8 and S9). In the case of PFHxS (Figure 8E,F; see also Figures S8 and S9), and like the results from Study 1 (Figure 3C,D), exposure caused hyperactivity relative to the DMSO control. However, the observed pattern of hyperactivity was modestly different, with hyperactivity detected in the L2 ($17.6\ \ 31.4\mu M$), D1 ($17.6\mu M$), or D2 ($17.6\mu M$) periods (Figure 8E,F). Directly replicating the hyperactivity pattern observed in Study 1 (Figure 3A,B), developmental exposure to PFOS caused hyperactivity in the L1 ($1.7\ \ 3.1\mu M$), L2 ($1.7\ \ 3.1\mu M$), and D1 ($1.0\mu M$) periods but no effect on the D2 period (Figure 8I,J; see also Figures S8 and S9). Similarly, exposure to PFHpS also triggered L1 ($5.5\mu M$), L2 ($3.1\ \ 5.5\mu M$), and D1 ($3.1\ \ 5.5\mu M$) hyperactivity but no effect on the D2 period (Figure 8G,H; see also Figures S8 and S9).

Figure 7. Measures of developmental toxicity in zebrafish exposed to alkyl sulfonic acid PFAS. Zebrafish were semi-statically exposed to $1.7\ \ 100.0\mu M$ PFBS, PFPeS, PFHxS, PFHpS, or PFOS or 0.4% DMSO daily from $0\ \ 5$ dpf. At 6 dpf, larvae were assessed for developmental toxicity. DevTox assay scores for

(A) PFBS, (B) PFPeS, (C) PFHxS, (D) PFHpS, or (E) PFOS are shown. Significance relative to the 0.4% DMSO control was determined by a Kruskal-Wallis ANOVA with a Dunn's multiple comparison test (**$p < 0.0001$). If a test for linear trend was significant ($p < 0.05$), with developmental toxicity observed at the highest concentration tested, nonlinear regression was performed with Hill slope curve fitting for half-maximal EC_{50} value determinations. $n = 8$ larvae per concentration per chemical tested. Note: ANOVA, analysis of variance; DevTox, developmental toxicity; DMSO, dimethyl sulfoxide; dpf, days post fertilization; EC_{50}, half maximal effective concentration; PFAS, per- and polyfluoroalkyl substance; PFBS, perfluorobutanesulfonic acid; PFHpS, perfluoroheptanesulfonic acid; PFHxS, perfluorohexanesulfonic acid; PFOS, perfluorooctanesulfonic acid; PFPeS, perfluoropentanesulfonic acid.

Figure 8. Locomotor activity in zebrafish exposed to alkyl sulfonic acid PFAS. Zebrafish were semi-statically exposed to $5.5\ \ 100.0\mu M$ PFBS, $3.1\ \ 5.5\mu M$ PFPeS, $3.1\ \ 31.4\mu M$ PFHxS, $1.7\ \ 9.8\mu M$ PFHpS, or $0.5\ \ 3.1\mu M$ PFOS daily from $0\ \ 5$ dpf. For all chemicals except PFPeS, 14–25 larvae were tested per chemical concentration and the same DMSO control larvae ($n = 327$) were used. PFPeS was tested separately ($n = 21\ \ 22$ larvae per concentration; 186 DMSO control larvae were evaluated). At 6 dpf, larvae were assessed for developmental toxicity. Morphologically normal larvae with inflated swim bladders were subjected to behavioral testing. (A, C, E, G, I) Distance moved (cm) each 2-min period or (B, D, F, H, J) mean distance moved during the light 1 (L1), light 2 (L2), dark 1 (D1), or dark 2 (D2) 10-min periods are shown. Repeated measures ANOVA models were run separately by period (L1, L2, D1, or D2). If a significant effect of concentration was detected ($p < 0.0125$), within-period pairwise comparisons to control were computed using *t*-tests with a Dunnett adjustment for multiple comparisons (*$p < 0.05$). ANOVA, analysis of variance; D, dark phase; DMSO, dimethyl sulfoxide; dpf, days post fertilization; L, light period; PFAS, per- and polyfluoroalkyl substances; PFBS, perfluorobutanesulfonic acid; PFHpS, perfluoroheptanesulfonic acid; PFHxS, perfluorohexanesulfonic acid; PFOS, perfluorooctanesulfonic acid; PFPeS, perfluoropentanesulfonic acid.

Comparison of Toxicity Phenotypes in Zebrafish Developmentally Exposed to PFAS

Collective analysis of developmental toxicity and developmental neurotoxicity data sets revealed a shared toxicity phenotype for sulfonic acid PFAS that contain five or more fluorinated carbons (e.g., PFPeS, PFHxS, PFHpS, and PFOS) that was generally characterized by abnormal ventroflexion of the tail and failed swim bladder inflation and, at nonteratogenic concentrations, hyperactivity in the L1, L2, and D1 periods (Figure 9A). Because PFPeS was more potent for developmental toxicity ($EC_{50} = 48.8 \mu M$; $LOEC = 56.0 \mu M$), relative to PFHxS ($EC_{50} = 227.9 \mu M$; Study 2 $LOEC = 56.0 \mu M$) and PFHpS ($EC_{50} = 168.1 \mu M$; $LOEC = 31.4 \mu M$), we did not identify a linear relationship between sulfonic acid carbon chain length and EC_{50} values for developmental toxicity ($R^2 = 0.027$) (Figure 9B). In the DNT assay however, sulfonic acid carbon chain length was correlated with Study 2 LOEC values for hyperactivity ($R^2 = 0.55$) (Figure 9C). These data also show that PFHxA has a unique toxicity phenotype consisting of hyperactivity in the L2, D1, and D2 periods with no observed developmental toxicity identified at the highest concentration tested (Figure 9A). Last, exposure to fluoroether PFAS (i.e., ADONA, GenX Free Acid, or PFESA1) failed to provoke developmental toxicity or developmental neurotoxicity in zebrafish.

Figure 9. Identification of shared phenotypes between structurally similar PFAS. (A) Heatmap depicting LOEC values for the DevTox assay and significant hyperactivity in the L1, L2, D1, and/or D2 periods of the DNT assay (Studies 1, 2, and 3). If chemicals were replicated in Study 1 and Study 2, the lowest observed LOEC value was used. Linear regression of (B) Study 3 DevTox assay EC_{50} or (C) Study 3 DNT assay LOEC values for aliphatic sulfonic acid PFAS. Note: ADONA, 4,8-dioxa-3H-perfluorononanoate; D, dark period; DevTox, developmental toxicity; DNT, developmental neurotoxicity; EC_{50}, half maximal effective concentration; GenX Free Acid, perfluoro-2-propoxypropanoic acid; L, light period; LOEC, lowest observed effect concentration; PFAS, per- and polyfluoroalkyl substances; PFBS, perfluorobutanesulfonic acid; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHpS,

perfluoroheptanesulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid; PFPeS, perfluoropentanesulfonic acid.

Discussion

PFAS are a class of ubiquitous environmental contaminants. There is insufficient toxicity data for the majority of PFAS used in industry and consumer products (OECD 2018). The initial goal of this study was to evaluate the developmental toxicity and developmental neurotoxicity of seven PFAS, including compounds that have been phased out of use but are still widely detected in human serum (i.e., PFOA, PFOS, PFHxS, and PFHxA). In addition, the study included several emerging fluoroether compounds (e.g., ADONA) for which there are limited developmental toxicity data (Gordon 2011; Rushing et al. 2017). Last, we tested PFESA1, a by-product associated with the synthesis of polymer products.

One major finding of this work is that exposure to several PFAS resulted in developmental neurotoxicity characterized by hyperactivity. Epidemiological studies report both positive (Ghassabian et al. 2018; Hoffman et al. 2010; Hoyer et al. 2015; Rappazzo et al. 2017) and negative (Lyall et al. 2018; Rappazzo et al. 2017; Stein and Savitz 2011; Stein et al. 2013) associations between PFAS exposures and neurodevelopmental outcomes. Similarly, exposure to PFAS has been reported to cause behavioral toxicity in some (Goulding et al. 2017; Johansson et al. 2008; Long et al. 2013; Sato et al. 2009; Wang et al. 2015), but not all (Butenhoff et al. 2009), animal studies. In those studies where a positive relationship was revealed, affected behavioral end points included hyperactivity (Goulding et al. 2017; Johansson et al. 2009), reduced habituation (Johansson et al. 2008), impairments in spatial learning and memory resulting from adult (Long et al. 2013) and prenatal exposures (Wang et al. 2015), and tonic convulsions in response to an ultrasonic stimulus (Sato et al. 2009). Molecular results obtained in animal studies suggest that PFAS exposures may disrupt dopaminergic and/or calcium signaling pathways during neurogenesis (Hallgren and Viberg 2016; Johansson et al. 2009; Lee and Viberg 2013; Liu et al. 2010a, 2010b; Zeng et al. 2011). Overall, because of contradictory evidence in human epidemiological and animal behavior studies, it remains unclear whether PFAS exposure is associated with adverse neurophysiological effects. To gain insight into this critical question, concentration-dependent automated behavioral data are needed to evaluate a variety of related and dissimilar PFAS structures. The zebrafish model represents an excellent alternative experimental system that can be used to address this growing research need because multiple chemicals can be evaluated in parallel using automated behavioral tests coupled with a powerful concentration–response design.

Legacy PFAS such as PFOA and PFOS have been extensively evaluated *in vitro* and in animal and epidemiological studies. However, PFAS are by no means a monolithic class of chemicals. They can be per- or polyfluorinated, straight or branched chained, and contain alkyl chains of varying lengths. PFAS may also contain ether linkages and either sulfonic acid or carboxylic acid R-group

moieties. In the United States, there are 602 PFAS in active commercial use (U.S. EPA 2019) and the OECD has identified 4,730 PFAS structures included in various publicly accessible databases (OECD 2018), most of which lack adequate toxicity data. The Zürich Statement on PFAS lays out a strategy for tackling the huge gap in our understanding of PFAS toxicity (Ritscher et al. 2018). Given the large number of PFAS in commerce and rather than cataloging the effects of individual chemicals, the statement calls for action on grouping PFAS (Ritscher et al. 2018). One obvious way to achieve this is to group chemicals by their toxicological activities and, perhaps in doing so, identify structural features that provoke the same toxicity phenotypes *in vivo*. In the current study, we identified three groups of PFAS toxicity outcomes in zebrafish. Aliphatic sulfonic acid PFAS with greater than four fluorinated carbons resulted in similar morphological and behavioral phenotypes, characterized by failed swim bladder inflation, abnormal ventroflexion of the tail, and, at nonteratogenic concentrations, hyperactivity in the L1, L2, and D1 periods. The second phenotype was unique to PFHxA, an aliphatic carboxylic acid PFAS. Exposure to PFHxA was negative for developmental toxicity but caused pronounced hyperactivity in the L2, D1, and D2 periods. The third group of chemicals consisted of three fluoroether PFAS (i.e., GenX Free Acid, ADONA, and PFESA1), all of which were negative in both toxicity assays.

In addition to grouping PFAS based on attributes such as structure or biological activity, the Zürich statement also recommends amassing data on the toxicokinetics and toxicodynamics of PFAS exposures, particularly those for which little toxicity data exist, with the goal of identifying safer PFAS that can be prioritized for commercial use (Ritscher et al. 2018). Interestingly, we observed a trend of reduced BCFs with increasing nominal concentrations of several PFAS (i.e., ADONA, PFESA1, PFOA, and PFOS). These data replicate a recent study conducted in larval zebrafish that showed an inverse relationship between BCF values and increasing concentrations of perfluorobutanoic acid (PFBA), PFHxS, PFOA, and PFOS, which was suggested to result from the saturation of substrate binding sites (Vogs et al. 2019). Targeted analytical chemistry also revealed detectable levels of ADONA, PFESA1, and PFOA in fish tissue at 6 dpf, indicating that these chemicals are negative for developmental toxicity and developmental neurotoxicity in zebrafish (up to $80\mu M$).

To our knowledge, this is the first published report showing that GenX Free Acid, a branched fluoroether PFAS with a carboxylic acid group directly adjacent to an ether linkage, is not stable in DMSO. This is significant because DMSO is a commonly used solvent for zebrafish and high-throughput *in vitro* and biochemical toxicity screening studies. Assessment of GenX Free Acid diluted in DI water showed that, although this compound was detectable in zebrafish tissue at the end of the 6-d study period, it was negative for developmental toxicity and developmental neurotoxicity. Collectively, these results suggest that, at least for the types and concentrations tested in the current study, larger fluoroether compounds (i.e., ADONA, GenX Free Acid, and PFESA1) were nontoxic in zebrafish. More work should be performed to explore whether this finding can be extended to other large fluoroether replacement PFAS (Wang et al. 2013).

Compared with previously reported morphological and behavioral effects following exposure to PFBS, PFHxS, PFOS, or PFHxA (summarized in Table 5)

(Hagenaars et al. 2011; Huang et al. 2010; Jantzen et al. 2016; Khezri et al. 2017; Padilla et al. 2012; Spulber et al. 2014; Truong et al. 2014; Ulhaq et al. 2013a, 2013b), this study expanded our understanding of aliphatic PFAS toxicity to include data on PFPeS and PFHpS for the first time and reported novel results for PFHxA and PFHxS. Although not systematically designed to test a specific PFAS R-group (i.e., sulfonic or carboxylic acids), Ulhaq et al. (2013a) tested 4-, 8-, 9-, and 10-carbon carboxylic acid aliphatic PFAS and 4- and 8-carbon sulfonic acid aliphatic PFAS in a zebrafish developmental toxicity assay and proposed the idea that carbon chain length may be a determinant of PFAS toxicity in zebrafish. The work presented here systematically tested the effects of aliphatic sulfonic acid PFAS with 4, 5, 6, 7, or 8 fluorinated carbons. Because of the 5-carbon compound PFPeS, carbon chain length was not correlated with malformations in the DevTox assay ($\mathrm{PFOS} > \mathrm{PFPeS} > \mathrm{PFHpS} > \mathrm{PFHxS}$; PFBS was negative; $R^2 = 0.35$). PFPeS was nearly as potent as PFOS, the most potent chemical evaluated in our study. In comparison, in the DNT assay, increasing carbon chain length was associated with increasing potency for hyperactivity ($\mathrm{PFOS} > \mathrm{PFHpS} > \mathrm{PFPeS} > \mathrm{PFHxS}$; PFBS was negative; $R^2 = 0.55$). Collectively, these data raise two important points. First, sulfonic acid aliphatic PFAS can be grouped based on their ability to cause the same morphological and behavioral toxicity phenotypes in zebrafish (i.e., failed swim bladder inflation, abnormal ventroflexion of the tail, and, at nonteratogenic concentrations, hyperactivity). Second, although carbon chain length generally increases PFAS potency, this dogma cannot be universally applied to all structurally similar PFAS, as exceptions to the rule exist (i.e., PFPeS).

Table 5 Summary of key zebrafish toxicity data.

Compound	Class	DevTox assay phenotype	Ref	DNT assa phenotyp
ADONA	Polyfluoroether	Negative[a]	This study	Negative
GenX Free Acid	Branched polyfluoroether	Negative[a]	This study	Negative
PFESA1	Branched polyfluoroether	Negative[a]	This study	Negative
PFHxA	Aliphatic carboxylic acid	Negative	This study; Truong et al. 2014	Hyperactivi
		Negative	This study; Padilla et al. 2012; Truong et al. 2014	Negative
PFOA	Aliphatic		Hagenaars	

	carboxylic acid	Positive	et al. 2011; Jantzen et al. 2016; Ulhaq et al. 2013a	Hyperactiv
		Negative	This study; Truong et al. 2014	Negative
PFBS	Aliphatic sulfonic acid	Positive	Hagenaars et al. 2011; Ulhaq et al. 2013a	Hyperactiv
PFPeS	Aliphatic sulfonic acid	Positive[a]	This study	Hyperactivi
PFHxS	Aliphatic sulfonic acid	Positive	This study; Truong et al. 2014	Hyperactivi Negative
PFHpS	Aliphatic sulfonic acid	Positive[a]	This study	Hyperactivi
PFOS	Aliphatic sulfonic acid	Positive	This study; Hagenaars et al. 2011; Huang et al. 2010; Jantzen et al. 2016; Padilla et al. 2012; Truong et al. 2014; Ulhaq et al. 2013a, 2013b	Hyperactiv Hypoactivi

Note: Data on PFAS evaluated in the present study and previous work. ADONA, 4,8-dioxa-3H-perfluorononanoate; DevTox, developmental toxicity; DNT, developmental neurotoxicity; GenX Free Acid, perfluoro-2-propoxypropanoic acid; PFBS, perfluorobutanesulfonic acid; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHpS, perfluoroheptanesulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS,

perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid; PFPeS, perfluoropentaslufonic acid; Ref, reference.

[a]Indicates previously unreported findings.

Here, we obtained DevTox and DNT Assay data for PFOS and PFHxS in two separate studies. This provided a unique opportunity to evaluate the consistency of observed toxicity effects across independent experiments. In the case of PFOS, EC_{50} values for developmental toxicity were similar, but not identical, with identified values of $7.5\mu M$ in Study 1 and $28.2\mu M$ in Study 3. Variability in calculated EC_{50} values was also observed in the PFHxS data set ($92.7\mu M$ in Study 1; $227.9\mu M$ in Study 3). These discrepancies could reflect both inherent assay variability and the different concentrations ranges tested in Study 1 ($0.04\ \ 80\mu M$) relative to Study 3 ($1.7\ \ 100\mu M$). In addition, the EC_{50} value calculated from Study 1 was based on a single positive concentration and, therefore, may not be as reliable as the value determined in Study 3. Last, the OECD Fish Embryo Acute Toxicity Test (No. 236) indicates that 20 animals should be tested across five concentrations of test chemicals to evaluate developmental toxicity (OECD 2013). Although this study assessed developmental toxicity across a six-point concentration–response curve with a minimum of 44 replicates for the 0.4% DMSO control group, only 6–10 biological replicates per exposure group were used. Collectively, these design choices may have contributed to differences in detected EC_{50} values described above.

However, these data are in line with previously published DevTox assay data with reported PFOS EC_{50} values of $42.3\mu M$ (Padilla et al. 2012) and $3.5\mu M$ (Truong et al. 2014) and PFHxS EC_{50} values of $116.5\mu M$ (Padilla et al. 2012) and $114.7\mu M$ (Truong et al. 2014). Given that the strain, rearing temperature, chemical source, exposure regimen (i.e., static vs. semi-static), and end point evaluation protocol varied across studies, these data are generally consistent and show that exposure to PFOS or PFHxS causes developmental toxicity in zebrafish. In the DNT assay, highly consistent LOECs were observed for PFOS ($1.8\mu M$ in Study 1; $3.1\mu M$ in Study 3) and PFHxS ($25.1\mu M$ in Study 1; $31.4\mu M$ in Study 3). However, although the pattern of the hyperactivity was identical across studies for PFOS with observed hyperactivity in the L1, L2, and D1 periods, it varied following exposure to PFHxS, where elevated locomotor activity was observed in the L1, L2, and D1 periods in Study 1 and the L2, D1, and D2 periods in Study 3. Regardless, in line with previously published work (Huang et al. 2010; Khezri et al. 2017; Spulber et al. 2014), exposure to nonteratogenic concentrations of PFOS or PFHxS consistently triggered behavioral hyperactivity. Although more work is needed to understand the biological relevance of disparate xenobiotic-induced locomotor activity phenotypes, this represents a powerful approach for grouping chemicals based on shared toxicity phenotypes.

From a developmental toxicity perspective, we and others have showed that exposure to sulfonic acid alkyl PFAS with at least five carbon atoms can cause

developmental lethality and elicit conserved malformations at nonteratogenic concentrations consisting of swim bladder inflation failure and dorsoflexion of the tail (Hagenaars et al. 2011; Huang et al. 2010; Jantzen et al. 2016; Padilla et al. 2012; Truong et al. 2014; Ulhaq et al. 2013a). Similar to zebrafish, neonatal rodents exposed to PFOS die in the postnatal period (Conley et al. 2019; Grasty et al. 2005). Although humans do not have a swim bladder, the organ shares functional, structural, ontological, and transcriptional similarities with the human lung (Winata et al. 2009). In zebrafish, swim bladder inflation is used for buoyancy and functions as a site for gas interchange at the air–mucus interface, with surfactant composition similar to the lung (Agier et al. 2019; Lapennas and Schmidt-Nielsen 1977; Robertson et al. 2007; Sullivan et al. 1998; Zeng et al. 2011). Toxicologically, neonatal rodents that died following prenatal exposure to PFOS presented with noted changes in lung histology (Grasty et al. 2005). In humans, epidemiologic evidence has shown that exposure to PFAS during pregnancy can result in decreased lung function in children (Agier et al. 2019). Although these similarities are intriguing, more work is needed to determine the relevance of zebrafish swim bladder defects for human health risk assessment.

Overall, this study rapidly assessed the developmental toxicity and developmental neurotoxicity of 10 PFAS, including some compounds that have never been previously tested in animal studies. However, limitations of the study warrant comment. In human NHANES data, mean PFAS levels are often higher in males relative to females (Calafat et al. 2007a, 2007b). We did not capture sex-specific outcomes in early life stage zebrafish. In addition, here we used locomotor activity in a light/dark behavior test as a functional readout of neurodevelopment. This is just one behavioral end point among many. Future studies should examine the effect of PFAS on other zebrafish behavior end points such as habituation to further support the developmental neurotoxicity effects reported here. Here, 0.4% DMSO was used for all exposure groups, except GenX Free Acid which was diluted in DI water. This concentration of DMSO is commonly used in zebrafish chemical screens for developmental toxicity (Padilla et al. 2012) and does not affect the larval zebrafish photomotor response (Kokel and Peterson 2011) or light/dark swimming response (Teixidó et al. 2019). However, it should be noted that effects on the transcriptome (Turner et al. 2012) and metabolome (Akhtar et al. 2016) have been reported in zebrafish exposed to 0.1% DMSO. There is also evidence that elevated DMSO concentrations can facilitate increased uptake of chemicals into the perivitaline space (Kais et al. 2013). Therefore, although all chemical exposure data were statistically compared with a DMSO control, it is possible that the concentration of solvent used in the current study affected chemical uptake and the assessed end points.

These data raise some interesting questions for future research. Namely, do behavioral hyperactivity effects persist in older animals? One intriguing study observed hyperactivity in 14 dpf zebrafish developmentally exposed to PFOA or PFOS from 0 5 dpf (Jantzen et al. 2016), suggesting that early life perturbation of neuronal circuitry controlling the light/dark behavioral apparatus may persist at later life stages. Another key area that needs to be explored is the mechanism(s) by which PFAS cause locomotor hyperactivity in zebrafish. Last, humans and wildlife are exposed to a complex mixture of PFAS (Boiteux et al. 2016; Gebbink et al. 2017; Strynar et al. 2015), many of which may cause additive or synergistic disruption of neurodevelopmental signaling pathways (Khezri et al. 2017). Future

research should consider testing groups of related PFAS in environmentally relevant mixtures. From a dosimetry perspective, we observed tissue doses of PFAS that have been measured in, for example, the Danish National Birth Cohort (Fei et al. 2007). To truly understand the relevance of zebrafish toxicity data for human risk assessment, there is an urgent need for physiologically based pharmacokinetic models that compare PFAS doses measured in whole-body zebrafish homogenates to maternal serum levels. Finally, analysis of larval behavior data is not standardized. Here, we opted to use a repeated measures ANOVA and parametric mixed model analysis to account for the complex data structure that contains multiple, repeated locomotor activity values for each animal over the 40-min testing period (Catron et al. 2019a; Irons et al. 2013; Phelps et al. 2017; Stevens et al. 2018). However, zebrafish behavior data are not normally distributed and may often be below the LOD. We therefore also analyzed the behavior data using a square root transformation and multiple value imputation strategy (Pleil 2016a, 2016b). Based on the LOD, a large percentage of individual values were imputed. Although the order of the raw data was preserved in the final corrected distributions, the large percentage of imputed values present in the light period data should be noted. However, the combined use of square root transformation and multiple value imputation allowed key regression assumptions (i.e., homoskedasticity and normality of residuals) to be met and the subsequent use of mixed-effects models further allowed the appropriate examination of repeated measures for individual zebrafish. Overall, the imputation-based analysis strategy was used to buttress findings generated from untransformed zebrafish behavior data while appropriately accounting for nonnormal distributions. Although both approaches revealed PFAS-dependent hyperactivity, standardized methods for the analysis of fish behavior data that account for repeat measurements and nonnormal data distributions are needed.

Taken together, and in keeping with recommendations by the Zürich Statement (Ritscher et al. 2018), we used zebrafish toxicity data to group PFAS based on their ability to cause developmental toxicity and/or developmental neurotoxicity. We specifically identified aliphatic sulfonic acid PFAS as a particularly bioactive class of PFAS, thereby identifying relationships between chemical structures and *in vivo* phenotypes that may arise from putative shared mechanisms of PFAS toxicity. We also used analytical chemistry to reveal that GenX Free Acid is unstable in DMSO, a solvent widely used for zebrafish and *in vitro* screening studies. These data show that this emerging PFAS, in addition to other branched and/or fluoroether PFAS examined here, is negative for developmental toxicity and developmental neurotoxicity in zebrafish, possibly identifying a less bioactive group of PFAS (at least in the context of fish toxicology). Finally, this study supports the use of *in vivo* developmental neurotoxicity testing when evaluating this class of widely occurring environmental contaminants.

Acknowledgments

S.G. participated in study design, performed zebrafish experiments, analyzed data, prepared figures, and assisted with manuscript preparation. A.S. performed targeted analytical chemistry experiments. J.R.S. participated in study design, data interpretation, and analyzed behavior and analytical chemistry data. X.M.H. performed zebrafish experiments. J.S. performed behavior statistical analyses. T.C., J.M., E.H., and M.S. participated in study design and data interpretation.

T.T. designed the study, assisted with zebrafish experiments, analyzed and interpreted data, and wrote and rebutted the manuscript. All authors reviewed the manuscript.

We thank J. Hedge and the U.S. Environmental Protection Agency (EPA) zebrafish facility staff for fish husbandry and L. Adams for performing a data quality review. We are grateful to C. Lau, D. Villeneuve, and S. Hunter for their review of the manuscript. This manuscript has been reviewed by the U.S. EPA and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. This work was supported by the U.S. EPA Office of Research and Development.