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Perfluorohexanoic acid toxicity, part I: Development of a chronic human health toxicity value for use in risk assessment

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ABSTRACT

Perfluorohexanoic acid (PFHxA) is a short-chain, six-carbon perfluoroalkyl acid (PFAA) and is a primary impurity, degradant, and metabolite associated with the short-chain fluorotelomer-based chemistry used globally today. The transition to short-chain fluorotelomer-based products as a cornerstone in replacement fluorochemistry has raised questions regarding potential human health risks associated with exposure to fluorotelomer-based substances and therefore, PFHxA. Here, we present a critical review of data relevant to such a risk assessment, including epidemiological studies and *in vivo* and *in vitro* toxicity studies that examined PFHxA acute, subchronic, and chronic toxicity. Key findings from toxicokinetic and mode-of-action studies are also evaluated. Sufficient data exist to conclude that PFHxA is not carcinogenic, is not a selective reproductive or developmental toxicant, and does not disrupt endocrine activity. Collectively, effects caused by PFHxA exposure are largely limited to potential kidney effects, are mild and/or reversible, and occur at much higher doses than observed for perfluorooctanoic acid (PFOA). A chronic human-health-based oral reference dose (RfD) for PFHxA of 0.25 mg/kg-day was calculated using benchmark dose modeling of renal papillary necrosis from a chronic rat bioassay. This RfD is four orders of magnitude greater than the chronic oral RfD calculated by the U.S. Environmental Protection Agency for PFOA. The PFHxA RfD can be used to inform public health decisions related to PFHxA and fluorotelomer precursors for which PFHxA is a terminal degradant. These findings clearly demonstrate that PFHxA is less hazardous to human health than PFOA. The analyses presented support site-specific risk assessments as well as product stewardship initiatives for current and future short-chain fluorotelomer-based products.

1. Introduction

Per- and polyfluorinated substances (PFAS) have unique chemical properties that, during the past 50 years, have found wide ranging applications in industry (e.g., aerospace, metal plating, electronic, medical, firefighting) and commercial products (e.g., surface coatings for textiles and leather, carpets, some food contact substances, and non-stick cookware) (Buck et al., 2011). Although PFAS represent many different chemicals with widely variable chemical structures (Wang et al., 2017), there has been much focus on terminal degradation products from historical use, such as perfluorocarboxylic acids (PFCAs), the most notable being perfluorooctanoic acid (PFOA). Long-chain PFCAs, such as PFOA, are defined as having eight or more carbons (OECD, 2018a), have been shown to be persistent, bioaccumulative and toxic (PBT), and are found widely in the environment and in human serum (Kato et al., 2018; OECD, 2013). Based on these concerns, the major fluorochemical manufacturers initiated (2006) and completed (2015) a global stewardship program to eliminate long-chain PFCAs such as PFOA and potential precursors from emissions and products by year-end 2015 (USEPA, 2016a,b; USEPA, 2016b). To continue to provide the unique function and performance properties, manufacturers shifted production to short-chain homologues based on data indicating they had a significantly different and more favorable environmental and human health profile (Buck et al., 2011). For fluorotelomer-based products, this meant shifting to products that contained a six-carbon perfluoroalkyl moiety (Buck et al., 2011). As such, this brought focus to a primary potential impurity, degradant, and metabolite from short-chain fluorotelomer-based products, the short-chain PFCA, perfluorohexanoic acid (PFHxA).

As a result of the shift to short-chain fluorotelomer-based products,

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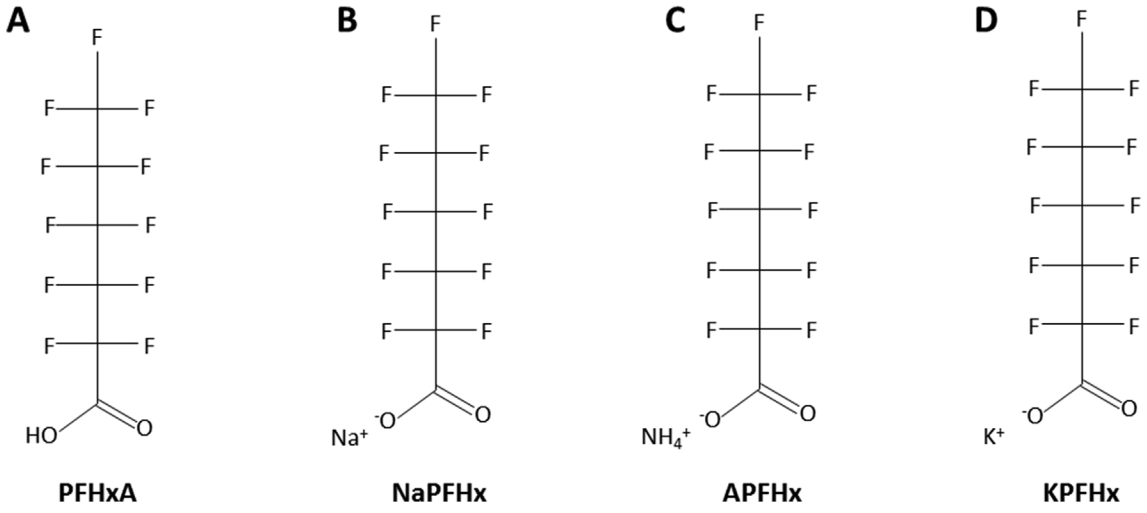

Fig. 1. Structure of test substances used in studies presented: (A) Perfluorohexanoic acid, PFHxA (CASRN: 307-24-4), (B) sodium perfluorohexanoate, NaPFHx (CASRN: 2923-26-4), (C) ammonium perfluorohexanoate, APFHx (CASRN: 21615-47-4), and (D) potassium perfluorohexanoate, KPFHx (CASRN: 3109-94-2).

concerns have been raised regarding their safety and availability of sufficient data for the products and their degradation products including PFHxA (Scheringer et al., 2014; Wang et al., 2013, 2015; Blum et al., 2015). Short-chain PFCAs, such as PFHxA, are environmentally persistent (Parsons et al., 2008; Wang et al., 2015), some are mobile (Venkatesan and Halden, 2014; Vierke et al., 2014), and some may accumulate in the leaves and fruits of plants (Felizeter et al., 2012, 2014; Krippner et al., 2014). At the same time, numerous studies have demonstrated that short-chain PFCAs, as compared to long-chain PFCAs, are more quickly eliminated from mammals via urinary excretion (Ohmori et al., 2003; Chang et al., 2008; Olsen et al., 2009; Gannon et al., 2011; Buck and Gannon, 2017), are not bioacummulative in fish (Conder et al., 2008), and are rarely detected in biomonitoring of the general population (Olsen et al., 2012; Russell et al., 2013; Kato et al., 2018).

PFHxA is an impurity, degradant, and metabolite from short-chain fluorotelomer-based products. PFHxA was also present in historic long-chain products from Electrochemical Fluorination (ECF) and telomerization (Prevedouros et al., 2006; Backe et al., 2013) and has been found at environmental contamination sites in groundwater and soil (Anderson et al., 2016). In 2017, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) proposed a human health chronic oral toxicity value (0.32 mg/kg-day) for PFHxA that is more than four orders of magnitude greater (i.e., less toxic) than the oral toxicity value derived by the U.S. Environmental Protection Agency (USEPA) for the drinking water health advisory for PFOA (0.00002 mg/kg-day) (ANSES, 2017; USEPA, 2016a). The ANSES work is currently the only human health toxicity assessment for PFHxA. However, it is not a comprehensive review or analysis of the available toxicity data, and does not use the most advanced risk assessment methodology available and customarily used by U.S. regulatory agencies. Currently, there are no U.S. federal cleanup standards, screening values, guidelines, or recent peer-reviewed human health risk evaluations that focus on PFHxA to provide insight into the chemical's potential public health risk. As such, a critical evaluation of the toxicological data for PFHxA is needed to assess whether this key impurity, degradant, and metabolite from short-chain fluorotelomer products has a more favorable human health risk profile when compared to PFOA. This assessment would provide the needed interpretation to facilitate risk assessment and risk communication initiatives in communities where environmental sampling demonstrates that PFHxA is present. An evaluation of PFHxA toxicological data will be informative to the relative potency and human health risk from exposure to, and assessment of, short-chain fluorotelomer precursors, and, the comprehensive presentation of the full PFHxA data set will inform read-across analogy to other short-chain PFCAs.

The peer-reviewed toxicology study data for PFHxA and PFOA, based on the review of available studies herein, is sufficient to make a meaningful comparison between these two PFCAs and to inform public health decisions on PFHxA, following the lead set by France (ANSES, 2017). The toxicological data set for PFHxA includes acute, subchronic, chronic, carcinogenic, and reproductive/developmental studies in rodents as well as a wide range of *in vitro* bioassays. The objective of this paper is to provide a comprehensive summary and evaluation of the current PFHxA toxicological data and use it to develop a human health-based toxicity value, analogous to an USEPA oral reference dose (RfD), for PFHxA. To understand and interpret the relative risk, the toxicological data for the short-chain PFCA, PFHxA, are compared to the toxicological data for the long-chain PFCA, PFOA, when comparable data are available. In this Part 1 paper, a chronic human health toxicity value for PFHxA is calculated that qualifies as a “tier three” value per USEPA policy (USEPA, 1989, 1993, 2003, 2013), and as such, can be useful for public health decision making, including derivation of screening levels for contaminated sites. According to USEPA policy and guidance (USEPA, 1989, 1993, 2003, 2013), tier three toxicity values must be recent, derived with transparent methodology and standard risk assessment methods, must have been peer-reviewed, and must be publicly available. In the companion Part 2 paper, the Tier 3 toxicity value is applied to examine a plausible range of risk-based standards for PFHxA following U.S. federal and state regulatory agency methodology (Anderson et al., 2019). These evaluations provide the first comprehensive review of PFHxA potential human health risk and will help inform global discussions regarding the safety of current short-chain fluorotelomer-based products.

2. Methods

The sufficiency of available toxicological data or risk assessment information that could be used to calculate a chronic RfD for PFHxA was first evaluated. An evaluation of the PFHxA toxicological literature was conducted by searching for and examining toxicity assay databases, general scientific literature, and any documents supporting regulatory agency PFAA toxicity assessments. PFHxA can exist in various ionic states; however, due to its low pK_a (< 1), PFHxA primarily exists in the environment as an anion. However, some laboratories report results for the acidic form of PFHxA and the standards used by some laboratories to perform toxicity testing include various PFHxA salts (Fig. 1). This is important, as the acid has been shown to be more irritating than

associated salts. However, regardless of the administered compound, once absorbed into the bloodstream, the $PFHx^-$ anion will form. Additionally, when the salt or acid exists in liquids, it will dissociate and the salt or acid will break off, resulting in the anion. Laboratories adjust for the H^+ cation or adjust the reported concentration to account for the mass of the counterion salt, neither of which has an impact on the data interpretation. This toxicity review included all studies that utilized PFHxA and its salts (Fig. 1).

Finally, a human-health based chronic toxicity value was calculated for PFHxA using up-to-date risk assessment methods and standard USEPA guidance (USEPA, 2002).

2.1. PFHxA toxicology literature review

Potential toxicity to humans may be evaluated by examining epidemiological data for humans and/or toxicity data for various animal species. Using various internet-based search engines and databases, all relevant PFHxA toxicological studies were located via searches of Google Scholar, the U.S. National Library of Medicine (NLM) and National Institutes of Health (NIH) PubMed, the Registry of Toxic Effects of Chemical Substances, and the NLM Toxicology Data Network. USEPA and NIH have also run PFHxA through their Tox21 high-throughput assays; data are available via the NLM PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and USEPA's Interactive Chemical Safety for Sustainability ToxCast database (http://actor.epa.gov/dashboard/) and are briefly discussed in Supplemental File 1 (Supplemental Section 2.2.6). The U.S. National Toxicology Program (NTP) conducted rodent bioassays and kinetic studies of several PFAAs, including PFHxA. The NTP data are not yet published, but are publicly available (NTP, 2018) and are reviewed and discussed below.

2.2. Dose-response evaluation and toxicity value derivation

Available literature was reviewed for applicability as the key study, based on study quality and adherence to general good laboratory practice (GLP) standards. Critical effects from the studies were identified to define points of departure (PODs). USEPA defines “critical effect” as the first adverse effect, or its known precursor, that occurs in the most sensitive species. A POD can either be the no-observed-adverse-effect level (NOAEL) or lowest-observed-adverse-effect level (LOAEL) for an observed incidence or change in response level, or the lower bound on a dose for an estimated response level from a dose-response model (USEPA, 2002).

In the late 1990s and early 2000s, USEPA's Office of Research and Development developed Benchmark Dose Software (BMDS) to estimate POD as an alternative to the NOAEL/LOAEL approach that was previously used for dose-response assessment and has recognized limitations. Benchmark dose (BMD) modeling provides a superior quantitative approach to derive a POD compared to the traditional NOAEL approach. BMDS fits mathematical models to the entire dose-response data, allowing utilization of the entire dose-response relationship rather than selecting one dose and response level to better characterize and quantify potential risks. When data are amenable to BMD modeling, this is the preferred methodology for deriving the POD (USEPA, 2012). USEPA's BMDS (version 2.7) was utilized to model dose response, in accordance with USEPA guidance (USEPA, 2012).

The BMD-based POD was then converted to a human equivalent dose (POD_{HED}) following standard USEPA guidance and allometric scaling of body weight (BW) to the 3/4th power (USEPA, 2011). As discussed in more detail below, there are not significant species-specific toxicokinetics for PFHxA that warrant toxicokinetic models to extrapolate between species (Russell et al., 2013).

Finally, uncertainty factors were applied to the POD_{HED} to calculate the final PFHxA RfD as described in Section 4.4. Uncertainty factors account for uncertainties associated with the available data and variability between different human populations. There are established guidance and protocols for the selection of uncertainty factors. Standard uncertainty factors include the following: UF_D for uncertainty in the underlying database; UF_H for intrahuman variability; UF_A for uncertainty associated with extrapolating from animals to humans; UF_S for uncertainty associated with a subchronic study if a chronic toxicity value is calculated; and UF_L if the derivation relied on a LOAEL rather than a NOAEL. The exact value of the chosen uncertainty factor depends on the quality of the studies available, the extent of the database, and scientific judgment. Default factors cover a single order of magnitude (i.e., 10), and a value of 3 is used in place of one-half power (i.e., $10^{0.5}$) (USEPA, 2002).

The oral RfD was derived using the following equation (USEPA, 2002):

$$RfD = \frac{POD}{UF_T}$$

Where:

POD = point of departure (mg/kg-day)
UF_T = uncertainty factor (total).

3. Results

3.1. PFHxA toxicology literature review

All available PFHxA toxicology data and studies are summarized below, organized by species (human then rodent data), and then by duration of exposure and major organ system.

3.1.1. PFHxA human epidemiological studies

The literature search identified four cross-sectional human epidemiology studies that included PFHxA (Table 1). It is likely that limited human observational studies have included PFHxA due to the low frequency of detection and low levels detected (see Part 2: Anderson et al. (2019) for a review of human biomonitoring studies).

Dong et al. (2013) investigated the relationship between serum levels of 11 PFAAs, including PFHxA, and levels of immunological markers (e.g., IgE, absolute eosinophil counts, eosinophilic cationic protein) in asthmatic Taiwanese children (N = 231) and nonasthmatic controls (N = 225). After adjusting for child age, sex, body mass index (BMI), parental education, month of survey, and tobacco smoke exposure, no association with serum PFHxA levels and immunological markers or asthma outcomes was detected.

Another cross-sectional study by Zhou et al. (2016) investigated the relationship between serum levels of nine PFAAs and sex hormone levels (e.g., testosterone and estradiol) in Taiwanese teenagers (N = 225). After adjusting for potential confounding factors (e.g., age, sex, BMI, smoking status, parental education, exercise, month of survey), a inverse association between serum PFHxA and testosterone levels was identified in Taiwanese boys, while no association between PFHxA and estradiol was detected for girls (Zhou et al., 2016). However, the authors note that menarche and other puberty indicators, as well as diurnal cyclicity, both of which can influence sex hormone levels were not accounted for in the study analysis.

Li et al. (2017) conducted a study of the general population in China (N = 202) and reported that exposure to PFHxA was positively associated with two biomarkers of thyroid autoimmune disease—thyroglobulin antibody (TGAb) and thyroid microsomal antibody (TMAb)—but not TSH, free T4 or T3. This positive finding was not observed for the other PFCAs included in the study (e.g., PFOA), nor is it consistent with evidence from rat studies of thyroid effects following exposure to PFHxA (Loveless et al., 2009; Iwai and Hoberman, 2014). Furthermore, the study authors did not statistically control for exposure to multiple PFAAs (carboxylates and sulfonates, such as perfluorooctane sulfonate, PFOS) in their analyses. Given that PFOS and PFOA accounted for approximately 70%–90% of the total sum of PFAAs

Table 1
Summary of PFHxA epidemiological studies.

Reference	Study Populations	Serum PFHxA		Results	
		Mean (ng/mL)[a,b]	FOD[a]	No Associations	Statistically Significant Associations
Dong et al. (2013)	Taiwanese children Control (N = 225; Age: 13.6 ± 0.7 years) Asthmatic (N = 231; Age: 12.9 ± 1.7 years)	0.3 (0.2)	97.0% (98.8%)	Immunological markers: IgE, absolute eosinophil counts, eosinophilic cationic protein, asthma severity scores	None
Fan et al. (2014)	C8 Health Study (N = 654 (GS); 27,450 (Control 1); 22,100 (Control 2)	1.7 (1.1 (C1 and C2))[c]	Not provided	[No other endpoints evaluated]	Gilbert Syndrome: ♀, ♂
Zhou et al. (2016)	Taiwanese children	0.2 ♀[d]	Not provided	Sex hormone levels: Estradiol (♀)	Sex hormone levels: Testosterone (♂; negative association)
	13–15 years old (N = 102 (♂); N = 123 (♀))	0.2 ♂[d]			Thyroid markers:
Li et al. (2017)	Chinese general population (N = 55 (♂); N = 147 (♀))	0.06 ♀ 1.1 ♂	53% (combined ♀ and ♂)	Thyroid markers: TSH, free T4, free T3	TGBa, TMAb (combined ♀ and ♂; positive association)

[a] Value in parentheses is for control group.
[b] Arithmetic mean unless noted otherwise.
[c] Geometric mean.
[d] Median; FOD = frequency of detection.

determined in blood in the cohort, the study does not provide clear definitve evidence of a link between PFHxA exposure and thyroid effects in the general population in China.

A study conducted using the C8 Health Study cohort investigated the relationship between serum levels of 10 PFAAs, including PFHxA, and Gilbert syndrome (GS; genetic disorder characterized by hyperbilirubinemia) (Fan et al., 2014). After adjusting for age, alcohol intake, BMI, education, exercise, gender, income, and smoking status, statistically significant differences in geometric serum PFHxA levels were noted for individuals who expressed the GS phenotype (both men and women). The homozygous genotype for GS is present in about 10% of the Caucasian population, but individuals do not always express the phenotype and remain clinically undiagnosed (Fan et al., 2014). Given that the prevalence rate for the GS phenotype was 2.6% for this study population (Fan et al., 2014), and that genetic testing was not part of the classification criteria, it is likely that some individuals in the control group were misclassified (e.g., normal levels of indirect bilirubin, yet carriers of the GS gene variant nonetheless). Therefore, this study likely exaggerates the degree to which serum PFHxA levels may be elevated among individuals with GS (including those who did not present with an abnormal liver function test but who have GS).

Overall, the epidemiology studies report some evidence of statistical associations between serum PFHxA levels and testosterone (Zhou et al., 2016), thyroid antibody markers (Li et al., 2017), and GS (Fan et al., 2014). However, all identified studies are cross-sectional in nature (i.e., can be used to identify associations only, not causal relationships), had other methodological weaknesses, and individuals had co-exposures to other PFAAs that were not controlled for in analyses. Therefore, the available human literature to date does not show a definitive association between PFHxA exposure and any human health disease.

In contrast to PFHxA, hundreds of epidemiology studies have investigated PFOA exposure in the general population, exposed communities, and occupational settings. Like PFHxA, most PFOA epidemiological studies tend to be cross-sectional in nature, and thus cannot be used to establish causation. Recent, in-depth reviews of these studies have been conducted by several government agencies. In 2016, USEPA concluded that there is “suggestive evidence of carcinogenic potential” for PFOA, and that there is potential for PFOA to affect liver function, risk of pregnancy-induced hypertension or preeclampsia, and low birth weight (USEPA, 2016a). In contrast, citing inconsistencies in findings from the published studies, the Australia Expert Health Panel recently concluded that there is no or limited evidence linking PFOA exposure to any human disease (Australia, 2018).

3.1.2. PFHxA animal bioassays

A robust suite of standard toxicity studies, including acute, subchronic (28- and 90-day), and chronic (2-year), have been conducted for PFHxA. In addition, there are seven publications that included an evaluation of toxicokinetics. Table 2 lists the available studies and study descriptions.

3.1.3. Acute toxicity studies

3.1.3.1. Oral toxicity. Two studies investigating the acute oral toxicity of PFHxA were identified (Loveless et al., 2009; Theunissen, 2004a). First, Loveless et al. (2009) dosed fasted female Crl:CD(SD) rats with a single oral (gavage) dose of sodium perfluorohexanoate (NaPFHx) at 175, 550, 1,750, or 5000 mg/kg BW. Rats were monitored for clinical signs of toxicity for up to 14 days. No mortality was observed in the 175 or 550 mg/kg treatment groups; one of four rats died on the day of dosing in the 1750 mg/kg treatment group; and all rats in the 5000 mg/kg treatment group died within 1 day after dosing. Clinical signs of toxicity occurred at the highest dose groups and included abnormal gait, ataxia, dehydration, high or low posture, lethargy, oral and nasal discharge, and wet or stained fur were observed in many of the surviving rats. Loveless et al. (2009) do not report the severity, dose-dependency, or day on which these effects were observed.

Table 2
PFHxA mammalian toxicity studies.

Study Type	Test Substance	Study Description	Reference
Acute (oral)	NaPFHxA	Single gavage doses of 175–5000 mg/kg administered to female Sprague-Dawley rats.	Loveless et al. (2009)
Acute (oral)	APFHx	Single gavage doses of 2000–3000 mg/kg administered to female Wistar rats.	Theunissen (2004a)
Acute (dermal)	APFHx	Single dose of 2000 mg/kg dermally applied to male and female Wistar rats.	Theunissen (2004b)
Acute (skin sensitization)	APFHx	Single dose applied to skin of rabbit, and monitored for up to 7-days.	Theunissen (2004c)
Acute (eye irritation)	APFHx	Single dose instilled in the eye of rabbits. Rabbits monitored for 28 days.	Theunissen (2004d)
Subchronic (28-day)	PFHxA	Repeat oral (gavage) dosing (62.6–1000 mg/kg-day PFHxA) of male and female Sprague-Dawley rats	NTP (2018)
Subchronic (90-day)	NaPFHx	Repeat oral (gavage) dosing (20–500 mg/kg-day) of male and female Sprague-Dawley rats. 30- and 90-day recovery period included.	Loveless et al. (2009)
Subchronic (90-day)	PFHxA	Repeat oral (gavage) dosing (10–200 mg/kg-day) of male and female Sprague-Dawley rats. Four-week recovery period included.	Chengelis et al. (2009a)
Reproduction/developmental	NaPFHx	One generation repeat oral (gavage) dosing (20–500 mg/kg-day) study in Sprague-Dawley rats.	Loveless et al. (2009)
Reproduction/developmental	APFHx	One generation repeat oral (gavage) dosing (7–175 mg/kg-day) study in Sprague-Dawley rats.	Iwai and Hoberman, 2014
Chronic toxicity/carcinogenicity (2-year)	PFHxA	Repeat oral (gavage) dosing in male and female Sprague-Dawley rats.	Klaunig et al. (2015)
Toxicokinetics	KPFHx	Male and female cynomolgus monkeys administered single IV dose of 10 mg/kg.	Noker (2001)
Toxicokinetics	PFHxA	Single IV and repeat (26-day) oral dosing studies in Sprague-Dawley rats and cynomolgus monkeys.	Chengelis et al. (2009b)
Toxicokinetics	APFHx	Single and repeat (14-day) oral dosing studies in CD-1 mice and Sprague-Dawley rats.	Iwai, 2011
Toxicokinetics	^{14}C-NaPFHx	Single and repeat (14-day) oral dosing studies in CD-1 mice and Sprague-Dawley rats.	Gannon et al. (2011)
Toxicokinetics	PFHxA	Single IV and gavage dosing of FVB/NJc1 mice.	Fujii et al., 2014
Toxicokinetics	KPFHx	Microminipigs administered a single dose (oral, capsule) of 3 mg/kg.	Guruge et al. (2016)
Toxicokinetics	PFHxA	Single IV or repeat oral (gavage) dosing studies in male and female Harlan Sprague-Dawley rats.	NTP (2018)
Toxicokinetics	Not applicable[a]	Estimation of serum elimination half-life in humans, and comparison of elimination kinetics in humans, mouse, rat, and monkey.	Russell et al. (2013)

[a] The human elimination half-life of PFHxA was determined in ski wax techniciians occupationally exposed to PFHxA precursors (i.e., 6:2 fluorotelomer alcohol or 6:2 fluorotelomer iodide).

In a GLP compliant OECD Guideline 423 (OECD, 2002) study, female Wistar rats (three per treatment) were orally (gavage) administered a single dose of ammonium perfluorohexanoate (APFHx) at 2000 or 3000 mg/kg and monitored daily for 14 days (Theunissen, 2004a). No mortality, effects on weight gain, or abnormalities upon macroscopic examination were noted at either dose, leading the author to conclude that PFHxA is not acutely toxic via the oral route at those dose levels.

Taken together, these studies indicate that the acute oral LD_{50} for rats is in the range of > 1750 to < 5000 mg/kg. USEPA (2016a) identified studies that reported an LD_{50} for rats in the range 250–1000 mg/kg for PFOA (Dean and Jessup, 1978; Glaza, 1997; Gabriel, 1976). Comparatively, the acute oral toxicity of PFOA is 3- to 20-fold greater than that of PFHxA.

3.1.3.2. Dermal toxicity. One GLP-compliant OECD Guideline 402 (OECD, 2017a) study was identified that investigated the acute dermal toxicity of APFHx (Theunissen, 2004b). A single dose of 2000 mg/kg was dermally applied to five male and female Wistar rats for 24-h followed by 14 days of observation. No mortality or abnormal macroscopic dermal effects were reported; however, skin irritation (e.g., scales and scabs) was noted near the site of application in most animals. Based on these results, PFHxA ammonium salt was not classified as acutely toxic via the dermal route.

Skin irritation following dermal application of APFHx has been investigated in rabbits (Theunissen, 2004c). In accordance with OECD Guideline 404 (OECD, 2015), test material was applied to the skin of a rabbit at three separate sites for 3 min, 1 h, and 4 h. Exposure caused erythema and oedema; however, these effects were not present 7 days after dosing. Accordingly, APFHx was not classified as a skin irritant.

Alternatively, perfluorohexanoic acid is a strong acid with a pK_a < 1, and is classified as a skin irritant (GHS Category 1B) by the European Chemical Agency based on its' acidic properties (ECHA, 2016). However, no experimental evidence of skin irritation is available.

Based on available data, and compared to PFHxA, APFHx is not expected to be a skin irritant or to be acutely toxic via the dermal exposure route. This is consistent with study results for long-chain PFAAs, such as PFOA, which have not been found to be highly irritating to skin (Kennedy, 1985).

3.1.3.3. Eye irritation. In accordance with OECD Guideline 405 (OECD, 2017b), APFHx was tested for acute eye irritation in rabbits (Theunissen, 2004d). APFHx was instilled into one eye of three different rabbits, and rabbits were monitored for eye irritation for 28 days. Corneal opacity and epithelial damage was noted, and was persistent in one rabbit for the entire study duration. These results led the authors to classify APFHx as "having irreversible effects on the eyes."

3.1.3.4. In vitro toxicity. The acute toxicity of PFAAs, including PFHxA, has also been tested *in vitro*. PFHxA was used in all *in vitro* studies in this section, which, as mentioned above, is acutely irritating. Several studies have shown that toxicity is dependent on chain length (Buhrke et al., 2013; Kleszczyński et al., 2007; Mulkiewicz et al., 2007). Using human colon carcinoma cells (HCT116), Kleszczyński et al. (2007) demonstrated that 24-h EC_{50} values (based on cell viability measured using the MTT assay) decrease with chain length (PFHxA [4154 μM] > PFHpA [1386 μM] > PFOA [937 μM] > PFNA [708 μM] > PFDA [283 μM] > PFDoDa [136 μM]). Similarly, Mulkiewicz et al. (2007) reported chain-length-dependent reductions in cell viability EC_{50} values in promyelocytic leukemia rat cells (IPC-81), rat glioma cells (C6), and *Vibrio fischeri*. PFHxA was less toxic (EC_{50} values 3–12 fold larger) than PFOA in every cell line tested. Finally, Buhrke et al. (2013) demonstrated that PFHxA is less cytotoxic than PFOA (IC_{50}s = 344 and 47 μM, respectively), and is a less potent inducer of cell proliferation in human hepatocarcinoma cells (HepG2). USEPA and NIH ran PFHxA through their Tox21 high-throughput bioassays; these data are discussed further in the section below on endocrine disruption and in Supplemental file 1 (Section 2.2.6). Collectively, these *in vitro* results clearly demonstrate that PFHxA is 3–12-fold less toxic than PFOA in acute exposures to

cultured cell lines.

3.1.3.5. General subchronic and chronic toxicity study descriptions. Given the number of studies and the large number of endpoints evaluated in each study, the general study descriptions are provided and endpoint-specific results are organized by major target organ and effect. The studies included are tabulated in Table 2.

3.1.3.5.1. Subchronic study descriptions. A 28-day subchronic toxicity study for PFHxA was recently conducted by the NTP, and findings have been made available to the public (NTP, 2018). The study findings have not yet been published in a peer-reviewed journal (as of December 2018). In this study, male and female Harlan Sprague-Dawley rats (10/treatment/sex) were orally (gavage) dosed with PFHxA at 62.6, 125, 250, 500, and 1000 mg/kg-day (corresponding to 378, 503, 1,297, 3,339, 10,899 ng/mL terminal plasma levels in males; and 129, 292, 475, 1,667, 6712 ng/mL in females) for 28 days. PFHxA treatment had no effect on survival in either sex or on terminal body weight in female rats; however, a ~13% reduction in terminal body weight was reported for male rats in the highest (1000 mg/kg-day) treatment group. A variety of clinical chemistry parameters increased (albumin-to-globulin ratio, bile salts, sorbitol dehydrogenase [male only]) or decreased (creatinine [male only], globulin, total bilirubin, indirect bilirubin [male only], total protein) in a dose-dependent manner in male and/or female rats. Effects on other parameters related to clinical chemistry were also noted, but are non-adverse due to their magnitude of change and/or lack of dose-dependency (e.g., alanine aminotransferase, alkaline phosphatase, aspartase aminotransferase, albumin, cholesterol, glucose). A variety of hematological parameters increased (reticulocytes, mean cell volume, mean cell hemoglobin, platelets, neutrophils [females only]) or decreased (hematocrit, hemoglobin, red blood cells, basophils [males only]) in a dose-dependent manner in male and female rats. Spleen weight increased in male (29%) and female (13%) rats treated with 1000 mg/kg-day. In addition, increased extramedullary hematopoiesis was noted in the spleen from male (≥500 mg/kg-day) and female (1000 mg/kg-day) rats. Additional organ (kidney, liver, and olfactory) specific effects are discussed further below in organ-specific toxicity sections.

Two 90-day subchronic toxicity studies for PFHxA have been conducted (Chengelis et al., 2009a; Loveless et al., 2009). Chengelis et al. (2009a) orally (gavage) administered PFHxA at 10, 50, or 200 mg/kg-day to male and female CRL:CD(SD) rats for 90 days. Ten rats of each sex were included in the 10 and 50 mg/kg-day groups, and 20 rats of each sex were included in the control and 200 mg/kg-day groups. Following 90 days of exposure, 10 rats in the control and 200 mg/kg-day treatment groups were monitored for an additional 4 weeks to determine if observed toxicological effects were reversible. PFHxA treatment did not affect animal survival, locomotor activity, any urinalysis parameters, or a functional observation battery at any dose in either sex. Body weight gains were generally reduced in male rats in all treatment groups throughout the 90-day exposure, whereas no significant effects on body weight were reported in females. Mean red blood cell parameters, including red blood cell count (males and females), hemoglobin (males only), and hematocrit (males only) were reduced in the 200 mg/kg-day treatment group, but were reversible after a 4-week recovery period. Several alterations in serum chemistry were also noted in the 200 mg/kg-day treatment group, including increased alanine aminotransferase and alkaline phosphatase in males (both reversible); reduced globulin in both sexes (reversible in females, but not males); and reduced cholesterol in males (did not recover). Additional organ (kidney and liver) specific effects are discussed further below in organ-specific toxicity sections.

Consistent with OECD Guideline 408 (OECD, 2018b), Loveless et al. (2009) dosed (gavage) 10 male and female Crl:CD(SD) rats per treatment group with NaPFHx at 20, 100, or 500 mg/kg-day for 90 days. A subset of rats was also evaluated after 30- and 90-day recovery periods. NaPFHx treatment had no effect on survival, food consumption, neurobehavioral activities, or a battery of functional observations in either sex at any dose. Reduced body weight gains were reported for males in the highest treatment group (500 mg/kg-day) on days 42–105 (i.e., 2 weeks post-dosing); however, no effects on body weight were reported for females. No changes in clinical chemistry were observed that were indicative of organ toxicity. Significant changes in aspartate aminotransferase, alanine aminotransferase, bilirubin, and total protein levels were not considered of toxicological importance due to their low incidence, lack of dose-dependency, or the direction of the change. Red blood cell count, hemoglobin, and hematocrit were reduced in both sexes in the highest treatment group; however, all alterations were reversible after the recovery period. Additional organ (kidney, liver, olfactory) specific effects are discussed further below.

3.1.3.5.2. Chronic study description. One study investigating the chronic toxicity of PFHxA was identified in which male and female Crl:CD(SD) rats were orally (gavage) dosed with PFHxA daily for 104 weeks (Klaunig et al., 2015). Male rats were dosed with 2.5, 15, or 100 mg/kg-day, while females were dosed with 5, 30 or 200 mg/kg-day. Sixty rats per sex were included in the low-dose treatment groups (2.5, 5, 15, 30 mg/kg-day groups), and 70 rats per sex were included in the highest treatment groups (100 and 200 mg/kg-day groups). PFHxA treatment had no effect on body weight or food consumption in either sex at any dose. PFHxA treatment did not affect male rat survival; however, a dose-dependent reduction in survival was observed for female rats, with a significant reduction in survival occurring in the highest treatment group (200 mg/kg-day). A number of significant alterations in locomotor activity and serum chemistry were reported for male and female rats; however, these changes did not correlate with histological alterations and were not dose-dependent or exposure-duration-dependent, and thus were not considered to be indicative of an adverse effect. Histological changes were noted in the kidney and liver and are discussed further below.

3.1.3.6. Organ and endpoint-specific toxicity study findings

3.1.3.6.1. Hepatic toxicity. In a 90-day subchronic toxicity study, Chengelis et al. (2009a) report a small increase (~15%) in relative liver weight in male rats in the highest PFHxA treatment group (200 mg/kg-day), but not in female rats. Absolute liver weights were not affected in either sex at any dose. Histopathological examinations noted hepatocellular hypertrophy in 7 out of 10 male, but not female, rats (200 mg/kg-day), which was characterized by granular material in the cytoplasm of enlarged hepatocytes, while necrosis and inflammation were noted in only a single animal. A small increase in hepatic peroxisomal β-oxidation activity was also detected in male rats in the highest treatment group (200 mg/kg-day). These effects were reversible after a 28-day recovery period. Collectively, these findings are consistent with an adaptive response following PPARα activation leading to peroxisome proliferation.

Similarly, Loveless et al. (2009) report increased relative and absolute liver weight in male and female rats administered NaPFHx at 500 mg/kg-day for 90 days. Mild hepatocellular hypertrophy and increased hepatic peroxisomal β-oxidation was reported in male (100, 500 mg/kg-day) and female (500 mg/kg-day) rats. Increased liver weight and hepatocellular hypertrophy were not completely reversible following a 90-day recovery period, and increased hepatic peroxisomal β-oxidation was persistent through a 30-day recovery period (not assessed in 90-day recovery group). No necrosis or inflammation was reported for either sex at any dose. Similar to the Chengelis et al. (2009a) study, Loveless et al. (2009) interpret the collective observations to be indicative of a non-adverse (adaptive) response.

In a recent NTP (2018) 28-day subchronic study of PFHxA administered to rats, dose-dependent increases in relative liver weight were noted in male (250, 500, 1000 mg/kg-day) and female (500, 1000 mg/kg-day) rats. Hepatocellular hypertrophy was observed in male rats treated with 500 and 1000 mg/kg-day, and female rats

administered 1000 mg/kg-day PFHxA, whereas cytoplasmic alterations were noted in both sexes treated with 1000 mg/kg-day. No necrosis was detected in either sex at any dose. A dose-dependent increase in liver acetyl-CoA activity (a marker of PPARα activation) was observed in male rats (250, 500, 1000 mg/kg-day; females not tested), which is consistent with a non-adverse, adaptive response that is not likely relevant to humans (Hall et al., 2012).

Increased hepatocellular necrosis (female only) and congestion (male only) have been reported for rats chronically (104 weeks) exposed to PFHxA at 200 mg/kg-day (Klaunig et al., 2015). However, these findings were not directly attributed to PFHxA treatment, as hepatocellular necrosis was primarily observed in animals that died prior to their scheduled necropsy without a dose-dependent pattern. Furthermore, Klaunig et al. (2015) note that the hepatocellular necrosis was consistent with ischemia caused by diminished hepatic blood flow, and liver function markers were not altered in this study.

Three subchronic studies in rats reported relatively consistent results for PFHxA and salts with respect to NOAEL and LOAEL values for common liver effect endpoints. Responses of the liver following PFHxA exposure are consistent with activation of PPARα-induced peroxisome proliferation resulting in hepatosome hypertrophy. Liver histological findings consistently reported minimal centrilobular hypertrophy in high dose (100–500 mg/kg-day males and 500 mg/kg-day female) rats (Loveless et al., 2009; Chengelis et al., 2009a; Klaunig et al., 2015). Hepatocytes were consistently described as enlarged and containing intracytoplasmic granular materials, consistent with peroxisome proliferation following PPARα activation. Wolf et al. (2008) reported that PFHxA activates PPARα in cells transfected with either the mouse or human PPARα receptor. Based on the diagnostic framework outlined by Hall et al. (2012), the lack of necrosis and inflammation suggests that these effects are not adverse and are unlikely to be relevant to human health. The majority of the hepatic effects reported for PFHxA can be considered non-adverse (adaptive) (see Supplemental Table S1).

Administration of PFOA over similar subchronic study durations yields LOAELs for increased liver weight in rats that are approximately 200- to 500-fold lower (0.96–1.0 mg/kg-day; Butenhoff et al., 2004a,b; Loveless et al., 2008) than those reported for PFHxA (Table S1), demonstrating that liver effects following PFHxA subchronic exposure occur at significantly higher doses than PFOA.

3.1.3.6.2. Renal toxicity. In a 90-day subchronic toxicity study (described above), Chengelis et al. (2009a) reported small increases (9%) in relative kidney weight in male rats dosed with 200 mg/kg-day PFHxA, while absolute kidney weight was not affected. A small, yet statistically significant increase (11.9%) in relative kidney weight was also noted in female rats in the 50 mg/kg-day, however, this effect did not occur in a dose-dependent manner, as no significant enlargement was reported in the 200 mg/kg-day female treatment group. Further, kidney enlargement was not accompanied by any notable histopathological findings. Therefore, despite the fact that the elevated absolute and relative kidney weights were not reversible after a 28-day recovery period the authors concluded that the response in the kidney was not indicative of an adverse effect. No further explanation was provided.

Similarly, increased (16%–17%) relative kidney weight in male (500 mg/kg-day) and female (500 mg/kg-day) rats exposed to NaPFHx for 90-days was reported by Loveless et al. (2009). Again, no histopathological alterations were detected in either sex or exposure group, and kidney effects were partially reversible in both sexes after a 30-day recovery period. The study authors reported a slight but statistically significant increase in urine volume in both male and female rats treated with 500 mg/kg-day NaPFHx, but no change in the 100 mg/kg-day group. Overall, the authors concluded that the absence of histological evidence of renal pathology or changes in serum chemistry parameters indicative of renal function impairment indicates that these kidney effects are not suggestive of an adverse effect associated with NaPFHx exposure.

In a 28-day subchronic study, NTP (2018) reports increased (12%–19%) relative kidney weight in the high dose male (500 and 1000 mg/kg-day) and female (1000 mg/kg-day) rats exposed to PFHxA. Furthermore, chronic progressive nephropathy was noted in female (1000 mg/kg-day) and male (62.6 and 250 mg/kg-day) rats, while no renal cysts were reported. Clinical chemistry markers, such as urea nitrogen, were unaffected by PFHxA treatment in either sex, while creatinine levels were slightly (~13%), yet significantly reduced in male rats in the highest treatment group (1000 mg/kg-day). No other indicators of kidney functional impairment were observed.

In a 2-year chronic toxicity study, male and female rats were treated with PFHxA at 0, 2.5, 15, 100 (male) or 0, 5, 30, 200 (female) mg/kg-day. Unlike the aforementioned 90-day subchronic toxicity studies, histopathologic alterations were noted in the kidneys of female (but not male) rats exposed to 200 mg/kg-day PFHxA for 104 weeks (Klaunig et al., 2015). Alterations included minimal to severe papillary necrosis and minimal to moderate tubular degeneration. However, these kidney effects coincide with a slight (14%) but significant reduction in survival. The study NOAEL for chronic oral kidney effects in female rats is 30 mg/kg-day and the LOAEL is 200 mg/kg-day.

Collectively, these studies indicate that high dose PFHxA exposure (≥200 mg/kg-day) can cause increased kidney weight; however, not all studies noted concomitant histopathological changes or clear dose-response and duration-response patterns. Comparatively, subchronic exposure to PFOA causes increased kidney weight in rats at 200- to 1000-fold lower doses (reviewed in USEPA, 2016a) (LOAEL of 1.0 mg/kg-day; Butenhoff et al., 2004a), demonstrating the higher renal toxicity of PFOA compared to PFHxA (Table 3).

3.1.3.6.3. Endocrine effects. A hypothesis-driven weight-of-evidence (WoE) approach was recently conducted to assess the potential endocrine disrupting activity of PFHxA (Borghoff et al., 2018). The review conducted by Borghoff et al. (2018) considered epidemiology studies, and *in vitro* and *in vivo* toxicity studies. Based on the lack of evidence for endocrine disruption by PFHxA in both *in vivo* mammalian and non-mammalian models, as well as the overall lack of endocrine bioactivity in *in vitro* studies, PFHxA was not classified as an endocrine

Table 3
Subchronic and chronic NOAELs and LOAELs for PFHxA for renal effects in rats.

Reference, Duration	Chemical	Endpoint	NOAEL (mg/kg-day)	LOAEL (mg/kg-day)	Dose-Response?	Histo-pathology	Interpretation
Chengelis et al. (2009a), 90 days	PFHxA	↑ kidney weight	50 ♂	200 ♂	Yes	none	Non-adverse
			> 200 ♀ (unbounded)	none	No		Unbounded NOAEL
Loveless et al. (2009), 90 days	NaPFHx	↑ kidney weight	100 ♂, ♀	500 ♂, ♀	Yes	none	Non-adverse
NTP (2018) 28 days	PFHxA	↑ kidney weight	250 ♂	500 ♂	Yes	none	Unclear
			500 ♀	1000 ♀	Yes		Unclear
Klaunig et al. (2015), 104 weeks (chronic)	PFHxA	Histological changes	> 100 ♀ (unbounded)	none	No	none	Unbounded NOAEL
			30 ♀	200 ♀	Yes	Papillary necrosis, tubular degeneration	Adverse

disruptor. This is in contrast with PFOA, wherein epidemiology data and rodent data show potential associations with PFOA exposure and effects on steroid hormones, including development of puberty in females, and decreases in fecundity and fertility in females (USEPA, 2016a). PFOA rodent bioassays have also reported numerous effects on hormone disruption and hormone-related endpoints, including thyroid, estradiol, and androstenedione (see review in USEPA, 2016a). Section 4 of Supplemental file 1 contains further discussion of the endocrine effects (or lack thereof) of PFHxA, including a summary of the ToxCast/Tox21 high throughput screening assay results (see Supllemental Table S2).

3.1.3.6.4. Reproductive and developmental effects. The reproductive and developmental toxicity of PFHxA and salts has been investigated in mammalian (Iwai and Hoberman, 2014; Loveless et al., 2009) and non-mammalian models (ECHA, 2015; Frey et al., 2010). In a study conforming to OECD Guideline 414 (OECD, 2018c), Loveless et al. (2009) dosed (gavage) 22 female Crl:CD(SD) rats per treatment group with 20, 100, or 500 mg/kg-day NaPFHx on gestational days (GD) 6–20. A 5% reduction in food consumption, and reduced (19%–26%) maternal body weight gains between GD 6–20 were noted in the highest exposure group. No skeletal or soft tissue alterations were noted in fetuses from any treatment group; however, a 10% reduction in fetal body weight, which coincided with maternal toxicity (i.e., reduced body weight gains and food consumption), was noted in the highest treatment group.

A one-generation reproductive study (OECD Guideline 415; OECD, 1983) was also conducted by Loveless et al. (2009). Females were orally (gavage) dosed with NaPFHx (20, 100, 500 mg/kg-day) for 70 days prior to cohabitation with dosed males, and then treatment was resumed throughout gestation and lactation, for a total of approximately 126 days. F1 offspring were examined at weaning and at 6 weeks post-weaning to assess developmental landmarks. No maternal or paternal mortality was observed at any dose; however, several clinical signs of generalized toxicity were reported, including stained skin/fur in males and females (500 mg/kg-day); reductions (12%–29%) in overall male body weight gain (100 and 500 mg/kg-day); and reduced maternal bodyweight gains during first week of gestation (500 mg/kg-day). Exposure to NaPFHx had no effect on mating, fertility, gestation length, number of implantation sites, estrous cyclicity, various sperm parameters, litter size, sex ratio, pup clinical observations, pup survival, or F1 developmental landmarks. A 17%–18% reduction in pup weight in the 500 mg/kg-day treatment group was reported throughout lactation; however, body weight gains in post-weaning F1 adults were unaffected by NaPFHx treatment. Based on reduced body weight, the adult rat NOAEL is 20 mg/kg/day, while the pup NOAEL is 100 mg/kg-day; however, reductions in pup body weight coincided with signs of overt maternal and paternal toxicity.

In another GLP compliant study designed to investigate the reproductive and developmental toxicity of PFHxA, Iwai and Hoberman (2014) treated (gavage) female (20/group) Crl:CD1(ICR) mice with APFHx on GDs 6–18. Results of phase 1 of the study (100, 350, and 500 mg/kg-day APFHx) resulted in mortality in mid- and high-dose groups, which negated the utility of the study for purposes of evaluating reproductive and developmental endpoints. A second phase was conducted utilizing lower doses of 7, 35, and 175 mg/kg-day. In the second phase of the study, APFHx treatment had no effect on F0 maternal survival, body weight (or gains), pregnancy rates, number of dams delivering litters, number of implantation sites, gestation index, litter sizes, or the sex ratios of litters. In the F1 generation, maternal treatment with APFHx did not affect pup body weight gains, survival through postweaning (out to post-partum day (PPD) 42), or sexual maturation. In F1 pups from the highest maternal treatment group (175 mg/kg-day), a 1.2% (3/241 pups) increase in stillbirths, a 1.7% increase in pup mortality on PPD 0, and a 12.5% decrease in pup body weight on PPD 0 were reported; however, effects were not dose-dependent and pup weight recovered by PPD 4. Two clinical observations were also reported for pups from the highest exposure group. Corneal opacity was observed in two pups, one in each of two different litters for an overall incidence of 0.8% (2/238 pups), while microphthalmia (undersized eye) was also reported for two pups from two separate litters. However, a recent reanalysis of the stillbirth data, and a comparison to the historical controls from the same laboratory demonstrates that the 1.2% incidence of stillbirths in the 175 mg/kg-day treatment group are well within the range of stillbirths reported for historical controls (average stillbirth rate of 1.3% from 23 studies) (Iwai et al., Inreview at the *Int J Toxicol*). Furthermore, several studies have demonstrated that corneal opacity and microphthalmia occur spontaneously in CD-1 mice at rates similar to those reported by Iwai and Hoberman (2014), and thus the low incidence (0.8%) of ocular effects are unlikely to be related to APFHx exposure (Iwai et al., In review at the *Int J Toxicol*). Collectively, this reanalysis indicates that the low incidence of mortality and stillbirths, ocular effects, and transient non-dose-dependent reduction in body weight on PPD 0 are unrelated to APFHx treatment. Therefore, the study results support an unbounded NOAEL of 175 mg/kg-day (maternal exposure) for developmental effects in mice exposed to APFHx.

No signs of male reproductive toxicity were reported in a recent 28-day subchronic oral gavage rat study (NTP, 2018). PFHxA had no effect on testes, epididymis, or cauda epididymis weight, or testicular spermatid count or sperm motility in males dosed up to 1000 mg/kg-day. A reduction (25%) in cauda epididymis sperm count was noted; however, this reduction was no longer significant after normalizing to cauda epididymis tissue weight. Similarly, no changes in uterus weight were noted in females dosed up to 1000 mg/kg-day, however, dilation and bilateral dilation were noted in the uterus of female rats in the 62.6, 250, and 500 mg/kg-day treatment groups. Estrous cyclicity was also mildly perturbed by PFHxA, with the estrous and diestrous stages of female rats exposed to 125, 250, or 500 mg/kg-day PFHxA (62.5 and 1000 mg/kg-day treatment groups not tested) extended, the mean cycle length shortened with increasing dose. However, the number of cycles in female rats was not affected by PFHxA exposure at any dose level, and other 90-day subchronic rat studies reported no changes in estrous cyclicity following treatment with PFHxA (Loveless et al., 2009; Kirkpatrick, 2005). Due to high variability in stages of the estrous cycle, it is an unreliable measure of potential reproductive toxicity (Morrissey et al., 1988). Thus, the overall lack of observed effects in key reproductive endpoints in both male and female rats supports a conclusion that PFHxA exposure is not a likely reproductive toxicant at doses as high as 1000 mg/kg-day.

Collectively, results from *in vivo* mammalian studies indicate that PFHxA and salts are neither a reproductive nor a developmental toxicant, which is further supported by OECD guideline studies conducted in non-mammalian models (Supplemental file 1, Section 1). In contrast, developmental toxicity (reduced ossification of the proximal phalanges (forelimb and hindlimb) and accelerated puberty in male pups from the Lau et al. (2006) developmental mouse study was chosen by USEPA as the critical effect for derivation of the PFOA toxicity value and drinking water health advisory (USEPA, 2016b).

3.1.3.6.5. Olfactory toxicity. Olfactory lesions, including degeneration/atrophy of the olfactory epithelium, have been reported in several subchronic oral gavage toxicity studies (Loveless et al., 2009; NTP, 2018). Nasal atrophy was not reported in the Chengelis et al. (2009a) subchronic gavage study and not assessed in the Klaunig et al. (2015) chronic gavage study. Loveless et al. (2009) reported minimal to mild degeneration and atrophy of the olfactory epithelium in male and female rats exposed (gavage) to NaPFHx at 100 or 500 mg/kg-day for 90 days (Loveless et al., 2009). These effects were mostly reversible following both 30- and 90-day recovery periods. NTP (2018) reported minimal to mild, non-neoplastic, olfactory epithelium degeneration, hyperplasia, and inflammation in male and female rats exposed to 250 and 500 mg/kg-day, while some lesions in the highest exposure group (1000 mg/kg-day) were classified as moderate to marked in male and

female rats. From these studies, the subchronic NOAELs for non-neoplastic nasal lesions are 20 (Loveless et al., 2009) and 125 mg/kg-day (NTP, 2018). However, patterns of nasal lesions in Loveless et al. (2009) are concistent with exposure to acidic or irritating test substance following gavage error and reflux, and do not appear to be due to toxicity following systemic distribution of PFHxA. Patterns of nasal lesion formation were not provided in study reports for the 28-day oral gavage NTP (2018) studies, thus, it is presently unclear whether or not nasal lesions in this study are consistent with gavage error and reflux.

Given that nasal lesions were inconsistently reported in toxicity studies, mostly reversible (Chengelis et al., 2009a), and that the patterns of nasal lesions suggest that they were due to exposure to acidic or irritating test substance through reflux and not systemic distribution, we concluded that nasal lesions were not a human-relevant adverse health effect, and these effects were not further considered for development of an RfD. More detailed discussion of the human health relevance of nasal lesions is included in Section 5 of Supplemental File 1.

3.1.3.6.6. Cancer. One laboratory bioassay investigating the carcinogenicity of PFHxA was identified (Klaunig et al., 2015; described in detail in Section 2.2.2). No increase in neoplasms (in any organ) was detected in male or female rats in any treatment group following a 104-week exposure to PFHxA, resulting in unbounded NOAELs of 100 mg/kg-day for male rats and 200 mg/kg-day for female rats.

Multiple studies with PFHxA demonstrate that PFHxA in not genotoxic or mutagenic, consistent with similar studies conducted with other PFAAs (USEPA, 2016a,b). The genotoxicity of PFHxA has been investigated *in vitro* (Eriksen et al., 2010; Buhrke et al., 2013; Loveless et al., 2009; NTP, 2018). Eriksen et al. (2010) investigated reactive oxygen species (ROS) production as a potential mechanism of inducing genotoxicity in human liver HepG2 cells across a 3-h exposure to PFHxA, PFOA, PFOS, PFNA, and PFBS (doses ranged from 0.4 μM to 2 mM). PFOA induced a 1.52-fold increase in ROS, while PFOS induced a 1.25-fold increase in ROS; however, induction of ROS formation did not occur in a dose-dependent manner, nor did either compound induce detectable DNA damage in the comet assay. Furthermore, PFHxA did not induce ROS production or DNA damage at any dose (Eriksen et al., 2010).

PFHxA has also been evaluated for mutagenicity in the bacterial reverse mutation (Ames) assay according to OECD Guideline 471 (OECD, 1997a; Buhrke et al., 2013; Loveless et al., 2009; NTP, 2018). None of these studies found PFHxA to be mutagenic in *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537) or *Escherichia coli* (WP2uvrA, pKM101) either with or without metabolic activation of PFHxA (Aroclor-induced rat liver S9 microsomes were used as an exogenous metabolic activation system). Loveless et al. (2009) also tested NaPFHx in the chromosomal aberration assay in accordance with OECD Guideline 473 (OECD, 1997b) and found no chromosomal damage in human peripheral blood lymphocytes exposed to activated (S9 activation system) or non-activated PFHxA. In general agreement with this, NTP (2018) found no chromosomal aberrations in red blood cells harvested from female Harlan Sprague Dawley rats orally (gavage) exposed to PFHxA (doses ranged from 31.3 to 500.0 mg/kg). However, results from male rats were equivocal, with dose-dependent increases in micronuclei measured in one experiment, trending-increases observed in a second experiment, and no increases in micronuclei formation noted in the third experiment (NTP, 2018). Alternatively, Buhrke et al. (2013) conducted an *in vitro* micronucleus test with V79-cells in accordance with OECD Guideline 487 (OECD, 2010), and found that PFHxA did not induce micronuclei formation.

Lastly, we reviewed PFHxA data related to potential non-genotoxic carcinogenic modes of action. A comprehensive review of both *in vitro* and *in vivo* studies evaluating PFHxA activity across endocrine pathways shows that PFHxA is not bioactive in estrogen, androgen, aromatase, or thyroid receptor signaling pathways (Borghoff et al., 2018) and does not act as an estrogen or androgen receptor agonist or antagonist at environmentally relevant levels (Behr et al., 2018), which demonstrates that PFHxA is not likely to be a carcinogen via non-genotoxic mechanisms such as disruption of hormone signaling and increased cellular proliferation.

Although the mutagenicity data on PFOA are largely negative, evidence of carcinogenicity related to PFOA exposure in epidemiology studies (primarily studies of kidneys and testicular cancers), and positive animal carcinogenicity studies, has led USEPA to classify PFOA as having “suggesting evidence of carcinogenic potential” (USEPA, 2016a) and the International Agency for Research on Cancer to classify PFOA as “possibly carcinogenic to humans” (IARC, 2016).

3.2. Mechanisms of PFHxA toxicity

Activation of the peroxisome proliferator-activated receptor alpha (PPARα) is considered one of the primary mechanisms underlying the toxicity of at least some of the PFAAs; however, activation of other nuclear receptors (e.g., PPARɣ, constitutive androgen receptor, and estrogen receptor alpha [ERα] [Rosen et al., 2008, 2010, 2017]), mitochondrial toxicity, and oxidative stress are also thought to contribute to PFCA toxicity. Similarly to PFOA and other long chain PFAAs, PFHxA can act as a PPARα agonist, albeit with reduced potency (Bjork and Wallace, 2009; Vanden Heuvel et al., 2006; Wolf et al., 2012). To date, the mechanisms of toxicity for PFCAs in general, and for PFHxA specifically, are undefined. Therefore, mechanisms of action and the potential for rodent-specific mechanisms are not used to identify critical effects for human health toxicity and derivation of the toxicity values herein. Readers are referred to Section 2 of Supplemental file 1 for a comprehensive review of potential mechanisms of action.

3.3. Toxicokinetics and bioaccumulation

The toxicokinetics of PFHxA have been investigated in humans, mice, micromini pigs, monkeys, and rats (Chengelis et al., 2009b; Fujii et al., 2014; Gannon et al., 2011; Guruge et al., 2016; Han et al., 2011; Iwai, 2011; Russell et al., 2013; Buck and Gannon, 2017), and results from these studies are summarized below. Overall conclusions support that PFHxA is rapidly eliminated in mammals from the body and is not biologically persistent or bioaccumulative, and there are no significant species- or sex-specific differences in mammalian elimination kinetics when the alpha elimination rate and beta elimination rate are compared across mammalian species (Buck and Gannon, 2017).

3.3.1. Absorption and distribution

Following oral (gavage) exposure, PFHxA is rapidly absorbed and eliminated in male and female CD-1 mice, Sprague-Dawley rats, and cynomolgus monkeys, as demonstrated by 70% to nearly 100% recovery of the administered dose in the urine and feces 24 h after dosing (Chengelis et al., 2009b; Fujii et al., 2014; Gannon et al., 2011; Iwai, 2011). Further demonstrating rapid absorption, maximum serum concentrations (C_{max}) of radiolabeled ^{14}C-PFHx were achieved 15–30 min after oral (gavage) exposure to 2 or 100 mg/kg ^{14}C-PFHx in male and female CD-1 mice and Sprague-Dawley rats (Gannon et al., 2011). Tissue distribution and clearance were also investigated by Gannon et al. (2011). At 30–120 min after dosing, ^{14}C-PFHx was primarily detected in the plasma, kidneys, liver, and bladder of male and female mice and rats; however, ^{14}C-PFHx was also detectable to a lesser extent in many other tissues, including red blood cells, bone, bone marrow, brain, fat, heart, lungs, muscle, pancreas, pituitary gland, skin, spleen, testes, thymus, thyroid, and uterus. In general agreement, Iwai (2011) dosed male and female Sprague-Dawley rats and CD-1 mice with 50 mg/kg-day APFHx for 14-days, and reported very low or below detection limits of APFHx in most tissues 7 days after the final dose. However, APFHx remained quantifiable in blood and liver (concentrations 4–8-fold higher in liver than blood) samples from both species and sexes (Iwai, 2011). Similarly, Fujii et al. (2014) dosed male

and female FVB/NJcl mice with a single dose of PFHxA (IV: 0.313 μmol/kg; gavage: 3.13 μmol/kg), and then assessed tissue distribution 24 h later. Regardless of route of exposure or sex, PFHxA was below detection limits in the serum, liver, kidney, brain, and adipose tissue 24 h post-dosing.

In agreement with rodent studies, PFHxA has also been shown to be rapidly absorbed in micromini pigs orally administered a single encapsulated PFHxA dose of 3.0 mg/kg, with maximum blood concentrations achieved within 12 h (Guruge et al., 2016).

3.3.2. Metabolism

Like other PFCAs, PFHxA is a highly stable compound and is not metabolized. In agreement with this, Gannon et al. (2011) isolated primary hepatocytes from male and female Sprague-Dawley rats, and incubated the cells with 50 μM APFHx for 120 min. No change in the concentration of APFHx in the cell culture medium was detected, nor were any APFHx metabolites detected, demonstrating no significant metabolism of APFHx in this *in vitro* model. Furthermore, Gannon et al. (2011) did not detect any APFHx metabolites in urine, feces, or plasma samples collected from male or female CD-1 mice or Sprague Dawley rats following oral administration of APFHx, further indicating that APFHx does not undergo metabolism *in vivo*. This is consistent with data on other PFCAs, including PFOA, where no metabolism has been demonstrated (USEPA, 2016b).

3.3.3. Excretion

Studies have demonstrated that PFHxA is rapidly excreted, primarily in the urine, in mice, rats, and monkeys. Furthermore, serum elimination in mammals occurs in a biphasic pattern in which > 99% of the administered dose is rapidly excreted in the first (alpha) phase of elimination (Chengelis et al., 2009b; Fujii et al., 2014; Gannon et al., 2011; Han et al., 2011; Iwai, 2011; Russell et al., 2013). Rapid elimination kinetics are likely due to the fact that PFHxA exhibits minimal to no binding to proteins such as fatty acid binding protein and certain organic anion transporters that mediate renal reabsorption (in depth discussions of PFHxA protein binding is included in Section 3 of Supplemental file 1). Gannon et al. (2011) reported > 99% recovery of ^{14}C-PFHx in the urine of male and female rats in single or repeat dose groups 12 h after exposure, while recovery of > 99% of the dose in urine from mice occurred over 24 (female) to 48 h (male), with no appreciable excretion occurring in the feces of either species or sex. Tissue clearance in this study also occurred rapidly as ^{14}C-PFHx was below the limit of quantitation in most tissues in both sexes and species 24 h after dosing. One notable exception to this is that ^{14}C-PFHx was detected in the skin of male rats (but not female), and both sexes of mice (Gannon et al., 2011). Iwai (2011) report similar urinary excretion rates in mice and rats; however, excretion of APFHx in feces was detected. Regardless of the sex, species, or dosing regimen (single vs. 14-day repeat), APFHx was primarily excreted in the urine (73.0%–90.2%), and to a lesser extent the feces (7.0%–15.5%), with > 95% of the total dose recovered 24 h after dosing. Chengelis et al. (2009b) reported 77%–100% recovery of PFHxA administered as a single (intravenous) or 26-day oral (gavage) repeat dose in urine of male and female rats 24 h after the final dose, with no appreciable differences in urinary excretion between sexes. Finally, Fujii et al. (2014) recovered 68.8%–100% of the administered dose 24 h after FVB/NJcl mice were exposed to a single dose of 0.313 μmol/kg (IV) or 3.13 μmol/kg (gavage), with the majority of the dose recovered in urine (47%–100%), and to a lesser extent feces (4.7%–15.6%). Excretion of PFHxA also appears rapid in micromini pigs, as no PFHxA was detected in the blood, kidney, or liver after a 21-day depuration period (Guruge et al., 2016).

Although numerous studies have reported biphasic serum elimination patterns for PFHxA in mammals, only toxicokinetic studies conducted by NTP (2018) in rats and Noker (2001) in monkeys have calculated alpha and beta phase serum elimination half-lives. In the study by NTP (2018), male and female Harlan Sprague-Dawley rats were administered (IV or gavage) single doses (40–160 mg/kg) of PFHxA. PFHxA was rapidly eliminated from the plasma during the alpha phase, with first-order elimination half-lives ranging from 0.7 to 2.4 h for male and 0.3–1.4 h for female rats. In contrast, the beta phase occurred more slowly, with elimination half-lives ranging from 5.7 to 13.7 h for male and from 2.3 to 12.2 h for female rats. Similarly, Noker (2001) administered (IV) single doses (10 mg/kg) of KPFHx to monkeys, and determined the alpha phase elimination half-life to be 1.9 and 1.2 h for male and female monkeys, respectively, while slower beta phase elimination half-lives of 34.8 (male) and 19.4 (female) hours were noted. The rapid alpha elimination phase, followed by a slower beta phase highlights the importance of distinguishing the kinetics of the two elimination phases, and studies that fail to do so will overestimate the serum elimination half-life of PFHxA.

To demonstrate this, Buck and Gannon (2017) recently presented a reanalysis of data provided in several toxicokinetic studies (Chengelis et al., 2009b; Gannon et al., 2011; Guruge et al., 2016; Noker, 2001;

Table 4
Alpha and beta serum elimination half-lives of PFHxA in mammals.

Mammalian Species	Test Substance	Elimination Kinetics		Study
		α phase $t_{1/2}$ (hour)	β phase $t_{1/2}$ (hour)	
Mouse (0.03 kg)	^{14}C-NaPFHx	1.5	63	Gannon et al. (2011) [a]
Rat (0.25 kg)	^{14}C-NaPFHx	1.8	ND	Gannon et al. (2011) [a]
Rat (0.25 kg)	PFHxA	0.3–2.4	2.3–13.7	NTP, 2018 [b]
Monkey (~5 kg)	KPFHx	1.7	50	Noker (2001) [a,c]
Monkey (~5 kg)	PFHxA	1.7	50	Chengelis et al. (2009b) [a]
Micromini pig (~12 kg)	KPFHx	ND	64	Guruge et al. (2016) [a,d]
Domestic pigs (~100 kg)	PFHxA	1.6	99	Numata et al. (2014) [a]
Humans (~80 kg)	NA	ND	122	Nilsson et al. (2013) [a,e]

ND = not determined.

[a] Raw data from these studies were reevaluated by Buck and Gannon (2017) to determine α and β half-lives.

[b] Reported half-lives are as indicated in original study report.

[c] Noker (2001) determined α and β values based on serum data from up to 4-days post-exposure; Buck and Gannon (2017) reevaluated data out to 14 days post-exposure to better estimate the beta phase.

[d] The first serum PFHxA measurement was taken 24 h post-exposure, thus missing the rapid alpha phase of elimination. Buck and Gannon (2017) assume the half-life derived in this study is for the beta phase.

[e] Data from this biomonitoring study was used by Buck and Gannon (2017) to determine the elimination kinetics of PFHxA in occupationally exposed ski wax technicians.

Nilsson et al., 2013; Numata et al., 2014) and calculated alpha and beta serum elimination half-lives for PFHxA. Half-lives from this reanalysis are presented in Table 4, while elimination half-lives calculated by the original study authors are presented in Supplemental Table S3. Results from this reanalysis are in line with NTP (2018) and Noker (2001), and demonstrate a rapid alpha elimination phase ranging from 1 to 2 h for mice, rats, monkeys, and pigs, and a slower beta elimination phase ranging from 50 to 122 h for mice, rats monkeys, pigs, and humans. Furthermore, the reanalysis by Buck and Gannon (2017) demonstrates that more than 99% of the adsorbed dose of PFHxA is eliminated in the rapid alpha phase.

The serum elimination half-life of PFHxA has also been estimated for a group of eight professional ski wax technicians exposed to various volatile PFAAs. In particular, technicians were exposed to relatively high concentrations of 6:2 fluorotelomer alcohol, which is a known metabolic precursor of PFHxA (Nilsson et al., 2013). Following the end of ski season (i.e., end of occupational exposure), serum concentrations of PFHxA were monitored and serum elimination half-lives were estimated to range from 14 to 49 days, with a geometric mean of 32 days (Russell et al., 2013). However, Russell et al. (2013) note that it is likely that ongoing exposure to PFHxA or its precursors occurred during the elimination period, and thus the 32-day half-life value is a highly conservative, high-end, estimate. To account for potential ongoing exposure, Buck and Gannon (2017) reevaluated PFHxA serum data for ski wax technicians presented in Nilsson et al. (2013). The reanalysis was based on serum PFHxA data for three individuals in which large, rapid reductions in serum PFHxA levels were observed, which indicates no ongoing exposure to PFHxA during the elimination phase. Base on these data, an improved serum elimination half-life of 5.1 days was proposed for the beta phase of elimination.

3.3.4. Bioaccumulation

Bioaccumulation of PFCAs has been shown to be related to the length of a compound's fluorinated carbon chain, with shorter-chain PFCAs not predicted to bioaccumulate (reviewed in Conder et al., 2008). Due to the rapid and nearly complete elimination of PFHxA in mice, rats, and monkeys, PFHxA would not be expected to bioaccumulate. Furthermore, Russell et al. (2013) noted no apparent trend of bioaccumulation in professional ski wax technicians occupationally exposed to PFHxA over 4 years. In agreement with human and mammalian studies, PFHxA was not found to bioconcentrate or bioaccummulate in rainbow trout (Martin et al., 2003a,b).

3.4. Toxicity data conclusion

There are several human epidemiology studies and a significant number of animal laboratory bioassays of acute, subchronic, and chronic duration, including evaluations of the standard full suite of organs, cancer incidences, and potential reproduction and developmental effects. All of the observed effects related to PFHxA were either unlikely to be indicative of true adversity (e.g., nasal lesions, liver weight changes) or were mild (e.g., kidney histopathology) and/or reversible and noted at levels significantly higher than PFOA. Overall, the data clearly demonstrates that PFHxA has low acute and chronic toxicity, particularly when compared with PFOA.

4. PFHxA chronic human toxicity value (reference dose, RfD)

4.1. Selection of critical effect

The available PFHxA literature discussed above was reviewed for applicability as the key study, based on study quality and adherence to general good laboratory practice standards. Critical effects from the studies were identified to define PODs. Klaunig et al. (2015) noted renal papillary necrosis (17 out of 70 animals) and renal tubular degeneration (7 out of 70) in the 200 mg/kg-day female rats exposed to PFHxA daily for 104 consecutive weeks (i.e., chronic exposure in a rodent model). Although there were no reported effects in the high dose (100 mg/kg-day) males and no reported changes in female renal function parameters (plasma creatinine and blood urea nitrogen) in the high dose group, the kidney histopathological effects noted in the females may be a precursor to adverse effects on the kidney. Furthermore, kidney effects were noted in several of the subchronic studies in both rats and mice (NTP, 2018; Loveless et al., 2009; Chengelis et al., 2009a) (Table 4), indicating that the kidney may be a target organ for PFHxA exposure. None of the noted liver weight changes in the subchronic studies (Loveless et al., 2009; Chengelis et al., 2009a) appear to be truly adverse and were noted only at high administered doses of 500 mg/kg-day. No other adverse effect is available in the same dose range as the kidney histopathology findings from Klaunig et al. (2015); therefore, papillary necrosis and tubular degeneration noted in female rats exposed to PFHxA for 104 weeks were selected as critical effects.

4.2. Deriving the POD

BMD modeling is the preferred methodology for deriving the POD (USEPA, 2012). USEPA's BMDS (version 2.7) was utilized to model dose response for the female kidney histopathological findings (papillary necrosis and tubular degeneration), in accordance with USEPA guidance for dichotomous endpoints (USEPA, 2012).

For the modeled female kidney histopathology data (both papillary necrosis and tubular degeneration) from Klaunig et al. (2015), the benchmark response (BMR) for extra risk was set at 10% from the control mean. Default program parameters were used. The *p*-value to determine test acceptance or rejection was set at 0.1, in accordance with USEPA recommendations (USEPA, 2012). Model fit was assessed considering *p*-value for goodness-of-fit, the Akaike information criterion (AIC) value, scaled residuals near the range of the BMD, and visual inspection of the dose-response curves in the low-dose range. All available models for noncancer dichotomous data within the BMDS (Gamma, Logistic, Probit, Weibull) were run using default settings to aid in selecting a model that best describes the data. BMD modeling output is shown in Table S4 of Supplemental File 1. Neither the female papillary necrosis or tubular degeneration data sets provide particularly good data for BMD modeling as statistically significant reponses only occurred in the highest exposure group; however, both data sets were amenable to modeling. Benchmark dose limits (BMDLs) from successful models for each data set were all within a factor of 3, but did vary. Therefore, among the models with adequate fit to the data based on visual inspection, the model with the lowest AIC, lowest BMDL, and passing statistical fit to the data was the LogProbit Model of the papillary necrosis (USEPA, 2012). Fig. S1 in Supplemental file 1 shows the model fit.

The resulting $BMDL_{10}$ of 90.4 mg/kg-day for papillary necrosis was therefore selected as the POD.

4.3. Calculating the POD_{HED}

The preferred approach when using animal laboratory bioassays as the basis for human health risk assessment is to use chemical-specific physiologically-based toxicokinetic modeling to convert toxicologically equivalent doses of orally administered agents from laboratory animals to humans. Another approach may include using chemical-specific toxicokinetic and toxicodynamic information to derive chemical-specific adjustments. In lieu of robust chemical-specific toxicokinetic and toxicodynamic information, the accepted method to derive the human equivalent dose is by body-weight scaling to the 3/4 power (i.e., $BW^{3/4}$), which relies upon the known relationship between body weight and the metabolism of humans compared to rodents (USEPA, 2011). The use of $BW^{3/4}$ scaling for deriving an RfD is recommended when the observed effects are associated with the parent compound or a stable metabolite in the absence of available chemical-specific toxicokinetic

models (USEPA, 2011). However, for chemicals that exhibit species-specific pharmacokinetic properties such as long-chain PFAAs, chemical-specific adjustment using specific information about species differences is most appropriate. PFOA and PFOS exhibit marked differences in species-specific pharmacokinetics, mainly due to differences in elimination rates (due to higher renal reabsorption and serum protein binding in the human). However, the elimination rate for PFHxA has been shown to scale by body weight, and there are no known species-specific elimination mechanisms that dramatically alter PFHxA elimination kinetics between species (Russell et al., 2013). Allometric scaling by use of $BW^{3/4}$, combined with a reduced interspecies uncertainty factor (UF_A, see below), is the current best risk assessment methodology when deriving an RfD in the absence of available chemical-specific toxicokinetic models, and has been recommended by USEPA since 2011 (USEPA, 2011). This methodology is appropriate for PFHxA because it is well established that any potential toxicity would be due to the parent compound (i.e., there is no species-specific metabolism to consider), there does not appear to be species-specific pharmacokinetics associated with PFHxA, and it has already been established that elimination rates for PFHxA scale by body weight (Russell et al., 2013). The use of $BW^{3/4}$ is a more concise expression of the following equation:

Human equivalent dose = BMD from the animal study / $(BW_{human} / BW_{animal})^{1/4}$

Based on evaluation of Klaunig et al. (2015) Figure 2, the body weights of the female rats at study termination were approximately 450 g, which is consistent with reported adult female body weights for this rodent strain (Pettersen et al., 1996). Therefore, the BMD should be adjusted by $(80kg/0.45\,kg)^{1/4} = 3.65$. The resulting POD_{HED} is 90.4 mg/kg-day divided by 3.65 = 24.8 mg/kg-day.

4.4. Application of uncertainty factors

Finally, uncertainty factors were applied to the POD_{HED} to calculate the final RfD. Uncertainty factors are applied to account for limitations in the underlying data and are intended to ensure that exposures below the calculated value will be unlikely to result in adverse health effects in exposed human populations. The five individual uncertainty factors used by USEPA and international organizations (UF_D, UF_H, UF_A, UF_S, and UF_L, defined below representing the customary default values of 10 or 3) were combined to determine the final total composite uncertainty factor (UF_T) for PFHxA.

UF_H: intrahuman variability—A default value of 10 was selected for this factor to account for the variation in sensitivity among different human subpopulations. The default value is selected in the absence of any chemical-specific information that would inform a chemical-specific adjustment factor.

UF_A: interspecies extrapolation factor—A default value of 3 was selected for this factor to account for the toxicodynamic differences in equivalent dose between animals and humans. Toxicokinetic differences were accounted for in the conversion of the POD to a HED.

UF_S: subchronic-to-chronic duration factor—A value of 1 was selected for this factor, because the exposure duration from the key study (Klaunig et al., 2015) was chronic, defined by USEPA as at least 10% of humans' lifespans, which is more than approximately 90 days to 2 years in laboratory animal species (USEPA, 2002).

UF_L: LOAEL-to-NOAEL factor—A value of 1 was selected for this factor, as the final POD was based on a BMDL, not a LOAEL.

UF_D: database uncertainty factor—A value of 3 was selected for this factor. The underlying PFHxA database includes systemic toxicity, reproduction and developmental, and cancer evaluations. A complete database for consideration includes subchronic or chronic systemic toxicity in two species, a two-generation reproductive toxicity study, and developmental toxicity studies in two species (USEPA, 2002). For PFHxA, systemic toxicity data are available for two species (rat and mice) in both subchronic and chronic studies, two one-generation reproductive/developmental studies are available in one species, and a chronic carcinogenicity study is available in one species. A large number of *in vitro* and high-throughput assays strongly suggest that PFHxA is not carcinogenic, does not possess endocrine disruptor ability, and does not activate common molecular targets within a cell. The value of 3 was selected for the UF_D to account for the lack of an additional chronic toxicity study in a second species, the absence of developmental toxicity data in a second species, lack of a two-generation reproductive toxicity study, and for the fact that there are additional data gaps that may affect the determination of the critical effect and the POD, including specifically immune system and thyroid hormone data.

Based on the selection of these individual uncertainty factors for chronic exposure, the UF_T is rounded to 100 (USEPA, 2012), and then applied to the PFHxA POD_{HED}.

4.5. Derivation of the PFHxA chronic human health toxicity value

Using the available toxicity data and equation described in the "Methods" section above, we calculated a chronic RfD for PFHxA of 0.25 mg/kg-day (rounded to two significant figures). The chronic RfD was calculated from a POD of 90.4 mg/kg-day for papillary necrosis in female rats (Klaunig et al., 2015), derived using BMD methodology and converted to a POD_{HED} using allometric scaling of body weight shown to be appropriate for PFHxA (Russell et al., 2013) to result in a POD_{HED} of 24.8 mg/kg-day. A UF_T of 100 was applied. The final chronic tier 3 human health toxicity value for PFHxA is 0.25 mg/kg-day.

4.6. Confidence statement

Standard descriptors are often used by USEPA to characterize the level of confidence in a reference value, based on the likelihood that the value would change with additional data. According to USEPA, confidence in reference values is based on the quality of the studies used and completeness of the database, with more weight given to the latter (USEPA, 1994). For the PFHxA chronic toxicity value calculated here, the overall confidence is low, while confidence in the principal study (Klaunig et al., 2015) is high. The available rodent bioassays, including the key study (Klaunig et al., 2015), were high quality studies that included the appropriate quality control measures, evaluated the standard endpoints, and reported methodology and data in a transparent and reproducible manner. Furthermore, none of the female rats demonstrated signs of actual kidney impairment—use of the kidney histopathology results as the critical effect is a health protective determination. However, as noted above, the database for PFHxA is missing several studies, and it is uncertain if additional studies with lower administered doses would result in the identification of adverse effects for data-scarce endpoints such as immunotoxicity. PFHxA clearly has a different toxicity profile compared to PFOA; however, critical effects associated with PFOA such as thyroid and immune effects, have not been well studied in PFHxA. The application of the database uncertainty factor provides an additional health-protective adjustment to take into account the limited database addressing such endpoints. Furthermore, the reference value has been assigned a descriptor of "low confidence." This does not preclude the utility of the reference value for public health decision making, but does indicate that additional studies may be warranted.

5. Discussion

As a result of the shift to short-chain fluorotelomer-based products, concerns have been raised regarding their safety and availability of sufficient data for the products and their degradation products, such as PFHxA (Blum et al., 2015; Borg et al., 2017; Scheringer et al., 2014; Wang et al., 2015, 2017). A full suite of standard toxicity studies,

including acute, subchronic (28- and 90-day), and chronic (2-year), have been conducted for PFHxA, and numerous *in vitro* and high-throughput data sets are available. These data are comprehensively organized and reviewed for the first time herein. All of the observed effects related to PFHxA were mild and/or reversible and noted at levels significantly higher than PFOA. PFHxA has rapid human elimination kinetics compared to PFOA ($t_{½}$ ~32 days vs. 3.5 years), and is not considered bioaccumulative. The primary biological target related to high-dose PFHxA exposure in the rodent is the kidney; absent are effects on reproduction or development, cancer, liver, etc., which are commonly associated with PFOA exposure. Using BMD modeling and a total UF of 100 to account for human variability, inter-species extrapolation, and an incomplete database, an RfD of 0.25 mg/kg-day was calculated for PFHxA, which is four orders of magnitude greater than the USEPA's RfD for PFOA (0.00002 mg/kg-day; USEPA, 2016a).[1] Collectively, our review of the PFHxA toxicity literature indicates that PFHxA not only has a more favorable toxicological profile than PFOA, but the toxicological profile is different between the two chemicals. PFHxA is a more appropriate comparison to other short-chain perfluorocarboxylates than PFOA.

Additional PFHxA epidemiological data would be beneficial. Owing to its low frequency of detection, PFHxA is the subject of very few epidemiological evaluations. Additional evaluation of communities exposed to mixtures (e.g., legacy aqueous film-forming foam sites), including PFHxA will be important to inform potential PFHxA-specific effects and/or how PFHxA may influence human health risk when present in a complex mixture. As noted above, the laboratory animal bioassay data set is missing the standard bioassays in a second species for chronic and developmental studies; although PFHxA appears to be relatively inactive in all target organs except for the kidney, these additional data would be helpful to reduce uncertainty.

In conclusion, the work conducted herein provides the first comprehensive review of PFHxA toxicity literature available to date. A chronic human health toxicity value of 0.25 mg/kg-day is calculated, which qualifies as a “tier 3” toxicity value per USEPA policy and guidance (USEPA, 1989, 1993, 2003, 2013) and can be used to develop human health-based screening levels for risk assessment. In the companion Part 2 manuscript (Anderson et al., 2019), the RfD calculated here in Part 1 is applied using equations relevant to U.S. federal and state drinking water and groundwater standards. In addition, ranges of exposure levels and biomonitoring results are presented to illustrate that exposure to PFHxA occurs at low levels.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2019.01.019.

[1] The RfD for PFHxA is 0.25 mg/kg-day and is 4 orders of magnitude larger than the USEPA RfD (0.00002 mg/kg-day) for PFOA (USEPA, 2016a). Similarly, comparing the POD_{HED} for PFOA (0.0053 mg/kg-day) and PFHxA (24.8 mg/kg-day) also shows an approximately 4 orders of magnitude difference.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.yrtph.2019.01.019.

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