EPA Document No.
822D-21-003

DRAFT

DO NOT CITE OR QUOTE

EXTERNAL PEER REVIEW DRAFT
Draft Framework for Estimating Noncancer Health Risks Associated with Mixtures of Per- and Polyfluoroalkyl Substances (PFAS)

Draft Framework for Estimating Noncancer Health Risks Associated with Mixtures of Per- and Polyfluoroalkyl Substances (PFAS)

Prepared by:

U.S. Environmental Protection Agency
Office of Water & Office of Research and Development
Washington, DC

EPA Document Number: EPA 822D-21-003

NOVEMBER 2021

Notices

This document has been reviewed in accordance with U.S. Environmental Protection Agency (EPA) policy and approved for publication for the purpose of external peer review by the EPA Science Advisory Board (SAB).

This document provides a draft framework for estimating the likelihood of noncancer human health risks associated with mixtures of per- and polyfluoroalkyl substances (PFAS), based on longstanding EPA mixtures guidelines and guidance. This document is not a regulation and does not impose legally binding requirements on EPA, states, tribes, or the regulated community, and might not apply to a particular situation based on the circumstances. Based upon peer-review and/or evolving availability of information, EPA may change certain aspects of this document in the future.

Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Authors, Contributors, and Reviewers

Prepared by

Justin Conley, PhD
Colleen Flaherty, MS (co-lead)
Earl Gray, PhD
Brittany Jacobs, PhD
Jason C. Lambert, PhD, DABT (co-lead)
Alex Lan, MPH
Casey Lindberg, PhD
Kathleen Raffaele, PhD

Contributors

Kelly Cunningham, MS
Hannah Holsinger, MPH
Amanda Jarvis, MS
James R. Justice, MS

Internal Technical Reviewers

Andrew Kraft, PhD
Allison Phillips, PhD
Glenn Rice, ScD
Jane Ellen Simmons, PhD

Executive Direction

Elizabeth Behl
Eric Burneson, P.E.
Santhini Ramasamy, PhD
Jamie Strong, PhD
Russell Thomas, PhD
Tim Watkins, PhD

Formatting By

Tetra Tech, Inc.

Contents

Figures

Tables

List of Abbreviations and Acronyms

6:2 FTS	6:2 fluorotelomer sulfonic acid
AFFF	aqueous film forming foam
AhR	aryl hydrocarbon receptor
AOF	adsorbable organofluorine
AOP	adverse outcome pathway
AR	androgen receptor
ATSDR	Agency for Toxic Substances and Disease Registry
AUC	area under the concentration versus time curve
BMD	benchmark dose
CAR	constitutive androstane receptor
C-F	carbon-fluoride bond
CAA	Clean Air Act
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
CPSC CHAP	Consumer Product Safety Commission Chronic Hazard Advisory Panel
DA	dose addition
DLC	dioxin-like chemical
DWI	drinking water intake
E	duration-relevant exposure
EC	effect concentration
ED_X	effective dose in x percent of test animals
EOF	extractable or adsorbable organofluorine
EPA	U.S. Environmental Protection Agency
EU	European Union
FQPA	Food Quality Protection Act
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
GenX chemicals	hexafluoropropylene oxide (HFPO) dimer acid and HFPO dimer acid ammonium salt
GD	gestation day
HBWC	Health-Based Water Concentration
HED	human equivalent dose
HFPO	hexafluoropropylene oxide
HI	hazard index
HQ	hazard quotient
HTTr	high-throughput transcriptomics
IA	integrated addition
IC	index chemical
ICEC	Index Chemical Equivalent Concentration
$ICEC_{MIX}$	total mixture ICEC
ICED	index chemical equivalent dose
IRIS	Integrated Risk Information System
IVIVE	in vitro to in vivo extrapolation
KE	key event
LOAEL	lowest observed adverse effect level
MAC	maximum acceptable concentration
MCL	Maximum Contaminant Level
MCLG	Maximum Contaminant Level Goal
mg/kg	milligrams per kilogram

MIE	molecular initiating event
MOA	mode of action
MRL	minimum risk level
NAM	New Approach Methodology(ies)
NAS	National Academy of Sciences
NBP2	Nafion byproduct 2
ng/g	nanograms per gram
ng/L	nanograms per liter
NHANES	National Health and Nutrition Examination Survey
NOAEL	no observed adverse effect level
NPDWR	National Primary Drinking Water Regulation
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OP	organophosphate
ORD	Office of Research and Development
osRfV	organ-specific reference value
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-p-dioxins
PCDF	polychlorinated dibenzofuran
PFAA	perfluoroalkyl acids
PFAS	per- and polyfluoroalkyl substances
PFBA	perfluorobutanoic acid
PFBS	perfluorobutanesulfonic acid
PFCA	perfluoroalkyl carboxylic acid
PFDA	perfluorodecanoic acid
PFDoDA	perfluorododecanoic acid
PFDS	perfluorodecanesulfonate
PFECHS	perfluoroethylcyclohexane sulfonate
PFHpA	perfluoroheptanoic acid
PFHpS	perfluoroheptanesulfonic acid
PFHxA	perfluorohexanoic acid
PFHxS	perfluorohexanesulfonic acid
PFNA	perfluorononanoic acid
PFNS	perfluorononanesulfonic acid
PFOA	perfluorooctanoic acid
PFOS	perfluorooctanesulfonic acid
PFOSA	perfluorooctane sulfonamide
PFPA	perfluoropropanoic acid
PFPeA	perfluoropentanoic acid
PFPeS	perfluoropentanesulfonic acid
PFPIA	perfluoroalkyl phosphinic acid
PFPS	perfluoropropane sulfonic acid
PFSA	perfluoroalkane sulfonic acid
PFSIA	perfluoroalkane sulfinic acid
PFTA	perfluorotetradecanoic acid
PFTrDA	perfluorotridecanoic acid
PFUnA	perfluoroundecanoic acid
PIL	post-implantation loss
PND	post-natal day
POD	point of departure
PPARα	peroxisome proliferator activated receptor alpha
PPARγ	peroxisome proliferator activated receptor gamma
ppt	parts per trillion
PWS	public water system

RA	response addition
RfD	reference dose
RfV	reference value
RPF	relative potency factor
RSC	relative source contribution
SAB	Science Advisory Board
SARA	Superfund Amendments and Reauthorization Act
SDWA	Safe Drinking Water Act
SEM	standard error of the mean
T4	serum thyroxine
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TD	toxicodynamic
TEC	toxic equivalent concentrations
TEF	toxic equivalence factor
TEQ	toxic equivalents
TK	toxicokinetic
TOSHI	target organ specific hazard index
TSCA	Toxic Substances Control Act
TTD	target-organ toxicity doses
UCMR	Unregulated Contaminant Monitoring Rule
UF_L	LOAEL-to-NOAEL uncertainty factor
UF_S	extrapolation from subchronic to a chronic exposure duration uncertainty factor

EXECUTIVE SUMMARY

The U.S. Environmental Protection Agency (EPA) is issuing the *Draft Framework for Estimating Noncancer Health Risks Associated with Mixtures of Per- and Polyfluoroalkyl Substances (PFAS)*. This document is designed to communicate and illustrate the practical application of existent EPA chemical mixtures approaches and methods to two or more PFAS co-occurring in environmental media, such as drinking water. Specifically, this document describes an approach for providing a flexible, data-driven framework that facilitates practical component-based mixtures evaluation of two or more PFAS based on dose additivity. Descriptions of the Hazard Index (HI) approach (Tier 1) and Relative Potency Factor (RPF) and Mixture Benchmark Dose (BMD) (Tier 2) approaches are presented to demonstrate application to PFAS mixtures but they are not intended to provide a comprehensive treatise on the methods themselves; EPA mixtures guidelines and guidance (EPA, 1986, 2000) already exist for such a purpose. The EPA mixture assessment concepts and associated illustrative examples presented in this framework may inform PFAS evaluation(s) by federal, state, and tribal partners, as well as public health experts, drinking water utility personnel, and other stakeholders interested in assessing the potential noncancer human health hazards and risks associated with PFAS mixtures.

PFAS are a large and diverse structural family of compounds used in a myriad of commercial applications due to their unique physicochemical properties. Although PFAS have been manufactured and used broadly in commerce since the 1940s, particular concern over potential adverse effects on human health grew in the early 2000s with the discovery of perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) in human blood. Since that time, hundreds of PFAS have been identified in water, soil, and air. Many PFAS are environmentally persistent, bioaccumulative, and have long half-lives in humans, particularly the longer-chain species such as PFOA and PFOS. PFAS with fewer carbon atoms, such as perfluorobutanesulfonic acid (PFBS) and hexafluoropropylene oxide (HFPO) dimer acid and HFPO dimer acid ammonium salt (GenX chemicals), were subsequently developed and integrated into various consumer products and industrial applications because they have the desired properties and characteristics associated with this class of compounds but are more quickly eliminated from the human body than PFOA and PFOS; however, shorter-chained compounds are not necessarily less toxic to humans. The landscape of PFAS encountered in environmental media is often a diverse milieu of linear and branched parent species, metabolites, and/or abiotic degradants, leading to significant potential for PFAS mixture exposures in aquatic, terrestrial, and human populations.

As of November 2021, completed EPA human health assessments are available for four PFAS; these include PFOA (EPA, 2016a), PFOS (EPA, 2016b), PFBS (EPA, 2021a), and GenX chemicals (EPA, 2021b). EPA is in the process of updating the assessments for PFOA and PFOS, which are undergoing EPA Science Advisory Board (SAB) review along with this document (EPA, 2021c,d), and developing five additional PFAS toxicity assessments (perfluorobutanoic acid (PFBA), perfluorohexanoic acid (PFHxA), perfluorohexanesulfonic acid (PFHxS), perfluorononanoic acid (PFNA), and perfluorodecanoic acid (PFDA)), which are expected to be completed by 2024. In May 2021, the Agency for Toxic Substances and Disease Registry (ATSDR) published a "Toxicological Profile for Perfluoroalkyls" that included an

additional nine species that EPA has not yet formally assessed. However, beyond PFOA and PFOS, ATSDR derived quantitative minimal risk levels (MRLs) for only two additional PFAS: PFHxS and PFNA. A significant challenge in evaluating PFAS is the lack of hazard and dose-response data suitable for human health risk assessment for the large majority of individual PFAS. EPA and the National Institute of Environmental Health Sciences (NIHES) are actively engaged in research and testing to help address data gaps for a broad landscape of PFAS (approximately 150 species at the time of the drafting of this document). Until results from these ongoing research and testing efforts are available, the evaluation of potential toxicity/risk associated with mixtures of PFAS is limited to existing data under the purview of human health assessments by federal, state, and/or international entities. The application of the component-based methods presented in this PFAS mixtures framework document is demonstrated using the four PFAS for which EPA human health assessments are available; however, this framework allows for the integration of information derived from other health assessment data sources (e.g., other federal, state, international), available human epidemiological and experimental animal hazard and dose-response data, and information from New Approach Methodologies (NAMs). Opportunities for integrating additional PFAS into the context of a mixture assessment is expected to evolve over time and will depend on the decision context and availability of hazard and dose-response data from traditional and/or NAM-based assays and in silico platforms.

The PFAS "mixture" information selected for the illustrative examples of the HI, RPF, and Mixture BMD approaches in Section 4 of this document were based on final EPA toxicity assessments at the time of drafting this document: PFOA (EPA, 2016a), PFOS (EPA, 2016b), PFBS (EPA, 2021a), and GenX chemicals (EPA, 2021b), and is purely intended for demonstration of methodological application and is not intended to be used directly in risk assessment or remediation, or in a regulatory context. Further, the extent of the framework's utility for a particular programmatic application will need to be assessed within each specific decision context under different authorities and regulations.

1.0 Introduction and Background

1.1 Purpose

The U.S. Environmental Protection Agency (EPA) has initiated the process to develop a Maximum Contaminant Level Goal (MCLG) and National Primary Drinking Water Regulation (NPDWR) for per- and polyfluoroalkyl substances (PFAS) under the Safe Drinking Water Act (SDWA) (86 FR 12272, March 3, 2021). The agency is seeking comment from the EPA SAB on key scientific issues related to development of the NPDWR. As part of this proposed rulemaking, EPA has prepared this document, *Draft Framework for Estimating Noncancer Health Risks Associated with Mixtures of Per- and Polyfluoroalkyl Substances (PFAS)*, that illustrates the practical application of EPA chemical mixtures approaches and methods for two or more PFAS co-occurring in environmental media, such as drinking water. This draft framework is being submitted by EPA for scientific review by the SAB along with three other documents:

- EPA's *Proposed Approaches to the Derivation of a Draft Maximum Contaminant Level Goal for Perfluorooctanoic Acid (PFOA) (CASRN 335-67-1) in Drinking Water* (EPA, 2021c)
- EPA's *Proposed Approaches to the Derivation of a Draft Maximum Contaminant Level Goal for Perfluorooctane Sulfonic Acid (PFOS) (CASRN 1763-23-1) in Drinking Water* (EPA, 2021d)
- EPA's *Analysis of Cardiovascular Disease Risk Reduction as a Result of Reduced PFOA and PFOS Exposure in Drinking Water* (EPA, 2021e)

Each of the four documents of which EPA is seeking review will inform development of the MCLGs and NPDWR for PFOA and PFOS. EPA is moving expeditiously to develop the proposed MCLGs and NPDWR, therefore this draft document was developed concurrently with the three other draft documents for SAB review. While qualitative statements on health effects are consistent with the conclusions from the first two documents listed above, this framework does not fully incorporate the updated information described in the proposed approaches for deriving MCLGs for PFOA and PFOS (EPA, 2021c,d). Specifically, all example calculations presented in this document for PFOA and PFOS are based on information from previously issued, final EPA human health assessments and drinking water Health Advisories (EPA, 2016a,b,c,d). EPA will consider the collective input from the SAB on this framework document and the other three draft documents to prepare final documents that will inform the promulgation of MCLGs and NPDWRs.

This document provides a draft approach for a tiered, data-driven framework for estimating the likelihood of noncancer human health risks associated with oral exposures to mixtures of PFAS, based on longstanding EPA guidelines and guidance (EPA, 1986, 2000). Although the framework and illustrative examples provided in this document include examples for PFAS in water, the framework itself is not limited to specific media and may be useful for understanding the potential non-cancer health effects of PFAS mixtures under various authorities or decision contexts. The approach presented here is not intended to be used to assign groups or subclasses or otherwise classify PFAS. Rather, the framework is designed for practical application of EPA chemical mixtures approaches and methods for a particular exposure to gain insight on the potential health risk(s) associated with exposure to mixtures of PFAS. The EPA mixture assessment concepts and associated illustrative examples presented in this framework may

inform PFAS evaluation(s) by federal, state, and tribal partners, as well as public health experts, drinking water utility personnel, and other stakeholders interested in assessing the potential human health hazards and risks associated with PFAS mixtures.

The framework and example calculations presented here incorporate information for four PFAS for which EPA has finalized toxicological assessments: PFOA, PFOS, PFBS, and GenX chemicals. However, due to the constantly evolving science related to PFAS, the approach has the flexibility to consider information as it becomes available, including forthcoming EPA toxicological assessments, assessments from other sources (e.g., federal, state, international), available hazard and dose-response data in the public domain, and information from high throughput bioassays and other NAMs.

The draft document is not a regulation and does not impose legally binding requirements on EPA, states, tribes, or the regulated community, and might not apply to a particular situation based on the circumstances. Based upon peer-review and/or evolving availability of information, EPA may change certain aspects of this document in the future.

1.2 Background on PFAS

PFAS are a large group of anthropogenic chemicals that include PFOA, PFOS, and thousands of other chemicals. The universe of environmentally relevant PFAS, including parent chemicals, metabolites, and degradants, is greater than 9,000 compounds.[1] The Organisation for Economic Co-operation and Development (OECD) *New Comprehensive Global Database of Per- and Polyfluoroalkyl Substances (PFASs)* includes over 4,700 PFAS (OECD, 2018). Comparatively, the number of PFAS currently used in commercial products at the time of the drafting of this document is approximately 250 substances (Buck et al., 2021).

PFAS have been manufactured and used in a wide variety of industries around the world, including in the United States since the 1940s. PFAS have strong, stable carbon-fluorine (C-F) bonds, making them resistant to hydrolysis, photolysis, microbial degradation, and metabolism (Ahrens, 2011; Beach et al., 2006; Buck et al., 2011). The chemical structures of PFAS make them repel water and oil, remain chemically and thermally stable, and exhibit surfactant properties; these properties are what make PFAS useful for commercial and industrial applications and purposes, but these are also what make some PFAS extremely persistent in the human body and the environment (Calafat et al., 2007, 2019). Due to their widespread use, physicochemical properties, persistence, and bioaccumulation potential, many PFAS co-occur in exposure media (e.g., air, water, ice, sediment), and in tissues and blood of aquatic and terrestrial organisms, and humans.

There are many families or classes of PFAS, and each contains many individual structural homologues and can exist as either branched-chain or straight-chain isomers (Buck et al., 2011; EPA, 2021f). These PFAS families can be divided into two primary categories: non-polymers and polymers. The non-polymer PFAS include perfluoroalkyl and polyfluoroalkyl substances. Polymer PFAS include fluoropolymers, perfluoropolyethers, and side-chain fluorinated polymers (Table 1-1). For the proposed reporting and recordkeeping requirements for PFAS under the Toxic Substances Control Act (TSCA), PFAS are defined as "per- and polyfluorinated

[1] See EPA PFAS Master List of PFAS Substances (Version 2) available at: https://comptox.epa.gov/dashboard/chemical_lists/PFASMASTER

substances that structurally contain the unit R-(CF2)-C(F)(R′)R″. Both the CF_2 and CF moieties are saturated carbons and none of the R groups (R, R′ or R″) can be hydrogen" (86 FR 33926, June 28, 2021). It should be noted however that what defines or constitutes a PFAS may change over time under different purviews (e.g., federal, state, international).

Table 1-1. Two Primary Categories of PFAS[a]

PFAS Non-polymers	Structural Elements	Example PFAS Families
Perfluoroalkyl substances	Compounds in which all carbon-hydrogen bonds, except those on the functional group, are replaced with carbon-fluorine bonds	Perfluoroalkyl acids (e.g., PFOA, PFOS), perfluoroalkane sulfonamides, perfluoroalkane sulfonyl fluorides, perfluoroethers
Polyfluoroalkyl substances	Compounds in which all carbon-hydrogen bonds on at least one carbon (but not all) are replaced with carbon-fluorine bonds	Polyfluoroalkane sulfonamido derivatives, semifluorinated *n*-alkanes and alkenes, fluorotelomers, polyfluoroalkyl ether carboxylic acids
PFAS Polymers	**Structural Elements**	**Example PFAS Families**
Fluoropolymers	Carbon-only polymer backbone with fluorines directly attached	Polytetrafluoroethylene, polyvinylidene fluoride
Polymeric perfluoropolyethers	Carbon and oxygen polymer backbone with fluorines directly attached to carbon	F-($C_mF_{2m}O$-)$_nCF_3$, where the $C_mF_{2m}O$ represents -CF_2O, -CF_2CF_2O, and/or -$CF(CF_3)CF_2O$ distributed randomly along polymer backbone
Side-chain fluorinated polymers	Non-fluorinated polymer backbone with fluorinated side chains with variable composition	Fluorinated acrylate and methacrylate polymers, fluorinated urethane polymers, and fluorinated oxetane polymers

[a] Modified from Buck et al. (2011).

PFOA and PFOS belong to the perfluoroalkyl acids (PFAA) of the non-polymer perfluoroalkyl substances category of PFAS and are among the most researched PFAS in terms of human health toxicity and biomonitoring studies (for review see Podder et al., 2021). The PFAA family includes perfluoroalkyl carboxylic, phosphonic, and phosphinic acids and perfluoroalkane sulfonic and sulfinic acids (Table 1-2). PFAA are highly persistent and are frequently found in the environment (Ahrens, 2011; Brendel et al., 2018; Wang et al., 2017). Although EPA defines, specifically for purposes under the purview of TSCA, long-chain perfluoroalkyl carboxylate substances as having perfluorinated carbon chain lengths equal to or greater than seven carbons and less than or equal to 20 carbons (85 FR 45109, July 27, 2020), a more comprehensive delineation of what constitutes short-chain versus long-chain PFAAs is provided by the OECD (OECD, 2021). Specifically, the OECD established long-chain perfluoroalkyl carboxylic acids (PFCAs) as those species with eight or more carbons (seven or more carbons are perfluorinated), and short-chain PFCAs are identified as those with seven or fewer carbons (six or fewer carbons are perfluorinated). Conversely, long-chain perfluoroalkane sulfonic acids (PFSAs) are identified as those species with six or more carbons (six or more carbons are perfluorinated), and short-chain PFSAs are identified as those with five or fewer carbons (five or fewer carbons are perfluorinated) (see Table 1-3).

Table 1-2. Classification and Chemical Structure of Perfluoroalkyl Acids (PFAA)[a]

Classification	Functional Group	Examples
Perfluoroalkyl carboxylic acids (PFCAs) Or Perfluoroalkyl carboxylates (PFCAs)	-COOH	Perfluorooctanoic acid (PFOA)[b]
	$-COO^-$	Perfluorooctanoate
Perfluoroalkane sulfonic acids (PFSAs) Or Perfluoroalkane sulfonates (PFSAs)	$-SO_3H$	Perfluorooctane sulfonic acid (PFOS)
	$-SO_3^-$	Perfluorooctane sulfonate (PFOS)[b]
Perfluoroalkane sulfinic acids (PFSIAs)	$-SO_2H$	Perfluorooctane sulfinic acid
Perfluoroalkyl phosphonic acids (PFPAs)	$-P(=O)(OH)_2$	Perfluorooctyl phosphonic acid (C8-PFPA)
Perfluoroalkyl phosphinic acids (PFPIAs)	$-P(=O)(OH)(C_mF_{2m+1})$	Bis(perfluorooctyl) phosphinic acid (C8/C8-PFPIA)

[a] Modified from Buck et al. (2011).
[b] The anionic form is most prevalent in the aquatic environment.

Table 1-3. Classification of Short-Chain and Long-Chain PFAA[a]

Total # of carbons	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
# of fluorinated carbons	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
PFCAs	**Short-chain PFCAs**					**Long-chain PFCAs**		
	PFPA	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA
# of fluorinated carbons	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
PFSAs	PFPS	PFBS	PFPeS	PFHxS	PFHpS	PFOS	PFNS	PFDS
	Short-chain PFSAs			**Long-chain PFSAs**				

[a] Modification of Table 2-2 (ITRC, 2021)

Notes:

For brevity, Table 1-3 only includes PFAAs of 3–10 carbons; the long-chain class of PFCAs and PFSAs can be expanded considerably.

PFPA = perfluoropropanoic acid; PFBA = perfluorobutanoic acid; PFPeA = perfluoropentanoic acid; PFHxA = perfluorohexanoic acid; PFHpA = perfluoroheptanoic acid; PFOA = perfluorooctanoic acid; PFNA = perfluorononanoic acid; PFDA = perfluorodecanoic acid; PFPS = perfluoropropane sulfonic acid; PFBS = perfluorobutanesulfonic acid; PFPeS = perfluoropentanesulfonic acid; PFHxS = perfluorohexanesulfonic acid; PFHpS = perfluoroheptanesulfonic acid; PFOS = perfluorooctanesulfonic acid; PFNS = perfluorononanesulfonic acid; PFDS = perfluorodecanesulfonate.

Although many PFAS are manufactured in various salt forms (e.g., potassium (K+) PFBS), they typically fully dissociate to their protonated acid and/or anionic forms depending on their acid strength (pK_a value) in aqueous environmental media, soils, or sediments. Importantly, the protonated and anionic forms may have different physiochemical and environmental fate and transport properties.

1.3 Occurrence of PFAS Mixtures

As a result of improved monitoring and detection methods, co-occurrence of multiple PFAS has been reported in drinking water, ambient surface waters, aquatic organisms, biosolids (sewage sludge), and other environmental media.[2] PFOA and PFOS have historically been target analytes, which has partly contributed to their prevalence in environmental monitoring studies. Relatively recent monitoring studies, however, have begun to focus on additional PFAS via advanced analytical instruments/methods and non-targeted analysis (De Silva et al., 2020; McCord et al., 2019, 2020). The proposed framework for estimating the likelihood of human health risks associated with oral exposures to mixtures of PFAS (described in Section 4) is flexible to accommodate information for any PFAS mixture of interest, provided sufficient hazard and dose-response information is available.

EPA uses the Unregulated Contaminant Monitoring Rule (UCMR) to collect data for contaminants that are suspected to be present in drinking water and do not have health-based standards set under the Safe Drinking Water Act (SDWA). Between 2013 and 2015, EPA's third UCMR (i.e., UCMR 3) required all large public water systems (PWSs) (serving more than 10,000 people) and a statistically representative national sample of 800 small PWSs (serving 10,000 people or fewer) to monitor for 30 unregulated contaminants in drinking water, including six PFAS: PFOS, PFOA, PFNA, PFHxS, perfluoroheptanoic acid (PFHpA), and PFBS. UCMR 3 data demonstrated that two or more of those six PFAS co-occurred in 48% (285/598) of sampling events with PFAS detected, and PFOA and PFOS co-occurred in 27% (164/598) of sampling events with two or more PFAS detected (EPA, 2019b; Guelfo and Anderson, 2018). EPA found that 4% of PWSs reported results for which one or more of the six UCMR 3 PFAS were measured at or above their respective minimum reporting levels.[3] Outside of the UCMR 3 data collection, many states have undertaken individual efforts to monitor for PFAS in both source and finished drinking water. These results show occurrence in multiple geographic locations consistent with what was observed during UCMR 3 monitoring (EPA, 2021g).

PFAS mixtures have also been reported in U.S. ambient surface waters and in aquatic biota (Ahrens, 2011; Benskin et al., 2012; Burkhard, 2021; Nakayama et al., 2007; Remucal, 2019; Zareitalabad et al., 2013). Most environmental monitoring of PFAS in surface waters has focused on sites of historical manufacturing and known contamination (3M Company, 2000; Boulanger et al., 2004; Cochran, 2015; Hansen et al., 2002; Jarvis et al., 2021; Konwick et al., 2008; Nakayama et al., 2007). Simcik and Dorweiler (2005) consistently detected both PFOA and PFHpA in all 12 surface waters sampled across the U.S. Midwest, and PFOS in all but two locations. Sinclair and Kannan (2006) detected PFOA and PFOS in all effluent-dominated samples collected across New York State. In addition to PFOA and PFOS, Sinclair and Kannan (2006) also detected PFHxS; however, PFBS and perfluorooctane sulfonamide (PFOSA) were below detection limits in all samples. De Silva et al. (2011) detected PFOS and additional short chain PFAS (i.e., perfluoropentanoic acid (PFPeA) (C5), PFHxA (C6), PFHpA (C7), and PFOA (C8)) co-occurring as mixtures in all surface water samples (n = 32) collected across the five

[2] For a more detailed discussion of the occurrence of PFOA, PFOS, and other PFAS in potential human exposure sources see the RSC sections in EPA, 2021c and 2021d.

[3] The 4% figure is based on 198 PWSs reporting measurable PFAS results for one or more sampling events from one or more of their sampling locations. Those 198 PWSs serve an estimated total population of approximately 16 million (EPA, 2019b,c).

Laurentian Great Lakes. Relatively longer chain PFAS, including PFNA (C9), PFDA (C10), perfluoroundecanoic acid (PFUnA) (C11), PFBS, PFHxS, perfluoroethylcyclohexane sulfonate (PFECHS), and perfluoromethylcyclohexane sulfonate, were also quantified in at least 20 of the 32 samples collected from the Great Lakes.

PFAS mixtures in the environment can be linked to direct application of manufactured products that contain a specific mixture of PFAS. For example, aqueous film forming foam (AFFF) used in firefighting and training activities can contain hundreds of polyfluoroalkyl precursors (Ruyle et al., 2021). Anderson et al. (2016) quantified PFAS in ambient surface waters across 10 U.S. Air Force bases where there were known historic uses of AFFF. PFOA and PFOS largely co-occurred with one another and were detected in 88% and 96% of samples, respectively. Anderson et al. (2016) also detected PFBA, PFBS, PFPeA, PFHxA, PFHxS, and PFHpA in ≥ 80% of samples.

Environmental monitoring of PFAS in aquatic biota has primarily focused on fish. Generally, PFCAs are less bioaccumulative than PFSAs in aquatic systems, with longer chain PFAS being more bioaccumulative than short chain PFAS (Burkhard, 2021; Conder et al., 2008; Kannan et al., 2005). Within the United States, PFAS in aquatic biota have been measured in several estuaries and in the Laurentian Great Lakes region. Sedlak et al. (2017) measured PFAS in composite samples containing yellowfin gobies (*Acanthogobius flavimanus*), chameleon/cheekspot gobies (*Tridentiger trigonocephalus/Ilypnus gilberti*), northern anchovy (*Engraulis mordax*), shiner surfperch (*Cymatogaster aggregata*), and staghorn sculpin (*Leptocottus armatus*) that were collected from the San Francisco Bay estuary. PFOS and PFOSA were detected in nearly all composite samples and at relatively high concentrations (geometric mean PFOS = 3.9 nanograms (ng) per gram (g); geometric mean PFOSA = 3.2 ng/g). Other longer chain PFAS, including PFNA, PFDA, PFUnA, and perfluorododecanoic acid (PFDoDA), were also frequently detected in the fish composite samples, but at relatively low concentrations (geometric mean concentrations < 2.4 ng/g). Shorter chain PFAS, including PFBS, PFBA, PFHxA, and PFHpA, were not detected in any of the fish composite samples. Houde et al. (2006) measured whole body PFAS in six fish species in Charleston Harbor, South Carolina, and in five fish species in Sarasota Bay, Florida. Out of the six species from Charleston Harbor, PFOA, PFOS, PFNA, PFDA, PFUnA, PFDoDA, PFHxS, and PFOSA were all commonly detected in fish tissues. Charleston Harbor was the more developed of the two sites and had higher overall PFAS concentrations. PFOS and PFDoDA were the only two PFAS that were detected at elevated concentrations in the fish species residing in Sarasota Bay (Houde et al., 2006). De Silva et al. (2011) measured PFAS from lake trout (*Salvelinus namaycush*) samples collected in 2001 from each of the Great Lakes. Eight different PFAS (i.e., PFNA, PFDA, PFUnA, PFDoDA, perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTA), PFHxS, and PFOS) were detected in lake trout tissues across all of the Great Lakes, with PFOA, PFECHS, and perfluorodecanesulfonic acid (PFDS) also being detected in Lake Ontario (De Silva et al., 2011).

Within the United States, PFAS occurrence in invertebrate tissues, such as shellfish, has not been as extensively monitored as PFAS occurrence in fish. Kannan et al. (2005) measured PFAS in several species, including zebra mussels, from two rivers in southern Michigan (Raisin River, St. Claire River), and one in northern Indiana (Calumet River). Overall, PFAS concentrations in zebra mussels were lower than in fish. Nevertheless, PFOS and PFOSA were both detected in

zebra mussels in the Raisin River (PFOS concentration = 3.1 ng/g wet weight; PFOSA concentration = 2.7 ng/g wet weight). Interestingly, PFOA was not detected in zebra mussel tissues even though it was detected in elevated concentrations in the Raisin River water column (PFOA water concentration = 17.7 ng/liter (L)), suggesting that chemical-specific considerations (e.g., carbon chain length, functional group differences) affect bioaccumulation dynamics in aquatic organisms and resultant human exposures to PFAS mixtures via ingestion of fish and shellfish (Kannan et al., 2005).

1.4 Evidence of PFAS Exposure in Humans

Humans can be exposed to PFAS through a variety of sources, including food that is packaged in PFAS-containing materials, processed with equipment that use PFAS, or grown or raised in PFAS-contaminated soil or water (including livestock and seafood); commercial household products, including stain- and water-repellent fabrics, nonstick products, polishes, waxes, paints, and cleaning products; the fire suppressant, AFFF; production facilities or industries that use PFAS; and drinking water, where these chemicals have contaminated water supplies. Although humans may be exposed to PFAS via dermal and inhalation routes, the primary focus of this document is the oral route of exposure, including drinking water, food, fish/shellfish, and incidental soil/dust ingestion (Egeghy and Lorber, 2010; Lorber and Egeghy, 2011; Poothong et al., 2020).

The Centers for Disease Control and Prevention's National Health and Nutrition Examination Survey (NHANES) has measured blood serum concentrations of several PFAS in the general U.S. population since 1999. Results, from a nationally representative, cumulative biomonitoring study in which data were gathered from 1999–2000 through 2015–2016, documented measurable serum levels of PFOS, PFOA, PFHxS and PFNA in greater than 95% of participants, indicating widespread exposure to these PFAS in the U.S. population. PFOA and PFOS have been detected in up to 98% of serum samples collected in biomonitoring studies that are representative of the U.S. general population; however, blood levels of PFOA and PFOS dropped 60% to 80% between 1999 and 2014, presumably due to restrictions on their commercial use in the United States. Under EPA's PFOA Stewardship Program, the eight major companies of the perfluoropolymer/fluorotelomer industry agreed to voluntarily reduce facility emissions and product content of PFOA, precursor chemicals that can break down to PFOA, and related higher homologue chemicals, by 95% on a global basis by no later than 2010 and eliminate these substances in products by 2015 (EPA, 2021f). However, since the voluntary phase out of these longer-chain PFAS compounds in the United States, manufacturers are shifting to shorter-chain and alternative forms of PFAS compounds such as GenX chemicals. Additionally, other PFAS compounds were found in blood samples from recent (2011–2016) NHANES surveys, for example, PFDA, PFDoDA, PFHpA, PFHxS, PFNA, and 2-(N-Methyl-perfluorooctane sulfonamido) acetic acid. Studies of residents in locations of suspected PFAS contamination show higher serum levels of PFAS compared to the general U.S. population reported by NHANES (Yu et al., 2020). There is less publicly available information on the occurrence and health effects of these replacements for PFOA and PFOS and other members of the carboxylic acid and sulfonate PFAS families.

1.5 Brief Summary of State, National, and International Approaches to Address PFAS Mixtures in Water

In 2016, EPA finalized drinking water Health Advisories of 70 parts per trillion (ppt) for PFOA and PFOS, for the individual chemicals and when present as a mixture (EPA, 2016c,d) because the reference doses (RfDs) were based on similar developmental effects and numerically identical. Since then, some states have developed state-specific cleanup levels, drinking water or groundwater guidelines, advisories or standards for PFOS and PFOA. In some cases, the state values are the same as EPA's 2016 drinking water Health Advisory (70 ppt for the individual and/or combined concentration of PFOA and PFOS); in other cases, states have developed different values. As of July 2021, Alaska, Colorado, Connecticut, Delaware, Florida, Montana, New Hampshire, North Carolina, Ohio, and Rhode Island have followed an approach similar to EPA and have adopted or otherwise applied a value of 70 ppt (e.g., as a guideline, advisory, or enforceable standard for water resources) to account for the combined toxicity of PFOA and PFOS (Table 1-4).

Several states have included additional PFAS (beyond PFOA and PFOS) in their combined toxicity approach based on similarity in chemical structure and/or toxicity (Table 1-4). For instance, Connecticut has established a limit of 70 ppt for any combination of the following PFAS: PFOA, PFOS, PFNA, PFHxS, and PFHpA (CT DPH, 2021). In some cases, the combined concentration is set at the higher concentration of either PFOA or PFOS alone. For example, Illinois established a Health Advisory of 21 ppt for PFOA and 14 ppt for PFOS for non-carcinogenic effects, and the combined value is set at 21 ppt (Illinois EPA, 2019). Wisconsin has established a maximum concentration of 20 ppt for combined PFOA and PFOS (WI DHS, 2019), while Massachusetts and Maine derived a maximum concentration of 20 ppt for any combination of the following six PFAS: PFOA, PFOS, PFNA, PFHxS, PFDA and PFHpA based on "close similarities in chemical structure and similar toxicities for this subgroup of PFAS" (Maine DEP, 2021; Mass DEP, 2019). Similarly, Vermont established a limit of 20 ppt for the same PFAS as Massachusetts and Maine with the exclusion of PFDA based on several criteria, including that "PFHxS, PFHpA and PFNA … are considered sufficiently similar to PFOA and PFOS" (VT DEC, 2021).

International approaches to addressing multiple PFAS in drinking water have resulted in a range of proposed and promulgated standards, guidance values, and a variety of grouping methods (Table 1-4). Canada has adopted a method similar to the HI to estimate cumulative toxicity by adding the ratio of the PFOA concentration to its maximum acceptable concentration (MAC) to the ratio of the PFOS concentration to its MAC. If the sum of the ratios is equal to or lower than one, the drinking water is considered safe to drink. Australia has established a combined level of 70 ppt for PFOS and PFHxS, as a precaution, based on the assumption that PFHxS is similar in toxicity to PFOS (i.e., PFOS tolerable daily intake also applies to PFHxS). Several countries have expanded the combined toxicity approach to include a variety of other PFAS chemicals. For instance, Denmark has set a limit of 100 ppt to account for any combination of the following: C4–C10 PFCAs, PFBS, PFHxS, PFOS, PFOSA, and 6:2 fluorotelomer sulfonic acid (6:2 FTS). Sweden has adopted the same approach for PFOSA, which Sweden excludes, and set a maximum limit of 90 ppt. In both Denmark and Sweden, it is assumed that these PFAS are similar in toxicity to PFOS. Most recently, the European Union (EU) adopted a level of 100 ppt for the sum of 20 PFAS including C4-C13 PFSAs and C4-C13 PFCAs and a level of 500 ppt for

all PFAS, as measured by extractable or adsorbable organofluorine (EOF/AOF) (Cousins et al., 2020; EU, 2020). Further, Sweden and the Netherlands have evaluated the potential human health risk(s) associated with mixtures of PFAS using component-based methods consistent with the HI or RPF approaches presented in EPA's draft framework (Borg et al., 2013; RIVM, 2018).

Table 1-4. Summary of U.S. and International Approaches to Addressing the Combined Toxicity of Multiple PFAS in Drinking Water or Groundwater[a,b] (only combined PFAS approaches are presented)

Entity	Date	Conc (ng/L)	Sum of PFAS	Background
EPA (EPA, 2016c,d)	2016	70	PFOA and PFOS	Drinking water Health Advisory. Assumes dose additive toxicity of PFOA and PFOS.
Alaska (USA) (Alaska DEC, 2019)	2019	70	PFOA and PFOS	Application of EPA Health Advisory.
Colorado (USA) (CDPHE, 2020)	2020	70	PFOA and PFOS	Application of EPA Health Advisory.
Connecticut (USA) (CT DPH, 2017)	2017	70	Either PFOA and PFOS or the sum of PFOA, PFOS, PFNA, PFHxS and PFHpA	Application of EPA Health Advisory to the sum of five PFAS; assumes toxicity similar to that of PFOS and PFOA.
Delaware (USA) (DE DNREC, 2018)	2018	70	PFOA and PFOS	Application of EPA Health Advisory.
Florida (USA) (Florida Health, 2020)	2019	70	PFOA and PFOS	Application of EPA Health Advisory.
Illinois (USA) (Illinois EPA, 2021)	2019	21	PFOA and PFOS	Same approach as EPA Health Advisory but used different references doses (MRLs from ATSDR (2021)) and applied additional uncertainty factor for PFOS.
Maine (USA) (Maine DEP, 2021)	2021	20	PFOA, PFOS, PFNA, PFHxS, PFHpA, and PFDA	Based on similarities in chemical structure and toxicities of six PFAS to PFOS and PFOA. Same approach as EPA Health Advisory but includes an additional uncertainty factor.
Massachusetts (USA) (Mass DEP, 2019)	2019	20	PFOA, PFOS, PFNA, PFHxS, PFHpA, and PFDA	Based on similarities in chemical structure and toxicities of six PFAS to PFOS and PFOA. Same approach as EPA Health Advisory but includes an additional uncertainty factor.
Montana (USA) (MT DEQ, 2020)	2019	70	PFOA and PFOS	Application of EPA Health Advisory
New Hampshire (USA) (NHDES, 2021)	2016	70[c]	PFOA and PFOS	Application of EPA Health Advisory

Entity	Date	Conc (ng/L)	Sum of PFAS	Background
North Carolina (USA) (NC DEQ, 2021)	2020	70	PFOA and PFOS	Application of EPA Health Advisory
Ohio (USA) (Ohio EPA, 2019)	2019	70	PFOA and PFOS	Application of EPA Health Advisory
Rhode Island (USA) (RIDEM, 2017)	2019	70	PFOA and PFOS	Application of EPA Health Advisory
Vermont (USA) (VT DEC, 2021)	2019	20	PFOA, PFOS PFNA, PFHxS and PFHpA	PFHxS, PFHpA and PFNA are considered sufficiently similar to PFOA and PFOS. Difference to EPA Health Advisory is due to Vermont's calculation being based on infant consumption rates.
Wisconsin (USA) (WI DHS, 2019a,b)	2019	20	PFOA and PFOS	Based on ATSDR's 2021 intermediate MRL, with additional modifying factor of 10 for immunotoxicity; HI approach.
European Union (EU, 2020)	2020	100 500	100 ng/L for sum of 20 PFAS (C4–C13 PFSAs and C4–C13 PFCAs) 500 ng/L for "PFAS Total" – the total of all PFAS	"PFAS Total" proposed to be enforced through measurement of EOF/AOF once validated or 100 ppt for the sum of 20 PFAS considered to be a concern for drinking water (implementation January 12, 2023).
Denmark (Danish Environmental Protection Agency, 2015)	2015	100	C4–C10 PFCAs, PFBS, PFHxS, PFOS, PFOSA, and 6:2 FTS	Assumes all 12 PFAS are similarly toxic as PFOS. Rationale: PFOS is the most toxic and toxicity data are limited on PFAS other than PFOS and PFOA.
Sweden (Swedish Food Agency, 2021)	2014	90	C4–C10 PFCAs, PFBS, PFHxS, PFOS and 6:2 FTS	Assumes all 11 PFAS are similarly toxic as PFOS. Rationale: PFOS is the most toxic and toxicity data are limited on PFAS other than PFOS and PFOA.
Australia (Australian Government Department of Health, 2019)	2017	70	PFOS and PFHxS combined, if both present	Assumes PFHxS is similarly toxic as PFOS. Rationale: PFOS is the most toxic and toxicity data are limited on PFAS other than PFOS and PFOA.
Canada (Health Canada, 2018)	2018	200 600	PFOA PFOS	When PFOS and PFOA are found together in drinking water, a cumulative toxicity (HI) approach is applied.

[a] Modified from Cousins et al. (2020).

[b] As of July 2021, several states have passed or proposed compound-specific Maximum Contaminant Levels (MCLs) or Health Advisories, e.g., California, Michigan, Minnesota, New Jersey, New York, Pennsylvania, Texas, and Washington. Some states have applied the EPA Health Advisory to interpret narrative water quality standards under the Clean Water Act, e.g., Colorado, Montana. Only approaches using the sum of PFAS parameters are presented in this table.

[c] New Hampshire established MCLs of 12 and 15 for PFOA and PFOS, respectively; however, there is a court injunction at this time preventing the MCLs from being enforced. In the meantime, New Hampshire is applying EPA's 70 ppt Health Advisory.

1.6 Overview of Proposed Framework for Estimating Health Risks for PFAS Mixtures

This draft document describes a framework for estimating the likelihood of noncancer human health risks associated with mixtures of PFAS, based on longstanding EPA chemical mixtures guidance. To address concerns over health risks from multichemical exposures, EPA issued the *Guidelines for the Health Risk Assessment of Chemical Mixtures* in 1986 (EPA, 1986). The 1986 guidelines were followed in 2000 by the *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (EPA, 2000). These documents define a chemical mixture as "any combination of two or more chemical substances, regardless of source or of spatial or temporal proximity, that can influence the risk of chemical toxicity in the target population" (EPA, 1986, 2000); this definition is used in this framework document.

Several laws direct EPA to address health risks posed by exposures to chemical mixtures, including the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) of 1980, the Superfund Amendments and Reauthorization Act (SARA) of 1986, and amendments in 2002 (CERCLA, 2002; SARA, 2002) (commonly referred to as Superfund); the Clean Air Act Amendments of 1990 (CAA, 1990); the Safe Drinking Water Act Amendments of 1996 (SDWA, 1996); and the Food Quality Protection Act (FQPA) of 1996 (FQPA, 1996). Both the 1986 Chemical Mixtures Guidelines (EPA, 1986) and the 2000 Supplementary Chemical Mixtures Guidance (EPA, 2000) were developed, in part, to be responsive to these laws. When developing risk information for exposures to chemical mixtures, risk assessors and risk managers in EPA's programs currently implement environmental laws through regulations that rely on the guidance and methods articulated in the 1986 Chemical Mixtures Guidelines and the 2000 Supplementary Chemical Mixtures Guidance. This proposed framework does not supersede previously published EPA guidance on mixtures or longstanding EPA approaches used to assess cumulative effects of chemical mixtures under various environmental statutes (e.g., Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), FQPA).

The objective of this document is to provide a tiered, flexible, data-driven framework that facilitates practical component-based mixtures evaluation of two or more PFAS under an assumption of dose additivity. The approach is broken into two tiers: Tier 1 entails a HI approach that provides an initial indicator screening metric for a PFAS mixture of concern (Section 4.3); and Tier 2 is based on the RPF approach that is designed to provide a mixture toxicity estimate (Section 4.4), and an alternative approach that uses the DA (dose addition) model-based calculation (similar to the Berenbaum equation; Section 4.2.6 in EPA, 2000) of a mixture BMD (e.g., ED_{10}) for a PFAS mixture with a specific mixing-ratio of component chemicals (Section 4.5). The HI is a component-based mixtures methodology that facilitates estimation of potential aggregate toxicity associated with co-occurrence of chemicals in environmental media (e.g., water, soil) (EPA, 2000). The RPF method is more data intensive than the HI approach in that the mixture component chemicals typically must meet two requirements: (1) there are data to demonstrate or suggest that component chemicals share either a similar toxicological mode of action (MOA) or have a conserved toxicological target (e.g., share a common apical endpoint/effect) and (2) the dose-response functions for the effect of concern are similar over the exposure ranges most relevant to the decision context (EPA, 2000). This is illustrated in Section 4.4 using common target organs/pathways including developmental, thyroid, and liver effects. These same assumptions are also inherent when applying the Mixture BMD approach (Section

4.5). Considering that PFAS are an emerging chemical class of note for toxicological evaluations and human health risk assessment, MOA data may be limited or not available at all for many PFAS, including those approved for use under TSCA. As such, this draft framework proposes to focus the biological level of organization for evaluation of potential dose additivity on similarity of toxicity endpoint/effect/adverse outcome rather than similarity in MOA, which is consistent with EPA mixtures guidance (EPA, 2000).

The PFAS used to demonstrate the application of the HI and RPF approaches described in this draft framework are limited to those with final EPA toxicity assessments at the time of drafting this document: PFOA (EPA, 2016a), PFOS (EPA, 2016b), PFBS (EPA, 2021a), and GenX chemicals (EPA, 2021b). Thus, the information provided in the Mixture BMD approach examples is purely hypothetical and provided to illustrate application of the framework. Recognizing the evolving and dynamic nature of PFAS science, the approach described herein is flexible to allow for consideration of new or evolving dose-response data and peer reviewed toxicity assessments as they become available. Additionally, because publicly available traditional (in vivo mammalian) toxicity studies are limited to only a small fraction of the more than 9,000 known PFAS, this framework also provides suggestions for practical integration of validated NAMs such as toxicogenomics (e.g., high-throughput transcriptomics (HTTr), in vitro bioactivity) and in silico platforms (e.g., structure-activity, read-across) into the HI and RPF approaches. The illustrative examples in Section 4 are intended to demonstrate the component-based mixture approaches with available dose-response data from completed EPA human health assessments and hypothetical exposure information.

2.0 Background on EPA Mixtures Additivity Guidance

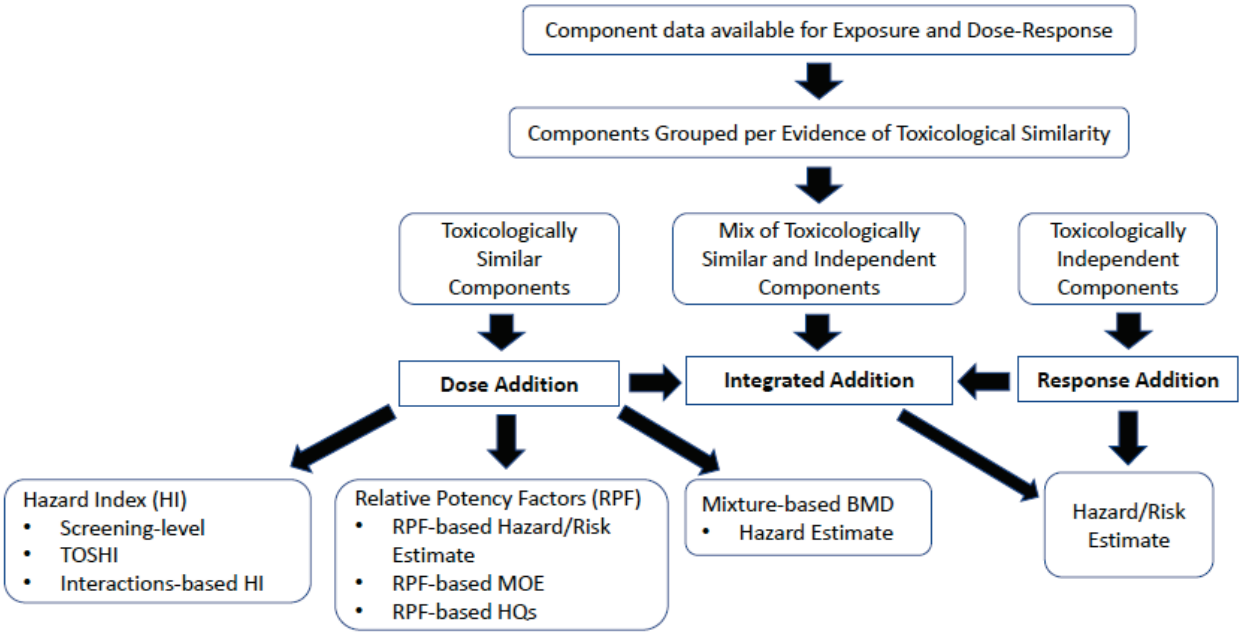
Exposure to mixtures of environmental chemicals occurs in human populations through ingestion, inhalation, and/or dermal contact with contaminated media (e.g., water, air, food). It should be noted that a "mixture" of chemicals may be a function of both co-occurrence in exposure media and/or internal bioaccumulation and persistence in biological matrices. In recognition of the need for methods and approaches that inform evaluation of potential health risks associated with chemical mixtures, EPA developed the 1986 Chemical Mixtures Guidelines and subsequently the 2000 Supplementary Chemical Mixtures Guidance (EPA, 1986, 2000). In those guidance documents, EPA proposed a tiered hierarchy of mixtures approaches where the preferred approach is to evaluate toxicity using hazard and dose-response data for a specific whole mixture of concern, or alternatively a sufficiently similar mixture. However, whole mixture data are rare; there are often too many chemical combinations and proportions in the environment (e.g., parent chemicals, metabolites, and/or abiotic degradants) introducing a level of complexity that is difficult to evaluate and characterize. Further, most controlled experimental toxicity data derive from single chemical exposures, or at best, small mixtures (i.e., limited number of component chemicals at fixed proportions/ratios). As such, EPA also developed multiple component-chemical based mixtures assessment approaches. Component-based methods are used more frequently than whole-mixture methods. These component methods are based on assumptions of how the chemicals behave when co-occurring. Although observed toxicity could be related to direct chemical-to-chemical interaction(s), the manner in which co-occurring chemicals induce toxicity in a coordinated or independent way is the basis for the concept of "additivity." Basic tenets of EPA mixtures additivity theory and practice are as follows:

- Additivity based methods are used to estimate the probability or magnitude of a given health outcome (e.g., incidence and/or severity, or change in magnitude, of a noncancer target organ effect) associated with exposure to mixtures of two or more component chemicals. In the 1986 and 2000 EPA mixtures guidelines and guidance documents, development of component-based mixture approaches were informed by two main concepts, simple similar action and simple independent action, as described by Bliss (1939) and Finney (1971).
- *Simple similar action* applies to mixtures of chemicals that *cause a common health effect* via *toxicologically similar* pathway(s). Under simple similar action (i.e., DA), the evidence associated with toxic responses to mixture component chemicals demonstrate or suggest coordinated (i.e., same/similar) pathway events. DA is generally applied when mixture chemicals are assumed to act through simple similar action.
- *Simple independent action* applies to mixtures of chemicals that cause a *common health effect* via *toxicologically independent* pathways. Under simple independent action (i.e., response addition (RA)), the evidence associated with toxic responses to different mixture component chemicals demonstrate or suggest independent pathway events. RA is generally applied when mixture chemicals are assumed to act through simple independent action.

2.1 Component-Based Mixtures Assessment Methods

Component-based methods that EPA has developed for evaluating potential additivity of dose, response, or both are shown in Figure 2-1. Based primarily on similarity in toxicity

endpoint/health effect of PFAS, this framework document focuses on the use of dose-additive, component-based methods (left side of Figure 2-1), specifically the HI, RPF, and Predictive Hazard Estimate (Mixture BMD approach). As noted above, the methods involve different assumptions for component chemical "mixtures" toxicity.



Notes:

Modification of Figure 4-3b (EPA, 2007a)

Component-based methods selection is based on the relevant evidence supporting toxicological similarity (DA) or toxicological independence (RA or effect summation). Integrated addition methods are reserved for mixtures of component chemicals that demonstrate a profile of both toxicological similarity and independence.

BMD = benchmark dose; HI = hazard index; HQ = hazard quotient; MOE = margin of exposure; RPF = relative potency factor; TOSHI = target-organ specific hazard index.

Figure 2-1. **Flow chart for evaluating chemical mixtures using component-based additive methods.**

An important property of DA-based methods is that they can aid in the prediction of effects of a mixture even when all of the individual component chemical exposures are at or below their individual no-observed-adverse-effect levels (NOAELs). In dose additivity models such as the RPF approach, the sum of the scaled index chemical (IC)[4] equivalent doses/concentrations for each component can exceed the equivalent threshold dose of the mixture and result in a

[4] An IC is that mixture component that is typically the most toxicologically well-studied member. The qualitative and quantitative hazard and dose-response data for an index chemical serve as an index or anchor against which all other components are compared. IC equivalent doses/concentrations represent scaled dose(s) of mixture components, based on potency for a given toxicity endpoint/health effect, in a corresponding dose of the index chemical.

detectable response, which has been supported experimentally (Jonker et al., 1996; Silva et al., 2002).

2.1.1 Application of Dose Addition as EPA's Default Assumption

Several in vivo studies have examined predicted mixture responses based on dose-addition models for specific groups of chemicals (e.g., Altenburger et al., 2000; Crofton et al., 2005; EPA, 2007a; Gennings et al., 2004; Hass et al., 2017; Howdeshell et al., 2015; Kortenkamp and Haas, 2009; Moser et al., 2005, 2012; Mwanza et al., 2012; Rider et al., 2008, 2009, 2010; Walker et al., 2005), focusing primarily on whether experimentally observed toxicity is consistent with modeled predictions of dose-additivity. Many of these studies examined groups of chemicals that are thought to target the same biological signal transduction pathways (Moser et al., 2012; Mwanza et al., 2012; Walker et al., 2005), while others have examined chemicals thought to target disparate pathways that lead to the same health outcome (NAS, 2008; Rider et al., 2009). In general, the results of such studies listed here, and many others, support the continued application of DA as EPA's default component-based mixture assessment approach. Further discussion and examples of the basis for use of dose additivity for PFAS is provided in Section 3.

3.0 Dose Additivity for PFAS

This section presents a review of in vivo chemical mixture studies for different biological pathways that provide information on how mixtures of chemicals with similar and dissimilar molecular initiating events (MIEs) and/or MOAs interact. As discussed in Section 3.2, evidence demonstrates that mixtures of chemicals that disrupt common pathways typically produce dose additive alterations. In studies that tested model prediction accuracy for mixture components that disrupted common pathways, DA models provided predictions that were better than or equal to Integrated Addition (IA) and RA predictions of the observed mixture effects (Section 3.2). Consistent with the conclusions of the National Academy of Sciences (NAS) (2008), a review of published studies in the literature (Section 3.2) did not contain a single case where RA was a better predictor of the adverse effects of a mixture than DA, even when the mixtures included chemicals like phthalates and androgen receptor (AR) antagonists with diverse MOAs (but common targets of toxic action). Taken together, this supports the health protective assumption that a mixture of chemicals with similar apical effects should be assumed to also act in a DA manner unless shown otherwise. Further, data demonstrating that PFOS, PFOA, and other PFAS disrupt signaling of multiple biological pathways resulting in common adverse effects on several biological systems including thyroid hormone levels, lipid synthesis and metabolism, developmental toxicity, and immune and liver function, are reviewed in Section 3.4. Finally, in Section 3.4, a summary is provided for two ongoing EPA Office of Research and Development (ORD) PFAS developmental toxicity mixture studies (of which one study uses a mixture of PFOA and PFOS) that provide robust evidence that PFAS behave in a DA manner.

3.1 Overview of Assessment Approaches for Chemical Mixtures

Over 30 years ago, scientists developed quantitative dose metrics and methods to assess the combined toxicity of mixtures of large classes of chemicals that disrupt a common pathway (NATO, 1988). Toxicity equivalence factors (TEFs) were initially developed in the mid-1980s for hundreds of dioxin-like polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs), and polychlorinated dibenzo-p-dioxins (PCDDs) based upon their potency relative to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Many of the lessons learned about assessing the effects of mixtures of dioxin-like chemicals (DLCs) also are applicable to assessing the effects of PFAS mixtures. Since that time, TEF-like approaches have been used to evaluate mixtures of other chemical classes. The emerging picture is that some chemicals, regardless of MIE or MOA, produce cumulative effects on common apical endpoints that generally are well predicted using DA models.

The general applicability of DA models is based on the review of studies specifically designed to evaluate how well different mixture models predict the way chemicals in a mixture interact to cause effects. Studies evaluating mixture effects typically include an evaluation of individual chemical dose response curves and apply this information to different statistical models of mixture interaction. The data from a number of studies (reviewed below) indicate that chemicals that produce common adverse effects will typically interact in a DA manner when they occur together in a mixture. Thus, the effects of any combination of co-occurring chemicals can be predicted when sufficient chemical dose-response data are available for all of the individual components within an environmentally relevant mixture. For example, the Consumer Product Safety Commission Chronic Hazard Advisory Panel (CPSC CHAP) on Phthalates used DA models to predict the hazard posed by mixtures of phthalates to pregnant women and children. In

their assessment, phthalate mixture exposures from NHANES data were used to predict individual hazard scores for each person and then determine the percentage of people who exceeded a point-of-departure (POD) (CHAP, 2014).

In the absence of an adequate database to evaluate cumulative mixture models, it should be assumed that any mixture acts in a DA cumulative manner if the individual chemicals produce common effects. This approach was fully endorsed by NAS (2008).

3.2 Examples of Chemical Classes and Toxicological Pathways Utilizing Mixture Assessment Approaches

3.2.1 Aryl Hydrocarbon Receptor Pathway and Toxic Equivalence Factors of Dioxin-Like Chemicals

In 2010, EPA published guidance for the use of TEFs for human health risk assessments of DLCs, which produce many of their adverse effects by acting as aryl hydrocarbon receptor (AhR) agonists (EPA, 2010). Hundreds of chemicals are AhR agonists including PCBs, PCDFs, and PCDDs. For DLC mixtures, EPA recommended use of the TEF methodology and the World Health Organization's TEFs to evaluate the risks associated with exposure to mixtures of TCDD and DLCs for human health (EPA, 1987, 1989, 2003) and ecological risk assessments (EPA, 2008). TEFs can be calculated for each DLC based on dietary dose or internal whole body toxic equivalent concentrations (TECs).

The total toxicity of a DLC mixture is based on toxic equivalents (TEQs) which are toxicity-weighted masses of mixtures of PCDDs, PCDFs, and PCBs. The TEQ for each chemical in the mixture is calculated by multiplying each toxic equivalence factor (TEF) by the corresponding chemical concentration in the mixture. The individual TEQs are then summed to calculate the TEQ of the mixture. The reported TEQ provides toxicity information about the mixture of chemicals and is more meaningful than reporting the total mass of DLCs in grams.

This approach assumes:

- Chemicals interact in a DA manner
- They all affect a common pathway via the AhR, among other pathways
- Synergistic and antagonistic interactions are uncommon within the group (Safe, 1994)
- TEFs and TEQs for AhR agonism cannot predict toxicities induced by these chemicals that perturb other biological pathways

TEF values have undergone several revisions (Van den Berg et al., 2006); in 2010, EPA published recommended TEFs for human health risk assessment for DLCs (EPA, 2010). Although the AhR is present in all classes of vertebrates, vertebrate species vary greatly in their sensitivity to environmental TEQ levels. Sensitive species include terns and cormorants (bill deformities), herons (embryo mortality), and mink (lethality and reproductive failure) (Beckett et al., 2008; Restum et al., 1998), for example. Adverse effects also occur in frogs (amphibians) (Gutleb et al., 2000), fish (Monosson, 2000), and snapping turtles (reptiles) (Bishop et al., 1998; Gale et al., 2002). EPA (2008) stated that the TEQ methodology was appropriate for evaluating risks to fish, birds, and mammals associated with AhR agonists.

Studies of AhR agonists in various species indicate:

- Species and tissues differ in sensitivity to the effects of the mixture
- Even though the AhR pathway is conserved, the adverse outcomes can vary greatly from species to species

One common effect of DLCs is a reduction in serum thyroxine (T4). Crofton et al. (2005) conducted a mixture study of 18 thyroid-disrupting DLCs consisting of 12 PCBs, 4 PCDFs, and 2 PCDDs at 6 dilutions of the highest dose, which contained ED_{30} concentrations of each chemical in the high dose. This mixture reduced serum T4 in a dose-related manner. The reduction in T4 was dose additive in the low dose range of interest, but the observed reduction in T4 in the high dose (46% reduced) exceeded DA predictions (28% reduced) by about 18%. In a review of the literature on the effects of mixtures on the thyroid axis, Crofton (2008) concluded "To date, the limited data from thyroid disrupting chemical mixture studies suggest that DA is reasonably accurate in predicting the effects on serum T4 concentrations."

3.2.2 Pyrethroids – Central Nervous System and Behavior

Pyrethroids act on the nervous system, and they all alter neuronal excitability and neuronal firing rate; however, there is uncertainty about whether all pyrethroids act via a narrowly defined "common mechanism of toxicity" or if they should all be included in a common mechanism group for assessment of cumulative toxicity. Wolansky et al. (2009) administered a mixture of 11 pyrethroid pesticides to adult male rat acutely by oral gavage using a fixed-ratio dilution design at eight dose levels and measured locomotor activity on the day of dosing. The reduction in exploratory activity by the mixture was accurately predicted by DA modeling. These pesticides may disrupt different MIEs, but they all converge on a common key event (KE) within an adverse outcome pathway (AOP) network. Exclusion of chemicals from a common mechanism group for cumulative toxicity assessment based upon purported differences in MIEs is not protective when they all converge in an AOP network on a common KE and induce a common adverse outcome in a DA manner.

3.2.3 Organophosphates and Related Pesticides – Lethality, Central Nervous System and Behavior

In the late 1950s Murphy and Dubois (1957) reported that O-ethyl O-p-nitrophenyl phenylphosphonothioate potentiated the lethality of malathion when the two chemicals were given simultaneously. Subsequently, all organophosphate (OP) pesticides in use were evaluated in binary mixture studies to determine if nonadditivity was a common outcome among this class of insecticides (reviewed by Moser et al., 2005; Padilla, 2006). An examination of the interactions of 43 pairs of OP insecticides revealed that 4 pairs showed greater-than-additive effects on lethality (Dubois, 1961). Moser et al. (2005, 2006) reported a range of responses with mixtures of 4 or 5 OPs. The ratios of the predicted-to-observed ED_{20}s and ED_{50}s of the mixtures indicated that several effects displayed small greater-than-additive effects (ratios = 1.2 to 2.6), a few were less than additive (ratio = 0.5 to 0.9), and most were dose additive (ratio = 1). In 2006, EPA concluded that DA was a reasonable approach for predicting the effect of exposure to OP mixtures (EPA, 2006).

Similarly, EPA (2007b) concluded that DA was a reasonable approach for estimating cumulative risk associated with joint exposure mixtures to another class of pesticides, the N-methyl

carbamate insecticides. Further, in 2018 ATSDR concluded that the "default assumption of dose-additive joint action at shared targets of toxicity (i.e., effects on neurological endpoints) be used for screening level assessments of the potential adverse health outcome from concurrent oral exposure to mixtures of pyrethroids, organophosphorus, and carbamate insecticides." (ATSDR, 2018).

3.2.4 Mixture Effects on the Female Reproductive Tract – Estrogen Agonists

Scientists have examined the effects of mixtures of estrogenic chemicals in the female rat using an uterotropic assay, an EPA Endocrine Disruptor Screening Program Test Guideline that is a sensitive in vivo test for estrogenicity (EPA, 2009). In this assay, immature or adult ovariectomized female rats are typically exposed to test chemicals for 3–4 days, after which uterine weights are taken. Exposures can be administered orally or through subcutaneous injections. Tinwell and Ashby (2004) exposed immature female rats for 3 days to several known xenoestrogens, either individually or as mixtures. In a reanalysis of the data, predictions of a DA model for a binary mixture of bisphenol A and genistein were consistent with the observed effects of the mixture with an average deviation of observed results versus the DA model of 4%. Similarly, Conley et al. (2016) found that the effects of mixtures of bisphenol S + methoxychlor, bisphenol AF + methoxychlor, and bisphenol F + bisphenol S + methoxychlor + bisphenol C + ethinyl estradiol, administered orally to female rats, produced effects that were comparable to predictions using DA models. Because the chemicals all stimulate uterine growth via a common estrogen receptor alpha pathway and produce a common effect, DA is the most appropriate model for mixtures of estrogenic compounds.

3.2.5 Mixture Effects on the Female Reproductive Tract – Phthalates in Utero

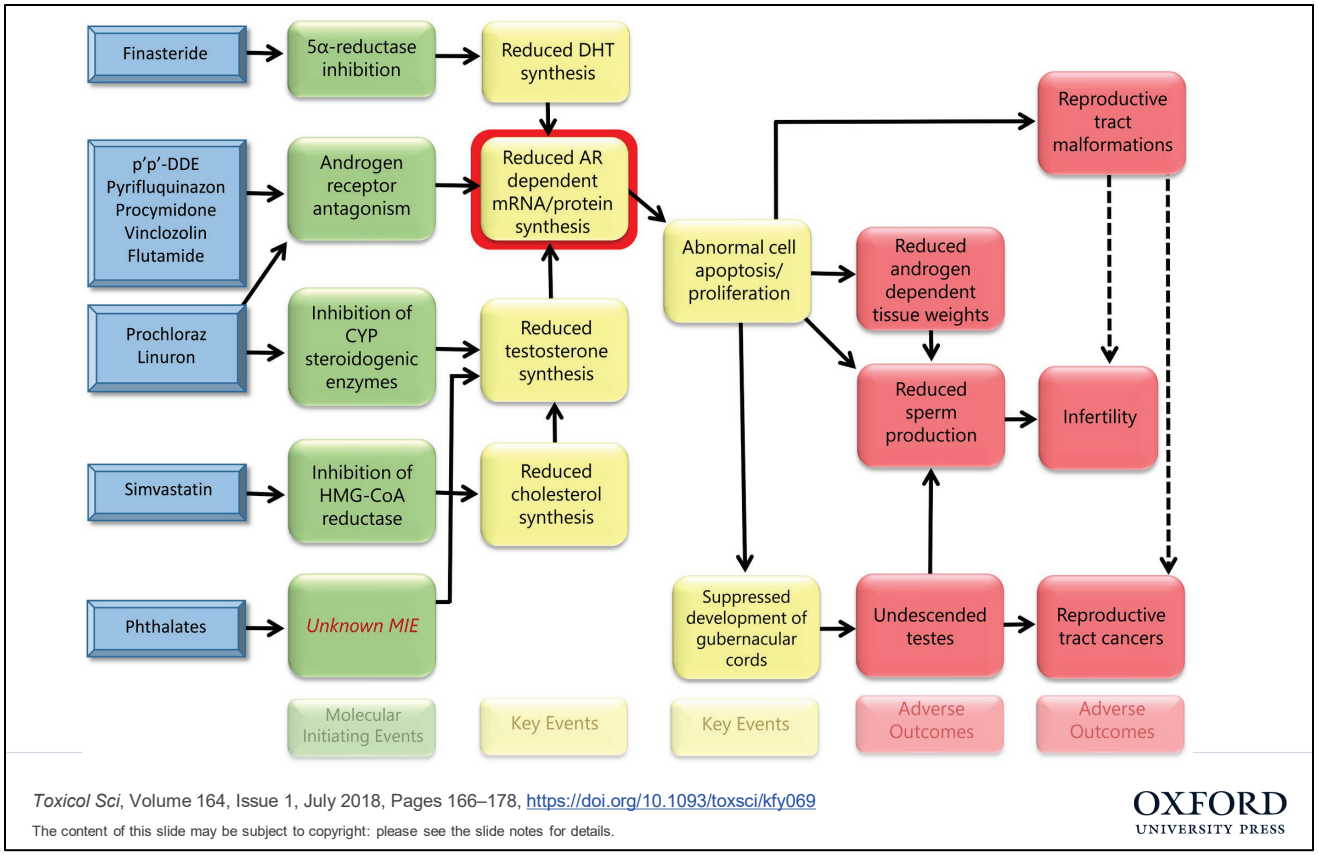
Hannas et al. (2013) reported that administration of a mixture of five phthalates (>520 mg total phthalate) to pregnant rats from gestational days 8 to 13 induced reproductive tract malformations in female rat offspring. These malformations included complete to partial uterine agenesis and agenesis of the lower vagina, an effect similar to a human congenital condition known as the Mayer-Rokitansky-Küster-Hauser syndrome that occurs in about 1 in 4,500 female newborns. The phthalate mixture was a fixed-ratio dilution and contained five phthalates that do not produce malformations in either female or male offspring when administered individually at the doses used in the mixture. These malformations have been seen in dibutyl-(500 mg/kg/d) and diethylhexyl (750 mg/kg/d) phthalate studies at a low incidence and at high doses but were not seen in similar studies with the other three phthalates. Although there was not enough individual phthalate data to compare DA and RA prediction models, it is clear these effects exceed RA (i.e., 0 + 0 + 0 + 0 + 0 = 75% for uterine agenesis) and is an example of "something from nothing" (Silva et al., 2002).

3.2.6 Male Reproductive Tract Development – Antiandrogens

Historically, it has been hypothesized that mixtures of chemicals with dissimilar MIEs would interact in a RA or IA manner. However, this conclusion is not currently supported by a large body of literature on the effects of chemical mixtures and was rejected by NAS (2008). Studies on the effects of mixtures on male reproductive development provide one of the larger databases supporting the use of DA models as the default model. These studies include chemical mixtures with common MIEs and those with multiple MIEs that converge on a common KE in multiple AOPs in an AOP network. These studies focus on chemicals that disrupt androgen signaling in

utero during the critical period of mammalian sexual differentiation. For over 20 years, scientists have examined the in utero effects of mixtures of chemicals that disrupt androgen signaling on the male reproductive tract (e.g., Gray et al, 2001; reviewed by Haas et al., 2007; Howdeshell et al., 2017; Metzdorff et al., 2007). These studies include defined binary or multi-chemical fixed-ratio dilution mixtures and were designed to compare the observed effects to DA, RA, and IA model predictions. The numbers of chemicals used in these studies range from 2 to 18, administered at a range of doses enabling one to discriminate additive from antagonistic or synergistic interactions. In all these studies, the DA model predicted the effects of the mixture on the male reproductive tract more accurately than IA or RA. Likewise, Metzdorff et al. (2007) concluded that the "Effects of a mixture of similarly acting anti-androgens can be predicted fairly accurately based on the potency of the individual mixture components by using the DA concept. Exposure to anti-androgens, which individually appears to exert only small effects, may induce marked responses in concert with, possibly unrecognized, similarly acting chemicals."

In addition, two recent studies were designed to specifically address a gap in the literature identified by the CPSC CHAP (Lioy et al., 2015). At the time of their review there were no published studies that addressed whether or not phthalate mixtures exhibited cumulative effects when administered at levels below the lowest observed adverse effect levels (LOAELs) of each individual chemical. In the first study, a mixture of 18 administered chemicals induced effects at dose levels about 80-fold below each chemical's individual LOAEL (Conley et al., 2018). These 18 chemicals disrupt androgen signaling via five different MIEs (Figure 3-1) and multiple AOPs that converge on common KEs resulting in common adverse reproductive effects in male rat offspring.



Notes:
Adapted from Conley et al., 2018
The bold outlined KE indicates the critical node that links the various MIEs to the downstream adverse outcomes.
DHT = dihydrotestosterone; AR = androgen receptor; CYP = cytochrome P450; HMG-CoA = 3-hydroxy-3-methyl-glytaryl coenzyme A.

Figure 3-1. AOP network for chemicals that disrupt AR-mediated cellular signaling leading to adverse effects on the development of male reproductive tract resulting from in utero exposure.

In the second study (Conley et al., 2021a), 15 chemicals (acting via at least 3 MIEs) demasculinized male rat offspring at dose levels 2- to 4-fold lower than the individual no observed effect levels for each chemical, and the DA models were always as good or better than RA or IA models. For example, 60% of male offspring were found to have penile malformations that resulted in infertility and this effect was accurately predicted by DA, whereas IA and RA predicted that none of the males would be malformed. This is not a unique observation; rather, it is a typical finding with male reproductive tract malformations.

The validity of using DA models for diverse mixtures of chemicals that disrupt androgen signaling and male rat development is supported by an examination of the effects of these chemicals at the cellular level. All these chemicals act via AOPs that converge on a common KE in an AOP network (Figure 3-1) that regulates the sequence of molecular events in cells that are involved in the development of the androgen-dependent tissues. Each of these chemicals reduces the number of AR dimers, AR/AR, activated by an androgen agonist. AR antagonists, like vinclozolin or procymidone, accomplish this by blocking androgens from binding to ARs and

pyrifluquinazon has been hypothesized to act by enhancing AR degradation (Gray et al., 2019; Yasunaga et al., 2013) Chemicals like the phthalates (di-n-butyl phthalate, di(2-ethylhexyl)phthalate, dipentyl phthalate, butyl benzyl phthalate, diisobutyl phthalate) reduce the levels of androgens available to the cell (Hannas et al., 2011; Howdeshell et al., 2008; Furr et al., 2014), and chemicals like finasteride inhibit the enzyme in the tissues that converts testosterone to dihydrotestosterone (a more active androgen that has higher affinity for the AR) (Clark et al., 1990). Fewer activated AR/AR heterodimers bind the promoter region on the DNA of androgen-regulated genes, androgen-dependent mRNA and protein synthesis levels are reduced, and growth and differentiation of androgen-dependent tissues in the fetus is inhibited. As a result, male offspring display agenesis or hypoplasia or malformations in androgen-dependent tissues. In summary, an examination of the events disrupted in the androgen signaling pathway by all these chemicals at the cellular-molecular level explains why one should expect the mixtures to behave in a DA manner. It is not important to the cell what MIE reduced the androgen signal to the gene; it is only important that the signal is reduced.

In summary, an examination of the literature on the effects of mixtures on male reproductive tract development is as follows:

- Mixtures of chemicals that disrupt common effects can be adequately modeled by DA.
- The chemicals acted in a DA manner regardless of whether or not they shared a common MIE.
- IA and RA models can grossly underestimate the hazard of a mixture of chemicals acting on a common KE or with a common apical effect.

3.3 Systematic Reviews of Mixtures Toxicity: Quantification of Deviations from Dose Additivity

Boobis et al. (2011) examined the literature from 1990 to 2008 that discussed synergy in mammalian test systems with an emphasis on “low dose” studies. Of the 90 papers identified, 43 papers had original data from which synergy could be examined, and only 11 studies reported the magnitude of the difference between the dose additive estimates of toxicity with the observed results. Of these 11 studies, 6 reported magnitudes of synergy that were generally less than 2-fold with a maximum value of 3.5-fold. As a result, the authors concluded that deviations from DA at low doses were not common.

The issue of the occurrence of greater-than-DA (sometimes referred to as synergistic) versus DA or less-than DA (sometimes referred to as antagonistic) interactions was recently reassessed by Martin et al. (2021). The authors conducted a systematic review and quantitative reappraisal of 10 years of a broad range of mixture studies from 2007 to 2017. Martin et al. (2021) identified 1,220 mixture studies, ~65% of which did not incorporate more than 2 components. They reported that “relatively few claims of synergistic or antagonist effects stood up to scrutiny in terms of deviations from expected additivity that exceed the boundaries of acceptable between-study variability,” and that the observed effects were not more than 2-fold greater than the predicted effects of the mixture predicted by DA.

3.3.1 Deviation from Additivity

Although the literature indicates that significant deviations from dose additivity are not common among mixtures containing chemicals that disrupt common targets via common AOPs or AOP

networks, it is important to note that greater-than-additive and less-than-additive interactions do occur with co-exposure to chemicals that affect different target organs or different, unrelated AOPs. There are numerous examples of chemical interactions that deviate from DA that are biologically relevant including mixtures in which one chemical alters the metabolism of the other chemical(s), including the ones provided below.

- Twenty years of research has identified at least 85 drugs whose metabolism is inhibited by a chemical in grapefruit, potentially resulting in serious side effects (Bailey et al., 2013). Furanocoumarins in grapefruit bind to the active site on the CYP3A4 enzyme causing irreversible inactivation that prolongs the half-life and AUC (the area under the concentration versus time curve) of some drugs, like some statins for example.
- The effects of metabolic alterations of chemical toxicity are not limited to drug-drug interactions. Hodgson (2012) published a comprehensive review of the effects of metabolism on the toxicity of a large number of pesticides and also described the metabolic mechanisms of chemical activation and/or inactivation.
- Imidazole and triazole fungicides, used as clinical medicines and pesticides, can influence sterol biosynthesis and retinoic acid metabolism, which have been associated with adverse patient outcomes (Yamazoe et al., 2020).

In addition to metabolic activity leading to synergistic or antagonistic interactions among chemical mixtures, there are other examples of deviations from DA. As with the examples in the list above, these do not include chemicals that disrupt common KEs, AOPs, AOP networks, or target organs. For example, adulteration of pet food with melamine and derivatives, including cyanuric acid, caused kidney failure and death of a large number of cats and dogs in the United States (Jacob et al., 2011). In addition, more than 54,000 infants and young children in China were treated for urinary problems and possible kidney stones related to the melamine contamination of infant formula and related dairy products with several confirmed deaths (WHO, 2008). Although individually these compounds present low toxicity, co-exposure can lead to the formation of melamine cyanurate crystals in the nephrons and eventual kidney failure in mammals.

3.4 PFAS Dose Additivity

PFOA and PFOS, as well as other PFAS with linear or branched alkyl or alkyl ether chains and sulfonic or carboxylic acid functional groups, share common toxicological impacts of exposure on multiple cellular receptors, tissues, life stages, and species (ATSDR, 2021; EFSA et al., 2018, 2020). As described above (Section 3.2), precedents of prior research conducted on mixtures of various chemical classes with disparate molecular mechanisms but common KEs or adverse outcomes support predictions of dose additive effects. Thus, in the absence of detailed molecular mechanisms for most PFAS, it is considered a reasonable health-protective assumption that PFAS which can be demonstrated to share one or more KEs or adverse outcomes will act with toxicological similarity to produce dose-additive effects from co-exposure. PFOA and PFOS have historically been the most studied and well-characterized PFAS, but recent work has also provided supportive evidence of similar effects of other straight chain compounds as well as emerging ether-linked compounds. Below is a brief overview of MIEs, KEs, and adverse outcomes that have been shown to be common to several PFAS and evidence which supports dose additivity. This overview highlights results from, among others, the NIEHS National Toxicology Program (NTP) 28-day repeat dose guideline toxicity studies of perfluoroalkyl

carboxylates (PFHxA, PFOA, PFNA, and PFDA) (NTP, 2019a) and perfluoroalkyl sulfonates (PFBS, PFHxS, and PFOS) (NTP, 2019b). The NTP studies provide high quality side-by-side comparisons of multiple PFAS from experiments conducted by a single lab with rigorous exposure characterization and multiple endpoints spanning MIEs, KEs, and AOPs. More comprehensive reviews of common PFAS toxicity endpoints in experimental animal studies and observational human studies can be found elsewhere (ATSDR, 2021; EFSA et al., 2018, 2020).

Mechanistically, demonstration of nuclear receptor activation constitutes a principle MIE in the description of PFAS-relevant AOPs. PFAS, such as PFOA and PFOS, have been shown to activate multiple similar nuclear receptors in both in vitro and in vivo studies, thus identifying multiple MIEs relevant to PFAS. In vitro activation of peroxisome proliferator activated receptor alpha (PPARα) (Behr et al., 2020; Ishibashi et al., 2019; Takacs and Abbott, 2007; Vanden Heuvel et al., 2006) and gamma (PPARγ) (Houck et al., 2021; Vanden Heuvel et al., 2006) is well described for multiple PFAS. Further, in vivo studies of tissue-specific gene expression patterns have also demonstrated activation of constitutive androstane receptor (CAR) for both PFOA and PFOS due to upregulation of CAR-dependent genes (Rosen et al., 2017) and liver-based profiles of PPARα-induced gene expression (Bjork et al., 2008; Rosen et al., 2007). Recently, PFOA and PFOS, along with PFHxA, PFNA, PFDA, PFBS, and PFHxS, were shown to upregulate the PPARα-inducible Acox1 and Cyp4a1 and the CAR-inducible Cyp2b1 and Cyp2b2 in adult male and female rat livers in 28-day repeat dose guideline studies (NTP, 2019a,b). From a molecular mechanism perspective, PFOA and PFOS both activate similar nuclear receptors and gene transcription pathways, along with several other studied PFAS including those listed above.

KEs downstream of the above potential MIEs are also shared between PFOA, PFOS, and other PFAS. In both rodent and non-human primate studies, serum lipids (cholesterol, triglycerides) are consistently reduced and markers of liver dysfunction (ALT, AST, and/or ALP) are consistently elevated in a dose-responsive manner (ATSDR, 2021; EFSA et al., 2018, 2020). Specifically, the NTP 28-day studies reported reduced serum cholesterol, triglycerides, and globulin and elevated serum ALT, AST (males only), ALP, and bile acids from exposure to PFHxA, PFOA, PFNA, PFDA, PFBS, PFHxS, and PFOS (NTP, 2019a,b). Further, all PFAS reduced total and free thyroxine (T4) (NTP, 2019a,b). In combination with the nuclear receptor activity and gene expression profiles, there is a pronounced similarity in the serum clinical chemistry and thyroid hormone-based KEs for PFOA, PFOS, and several other studied PFAS.

Similar adverse outcomes at the organ and whole animal levels have been described for PFOA, PFOS, and several other PFAS. Effects in developmental exposure studies with PFOA, PFOS, PFNA, and GenX chemicals in rats and/or mice have reported consistent effects on pups including reduced F1 survival/viability and reduced F1 body weight (Abbott et al., 2007, 2009; Blake et al., 2020; Butenhoff et al., 2004; Conley et al., 2021b; Das et al., 2015; Lau et al., 2003; Luebker et al., 2005a,b; Thibodeaux et al., 2003). All PFAS studied by NTP (2019a,b) increased rat liver weights and produced hepatocyte hypertrophy. PFOA and PFOS, and potentially other PFAS, have also been shown to produce functional immunotoxicity (i.e., reduced antibody response) in animal studies (NTP, 2016). Taken together, there is a broad spectrum of adverse effects in laboratory animals that are highly similar and plausibly associated with the common molecular mechanisms and KEs displayed by PFOA, PFOS, and several other PFAS.

Limited work has been conducted on combined exposure to PFAS in experimental systems. An in vitro mixture study of PPARα activation demonstrated cumulative effects of combined exposure to binary combinations of PFOA and PFOS, PFNA, PFHxA, and PFHxS that conformed to models of dose additivity (Wolf et al., 2014). Mammalian studies evaluating exposure to multiple PFAS are limited but two recent studies indicate that exposure to combined PFOA, PFOS, and PFHxS (Marques et al., 2021) and combined PFOA, PFOS, PFNA, PFHxS, and GenX chemicals (Roth et al., 2021) in mice produced numerous significant effects compared to control which were consistent with the spectrum of individual PFAS effects described above. Currently, developmental toxicity studies of PFAS mixtures are ongoing at EPA ORD. Conley et al. (2021a) presented preliminary data on an unpublished mixture study of PFOS, HFPO dimer acid (also known as GenX chemicals), and Nafion byproduct 2 (NBP2) (an emerging polyfluoroethersulfonic acid compound recently detected in human serum (Kotlarz et al. 2020)), which produced neonatal mortality that was accurately predicted by DA modeling, among other cumulative mixture effects. Further, a direct investigation of the cumulative in vivo mixture developmental toxicity of combined exposure to PFOA and PFOS was recently conducted by EPA ORD (see Appendix A). A series of experiments were designed to characterize the dose responses across several endpoints for PFOA and PFOS individually, followed by a mixture study of the two chemicals combined. Preliminary results identified numerous effects from each chemical individually as well as the mixture, including reduced maternal gestational weight gain, reduced pup body weight and pup viability, and increased maternal and pup liver weights. As a clear demonstration of cumulative mixture effects, individual exposures to 62.5 milligrams (mg) per kilogram (kg) PFOA and 2 mg/kg PFOS produced 12±7% (mean± standard error of the mean (SEM) and 8±6% post-implantation loss (PIL; a measure of fetal and pup mortality), respectively, while a combination of the two (62.5 mg/kg PFOA+2 mg/kg PFOS) produced 66±15% PIL. Further, when graphed as a function of oral PFOA dose, the dose response curves for the combination of PFOS+PFOA across multiple effects, such as pup body weight and maternal and pup liver weights, were significantly shifted towards effects at lower doses than the curves for PFOA alone, which means that there was an additive effect of PFOS (see Figure 2 in Appendix A). This study is ongoing with multiple analyses still to be conducted on samples collected during the studies. However, these preliminary results provide robust evidence of combined toxicity of PFOA, PFOS, and other PFAS on multiple developmental endpoints. Studies with PFOA alone and PFOS alone demonstrated that both chemicals independently produce numerous similar developmental adverse outcomes including increased PIL, reduced pup body weight, and increased pup and maternal liver weight (see Figure 2 in Appendix A). Co-exposure to PFOA and PFOS in combination produced cumulative mixture effects that are at least dose additive for most endpoints and support the combined toxicity of these compounds.

In summary, the data reported in the literature support an assumption of similarity in toxicity profiles for PFOA and PFOS and other PFAS with linear or branched alkyl or alkyl ether chains and sulfonic or carboxylic acid functional groups and a dose-additive assessment approach. Recent efforts to characterize in vivo mixture effects from combined exposure to multiple PFAS provide key supportive evidence that co-exposure produces dose additive effects on several endpoints within the range of “same/similar” endpoints that are shared across the spectrum of PFAS effects.

4.0 Introduction to Estimating Noncancer PFAS Mixture Hazard or Risk

4.1 Whole Mixtures Approach

The preferred hazard and dose-response knowledge base for any mixture of environmental chemicals would be derived from exposure to a whole mixture of concern. However, the exponential diversity of chemicals such as PFAS co-occurring in different component associations and proportions makes whole mixture evaluations extremely difficult and complex. That is, in the environment, due to differing fate and transport properties of chemicals, biotic (metabolism) and abiotic (degradation) processes, pH, ultraviolet radiation, media temperature, and so on, components commonly co-occur in an array of parent species, metabolites, and/or abiotic degradants making characterization of any given mixture complicated. In controlled experimental study designs, whole mixtures can be assembled with defined component membership and proportions. However, the relevance of toxicity associated with exposure to a defined mixture in a laboratory setting may not be translatable to mixtures of different component associations and proportions in the field. In the context of PFAS, increasing environmental evidence (e.g., environmental water, air, and soil sampling results) suggests that the complexities briefly summarized above with regard to the diversity of chemicals co-occurring in different component associations and proportions make evaluating each unique whole mixture of PFAS intractable, which is why component-based mixture approaches are considered particularly useful and appropriate for addressing the real problem of human exposure to multiple PFAS (see Sections 4.2–4.5).

EPA's *Supplemental Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (EPA, 2000) indicates that there may be opportunities to infer hazard and dose-response for a mixture of concern from a sufficiently similar mixture. A mixture is considered sufficiently similar to a mixture of concern when the components and respective proportions exist in approximately the same pattern. There are clearly gradations of expert judgment involved in what constitutes a "sufficiently similar mixture," but determinations should be based on a comparison of similarities or differences in the component's chemical fate and transport in the environment, persistence, bioaccumulative potential, kinetics, and toxicity profile. If no significant qualitative differences are identified in a systematic comparison of mixtures of chemicals, the hazard and dose-response information associated with the sufficiently similar chemical could be used as a surrogate for the mixture of concern.

4.2 Component-Based Mixtures Approach for PFAS

As a result of both the complexities associated with characterization and evaluation of whole mixtures (see Section 4.1 above) and the reality that most toxicological information for chemicals derives from exposure-response studies of individual species, component-based mixtures risk assessment is particularly relevant (see Figure 4-1). In addition, although the methodological approaches and associated illustrative examples in this framework are targeted at application to water, the concepts may facilitate evaluation of PFAS mixtures in other exposure media as well (e.g., soil, air). As outlined in earlier sections of this framework, while EPA component-based methods and approaches are available for evaluation of mixtures of chemicals under different assumptions of additivity (EPA, 2000), the currently available evidence on PFAS supports an assumption of dose additivity (see Section 3). The HI and RPFs are two component-

based mixture approaches based on dose additivity, that are well validated and actively used by EPA. These two approaches are discussed below and include illustrative examples that are based on PFAS with completed EPA human health assessments. An alternative Mixture BMD approach, generally based on the Berenbaum equation (see section 4.2.6 in EPA mixtures guidance (2000)), is also a dose additive approach that is described and illustrated with hypothetical examples. It should be noted that others have recently demonstrated the application of the HI and RPF approaches in the evaluation of PFAS (Bil et al., 2021; Mumtaz et al., 2021), lending confidence to the direction of this framework document in guiding formal component-based assessment of PFAS mixtures.

A pragmatic tiered approach to application of component-based evaluation of mixtures of PFAS with variable hazard and dose-response databases is presented in Figure 4-1. The entry point into the flow diagram follows the search, collection, and assembly of all available toxicity (including NAM-based data such as cell-based bioactivity) and exposure data available for all mixture component PFAS of potential concern. Off-the-shelf exposure duration-relevant human health hazard data and dose-response assessment values (e.g., POD, RfDs, MRLs) are available from federal (EPA, ATSDR), state, international, or other formal entities for a limited set of PFAS (e.g., EPA final assessments are currently available for PFOA, PFOS, PFBS, and GenX chemicals). Such human health reference or toxicity values may be leveraged to inform PFAS mixture assessment (i.e., using HI, RPF, and Mixture BMD approaches), however with clearly identified nuances and uncertainties that help contextualize such values (see discussion in Section 4.3). For many PFAS, no formal health assessments exist, however human epidemiological and/or experimental animal hazard and dose-response data may be available in the public domain. Should such data be available, de novo derivation of chronic (and/or subchronic) non-cancer toxicity values might be possible; it would be necessary to transparently communicate the targeted fit-for-purpose application and attendant uncertainty(ies) associated with such derived values.

Users of this framework may find that PFAS of interest may be data-poor (i.e., no usable traditional human health assessment relevant epidemiological or experimental animal study data are available). In such cases, NAM platforms or assays might provide opportunities to inform potential hazard and dose-response for PFAS mixture components. For example, read-across is a NAM approach that could potentially be leveraged to identify dose-response metrics (e.g., POD, effect concentration (EC_X), IC_X) for integration into the component-based mixtures assessment approaches presented in the subsections below. Analogue-based read-across, in general, is a process in which chemicals (i.e., analogues) with relatively replete toxicity databases are compared to a data-poor target chemical across similarity domains including structural, physicochemical, toxicokinetic (TK), and/or toxicodynamic (TD) similarity (Wang et al., 2012; Wu et al., 2010). Based on weight-of-evidence for similarity between a target chemical and candidate analogues, hazard and dose-response data (e.g., POD) are then adopted from a selected (single-best) analogue as surrogate for the data-poor target chemical. This read-across approach might facilitate incorporation of data-poor PFAS into the component-based methods presented in this framework, as (surrogate) data that informs similarity of toxic endpoint/health effect and dose-response could potentially: (1) be used in the derivation of a non-cancer RfD (using UFs appropriate for the data-poor target chemical) and subsequent calculation of a HQ, (2) be used in the calculation of RPF(s), or (3) the surrogate health-effect dose-response data could be BMD modeled and included in the calculation of an overall mixture BMD.

PFAS with identified hazard(s) and dose-response inputs (e.g., RfDs, MRLs), as described above, are first screened in Tier 1 of this framework using the HI approach (Section 4.3). In brief, duration-relevant exposure (E) and toxicity values (e.g., reference value (RfV)) for each mixture PFAS are used in a simple ratio (E/RfV) to calculate a hazard quotient (HQ). The component PFAS HQs are then summed to generate a mixture HI (see Equation 4-1). The HI approach is split into three general subtypes: (1) screening-level, (2) target-organ-specific hazard index (TOSHI), and (3) interactions-based HI (see Figure 2-1). In this framework, only the screening level HI and TOSHI are included as dose additive Tier 1 methods; data to inform deviations from dose additivity (e.g., interactions such as synergism or antagonism) are virtually non-existent for PFAS co-occurring in mixture, as such, an interactions-based HI is not feasible at present. The screening level HI involves the use of RfVs for each PFAS mixture component irrespective of health outcome domain. Since each mixture component HQ is calculated using a corresponding RfV, the mixture HI may represent a conservative indicator of potential mixture hazard. Conversely, the TOSHI approach is exactly as the name suggests, that is, it entails calculating component chemical HQs and corresponding mixture HIs for specific target-organ effects/endpoints using target-organ toxicity doses (TTDs) (note: some TTDs could also be the overall RfV for a given PFAS). A mixture HI approaching or exceeding 1.0 indicates potential concern for a given environmental media or site and is typically flagged for further evaluation using a more robust evaluation (e.g., Tier 2, RPF). The key to this first HI Tier is that it provides an initial indication of: (1) concern for the overall mixture and (2) potential driver PFAS (i.e., those PFAS with high[er] HQs). Potential "driver" PFAS may be prioritized for further evaluation in a RPF approach. Conversely, those PFAS with low(er) HQs (e.g., $\leq$ 0.0X) might be deprioritized for further evaluation as they may not have significant impact(s) on overall mixture risk. It should be noted that a user of this approach should consider the potential exposure (e.g., water concentration), the potency for effect (e.g., low[er] or high[er] PODs), and qualitative and quantitative uncertainty (i.e., totality of uncertainty factor application) for each PFAS mixture component in deciding if a given PFAS should move on to a more robust mixture evaluation (i.e., Tier 2).

In contrast to the HI, the RPF approach in Tier 2 provides a PFAS mixture risk estimate (see Section 4.4). Application of this approach is demonstrated using PFOA, PFOS, PFBS, and GenX chemicals as examples in this document; in practice, it could be expanded to other PFAS with sufficient hazard and dose-response information. In the illustrative examples provided in Section 4.4, potency for an effect across each mixture PFAS is normalized to a selected IC for three critical health effect domains – developmental, thyroid, and liver – resulting in three sets of RPFs. These three health effect domains were selected primarily because: (1) the effects were the basis for RfD derivation for each respective PFAS (i.e., PFOA/PFOS = developmental effect (EPA, 2016a,b); PFBS = thyroid effect (EPA, 2021a); and GenX chemicals = liver effect (EPA, 2021b)) and (2) each example PFAS has sufficient hazard and dose-response data across all three health effect domains to facilitate demonstration of the RPF methodology. In addition, these three health effect domains have been identified for other PFAS for which data are available. In practice, for application in a water context, each respective PFAS RPF is multiplied by its corresponding specific media concentration (e.g., water concentration), resulting in an Index Chemical Equivalent Concentration (ICEC). The ICECs across PFAS mixture components are summed to generate an overall mixture ICEC (see Equation 4-3), which is effectively a total concentration of the IC, for each health effect domain. In traditional EPA mixtures risk assessment practice, the mixture ICEC is then mapped to the dose-response function of the IC to

arrive at a "mixture response." In this framework, in the context of water, the mixture ICEC (i.e., total dose of IC) is compared directly to a Health-Based Water Concentration (HBWC) (e.g., Health Advisory, MCLG) based on the relevant health effect domain (e.g., developmental, thyroid, liver) for the IC. If the mixture ICEC for one or more of the three effect domains exceeds the corresponding IC HBWC then there may be cause for concern for the mixture at the reported/measured component water concentrations. Conversely, if the mixture ICEC for all three effect domains is below the IC HBWC, risk is not anticipated. Additionally, individual mixture PFAS with large(r) RPFs and corresponding ICECs should be flagged regardless of whether the total mixture ICEC is above or below an IC HBWC.

An additional option, represented as an alternative Tier 2 approach (Figure 4-1) in the decision flow diagram, entails calculation of a mixture BMD (see Section 4.5). In contrast to the Tier 2 RPF approach, there is no need for identification of mixture ICs, calculation of RPFs or ICECs, or existence of HBWCs. The approach results in a mixture BMD (i.e., POD) that could be converted into a mixture RfD using appropriate uncertainty factor application, and subsequently a corresponding mixture-specific HBWC (e.g., Health Advisory or MCLG). However, it is cautioned that such values would be specific to a given mixture of PFAS at defined component proportions (e.g., individual PFAS water concentrations).

As previously noted, the HI and RPF examples presented for PFOA and PFOS rely on information from EPA's 2016 health effects support documents (EPA, 2016a,b). To support the NPDWR for these chemicals, EPA has reevaluated the current toxicological literature knowledgebase and developed draft updated RfDs for PFOA and PFOS, which are currently undergoing review by EPA's SAB. This document was developed concurrently with the draft MCLG documents for PFOA and PFOS and does not reflect the updated health information (i.e., RfDs) presented in those specific documents.

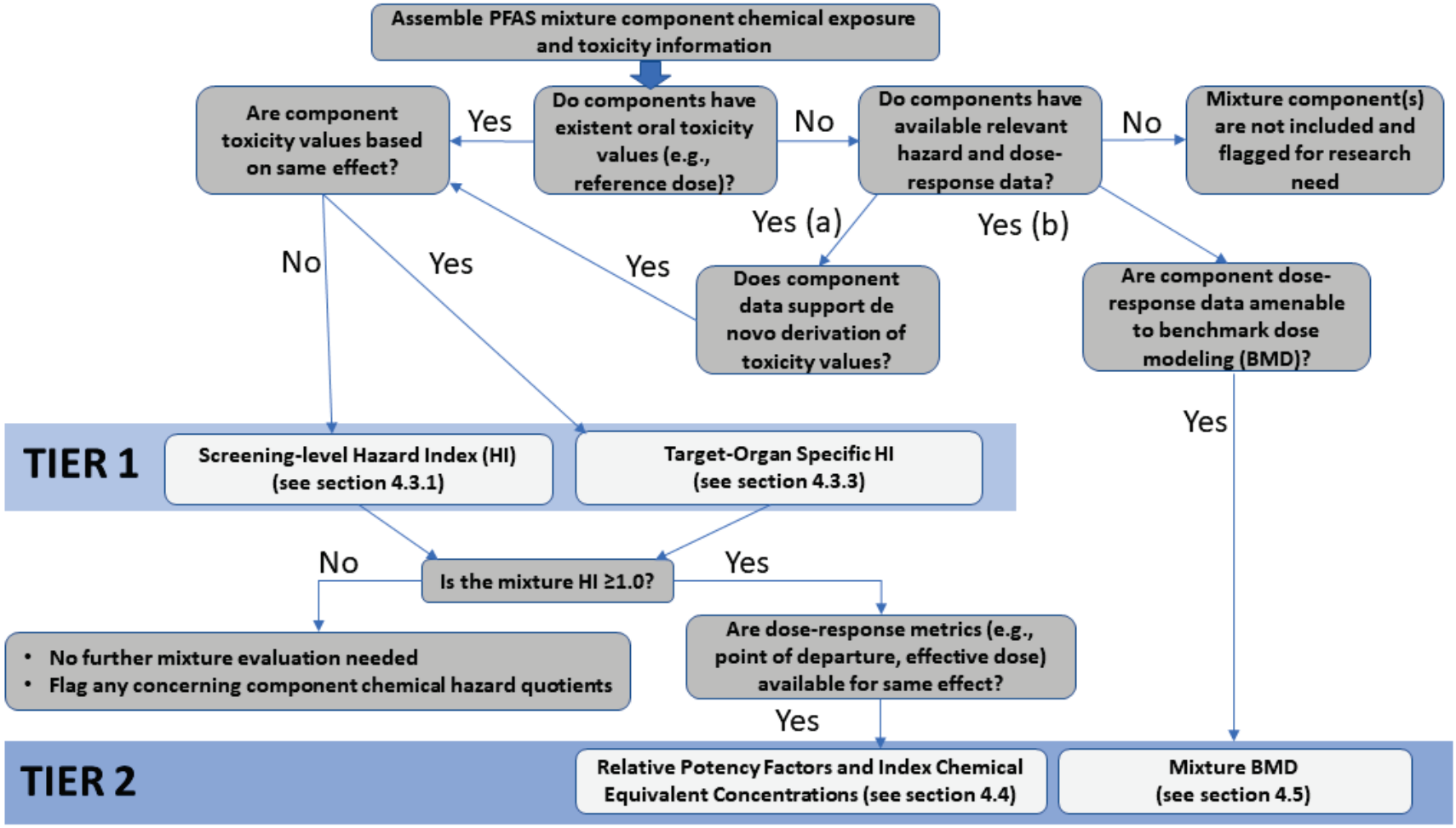


Figure 4-1. Flow diagram for proposed component-based approaches based on dose additivity to estimate noncancer health risk associated with PFAS mixtures.

4.3 Hazard Index

The HI is the most commonly used component-based mixture risk assessment method in EPA. Because the HI employs a population level exposure and human health assessment value, such as an oral RfD, this ratio provides an indication of potential health hazard(s). That is, the HI is a decision aid; it is not a mixture risk estimate in that it is not expressed as a probability, nor is it an estimate of specific toxicity (e.g., embryo toxicity). The HI is based on an assumption of DA among the mixture components (EPA, 2000; Svendsgaard and Hertzberg, 1994). In the HI approach, an HQ is calculated as the ratio of human exposure to a health hazard RfV for each mixture component chemical (i) (EPA, 1986). The HI is dimensionless, so in the HI formula, E and the RfV must be in the same units (Equation 4-1). For example, if E is the oral intake rate (mg/kg/d), then the RfV could be the RfD, which has the same units. Alternatively, the exposure metric can be a water concentration and the toxicity value is best represented as an HBWC, for example, an EPA drinking water Health Advisory (e.g., EPA 2016c,d) or MCLG, or a similar value (e.g., developed by a state). The component chemical HQs are then summed across the mixture to yield the HI, as illustrated in Equation 4-1.

$$HI = \sum_{i=1}^{n} HQ_i = \sum_{i=1}^{n} \frac{E_i}{RfV_i} \qquad (4\text{-}1)$$

Where:

HI = Hazard Index

HQ_i = Hazard Quotient for chemical i

E_i = Exposure, i.e., dose (mg/kg/d) or occurrence concentration, such as in drinking water (mg/L), for chemical i

RfV_i = Reference value (e.g., oral RfD or MRL (mg/kg/d), or corresponding health-based, media-specific value; e.g., such as an HBWC, that is, a drinking water Health Advisory or MCLG) for chemical i (mg/L)

Because the numerator of each component chemical HQ is the estimated population-level human exposure, the non-cancer health RfVs used in the denominator must be based on human toxicity. These RfVs are derived either directly from human epidemiological/occupational study PODs (or measured or modeled effective dose (ED_X) from exposure-response data in a cohort or population) or as human-equivalent PODs converted from experimental animal studies (e.g., conversion of a rodent POD to a human equivalent dose (POD_{HED}) using cross-species TK-based modeling or allometric body-weight scaling).

The HI approach in practical application may be subdivided into a "screening-level" HI and a "target-organ specific" HI (TOSHI). In the screening level HI, the RfV for each mixture component chemical is used in the calculation of a HQ, irrespective of the effect on which each component RfV is based (e.g., RfD for mixture chemical 1 may be based on liver effect, for chemical 2 thyroid effect, and chemical 3 developmental effect). The resultant screening-level HI is generally a conservative indicator because often the most sensitive health effects are used as the basis for each respective chemical HQ. Conversely, the TOSHI entails derivation of HQs for each mixture component chemical based on a "similar" effect. For example, in the case of a

liver-specific HI, for some mixture components the liver effect(s) may indeed be the basis for the RfD (e.g., GenX chemicals) whereas for other components, the liver might be among the least sensitive of effects (e.g., PFBS). To use this approach, organ-specific reference values (osRfVs) or TTDs are needed (note: these are the same type of non-cancer values, just with different naming conventions) for each mixture component of potential concern. For chemicals lacking hazard and dose-response data from traditional or NAM-based data streams for the selected effect, it may not be possible to determine their potential contribution to the mixture, which may result in an underestimation of the overall mixture risk.

In a screening-level context, an HI less than or equal to one is regarded as being of minor or no concern (recall that an RfV, like an oral RfD, represents an estimate at which no appreciable risk of deleterious effects exists), typically requiring no further analysis (EPA, 1986, 1991, 2000). An HI greater than one is generally regarded as suggestive of possible toxicity. Further analysis could provide a refined assessment of the potential for health effects associated with the individual chemicals and their contributions to the potential joint toxicity associated with the mixture (see Section 4.4).

In the case of PFAS, final peer-reviewed toxicity assessments are only available for a small proportion of the more than 9,000 environmentally relevant PFAS (e.g., see summary of EPA and ATSDR PFAS assessments in Table 4-1). EPA's primary source of peer-reviewed toxicity assessments is its Integrated Risk Information System (IRIS) program, but in some cases (e.g., when no IRIS assessment exists or there is a more current assessment from another authoritative source), the agency relies on assessments from other EPA program offices, and other state, national, and international programs. U.S. federal toxicological assessments, such as EPA's IRIS[5], Provisional Peer-Reviewed Toxicity Values (PPRTV)[6], EPA Office of Water toxicity assessments[7], TSCA risk evaluations[8], and ATSDR's ToxProfiles[9], undergo rigorous peer and public review processes; as a result, they are considered to be of high scientific quality. The chronic RfDs for PFOA (EPA, 2016a), PFOS (EPA, 2016b), PFBS (EPA, 2021a), and GenX chemicals (EPA, 2021b) represent the only currently available/final EPA toxicity values, although several more PFAS assessments are under development in EPA ORD (e.g., PFBA, PFHxA, PFHxS, PFNA, and PFDA; see Table 4-1 below) that can be considered in the future. Additionally, use of this approach could consider other PFAS toxicity values (e.g., ATSDR MRLs) for which EPA has not yet developed values.

[5] https://www.epa.gov/iris
[6] https://www.epa.gov/pprtv
[7] e.g., https://www.epa.gov/ground-water-and-drinking-water/supporting-documents-drinking-water-health-advisories-pfoa-and-pfos
[8] https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/risk-evaluations-existing-chemicals-under-tsca
[9] https://www.atsdr.cdc.gov/toxprofiledocs/index.html

Table 4-1. EPA and ATSDR Peer-Reviewed Human Health Assessments Containing Non-Cancer Toxicity Values (RfDs or MRLs) for PFAS that Are Either Final or Under Development (only final assessment values are provided)

Chemical	EPA Chronic Oral RfD	ATSDR Intermediate MRL[a]
PFOA	2016 RfD = 2 x 10^{-5} mg/kg/d; Draft updated toxicity assessment undergoing SAB review (EPA, 2021c)	2021 MRL = 3 x 10^{-6} mg/kg/d
PFOS	2016 RfD = 2 x 10^{-5} mg/kg/d; Draft toxicity assessment undergoing SAB review (EPA, 2021d)	2021 MRL = 2 x 10^{-6} mg/kg/d
PFNA	Under development in the EPA IRIS program	2021 MRL = 3 x 10^{-6} mg/kg/d
PFDA	Under development in the EPA IRIS program	N/A
PFBA	Public Comment and External Review Draft released August 2021	N/A
PFBS	2021 RfD = 3 x 10^{-4} mg/kg/d	N/A
PFHxA	Under development in the EPA IRIS program	N/A
PFHxS	Under development in the EPA IRIS program	2021 MRL = 2 x 10^{-5} mg/kg/d
GenX chemicals	2021 RfD = 3 x 10^{-6} mg/kg/d	N/A

[a] Note that MRLs and RfDs are not necessarily equivalent (e.g., intermediate duration MRL vs. chronic duration RfD; EPA and ATSDR may apply different uncertainty/modifying factors) and are developed for different purposes.
N/A = Not available

Some state health agencies publish toxicological assessments for PFAS that could potentially be used in HI calculations. For example, the Minnesota Department of Health publishes Toxicological Summaries that include the assessment of available toxicological information and subsequent development of oral toxicity values if adequate data are available (MN DOH, 2021). It should be noted that state or other (e.g., international) assessments may have varying levels of peer and public review and may reflect different risk assessment practices or policy choices as compared to EPA or ATSDR assessments.

There may be scenarios where a final peer-reviewed toxicity assessment for one or more component chemicals is not available for a mixture. In these cases, an evaluation of available hazard and dose-response information for PFAS in the mixture may be necessary under a HI approach. For instance, there may be a need to develop toxicity value(s) to estimate potential risks associated with site-specific/localized contamination from a PFAS mixture with a component(s) that may not be relevant to other areas, sites, or exposure sources, and/or has not been prioritized for assessment at the federal level. In such cases, the user of this framework might have a need to develop a targeted, fit-for-purpose assessment, if possible (i.e., based on availability of hazard and dose-response data, resources, and expertise). Excluding component PFAS that lack off-the-shelf toxicity values from further analysis could result in underestimation of the potential risk of the mixture. If de novo derivation of toxicity values is necessary, it is recommended that experts in hazard identification and dose response assessment be consulted, the associated uncertainties (e.g., data gaps) transparently characterized, and that the assessment undergoes independent external peer review. EPA has published several peer-reviewed guidance documents that may assist in efforts to derive chronic (or subchronic) oral RfDs for chemicals

with no available peer-reviewed toxicological assessment (for more information see EPA's Human Health Risk Assessment website at https://www.epa.gov/risk/human-health-risk-assessment).

To date, the majority of environmental chemicals including PFAS are data-poor, having no known or available information to inform hazard or dose-response in a screening/prioritization or assessment context. Considering that the number of legacy and new(er) chemicals present in commerce and the environment is in the tens of thousands, the generation of traditional animal toxicity data to support hazard identification and dose-response assessment would take decades and extraordinary numbers of animals and fiscal resources to complete. As human populations and biota are currently exposed to mixtures of chemicals such as PFAS, it is critical to identify methods, approaches, and platforms that can provide some reasonable context for potential human health hazard(s) and associated dose-response/potency for effects associated with exposure to multiples of PFAS (i.e., two or more co-occurring PFAS). A diverse set of resources has been developed over the past 15+ years that entails, in general, high(er)-throughput assays in cell culture (or cell free) systems, in silico computational prediction models, alternative animal species (e.g., zebrafish), and refined short-term laboratory rodent assays and databases and platforms to collate and deliver such data to end-users. These methods, assays, and platforms are collectively referred to as NAMs. In the absence of traditional animal bioassay and human epidemiological information, validated NAMs could potentially play a pivotal and transformational role in human health (and ecological) risk assessment, particularly in evaluating hazard and dose-response of PFAS that co-occur in mixtures.

Individually or in concert, NAMs such as toxicogenomics (e.g., HTTr, in vitro cell bioactivity) and in silico platforms (e.g., read-across) might inform identification or prediction of metrics that can be used in PFAS-specific hazard and dose-response assessment. For example, in vitro concentration-bioactivity data from resources such as ToxCast and Tox21 can be transformed into estimated in vivo exposure-response using in vitro to in vivo extrapolation (IVIVE) (Rotroff et al., 2010; Wambaugh et al., 2015; Wetmore et al., 2012, 2014). These administered human-equivalent dose datasets could potentially then be used to identify PODs (e.g., BMDs, NOAELs, LOAELs), with expert-driven application of appropriate uncertainty factors and corresponding non-cancer toxicity values. These NAM-based toxicity values could then be converted into corresponding HBWCs and used, with exposure data, to calculate HQs for data-poor PFAS.

A critical consideration in using NAM-based hazard and concentration/dose-response data is recognizing that for some high(er) throughput platforms or bioassays, perturbations of underlying biological pathways may not be readily identifiable as being directly related to specific apical toxic effects per se. That is, chemical exposures may elicit a myriad of perturbations or responses at the molecular, macromolecular, or cellular level, with some alterations being critical or key to eliciting an apical toxic effect level response, whereas many other alterations may seemingly have no relationship to toxic effect(s) (e.g., general stress, housekeeping). However, the dose-response relationship associated with non-apical perturbations or effects (e.g., cell-based bioactivity, transcriptomics) may be considered in an in vivo effect agnostic context. Specifically, although there may not be clear qualitative linkages between non-apical biological perturbations and a specific, apical tissue- or organ-level effect, corresponding dose-response relationships for biological perturbations have been shown to provide a reasonable quantitative approximation for dose-response (e.g., POD) associated with traditional apical

effects (Paul-Friedman et al., 2020; Johnson et al., 2020; Thomas et al., 2011, 2013). The implication for use of NAM data such as in vivo or in vitro cell-based bioactivity or transcriptomics, for example, is that pathway- or cell function-based response levels (e.g., effect concentration 50 (EC_{50}), inhibitory concentration 50 (IC_{50}), or other biologically supported response levels of interest), could potentially be leveraged and applied in the mixture component approaches proposed in this chapter (e.g., HI, RPF, mixture BMD), irrespective of direct linkage(s) to a phenotypic apical effect.

In summary, considering the lengthy and resource-intensive processes and study protocols (e.g., OECD Test Guidelines-type studies) typically involved in generating traditional repeat-dose bioassay data for human health assessment of chemicals, leveraging NAMs could potentially serve an important role for PFAS screening and assessment, including in a mixture context. It is recognized that practical application of NAMs in an assessment, whether for a single chemical or mixtures of chemicals, would be dependent on whether the results provide information that fits a decision context or purpose and this may not be intuitive. It is recommended that experts in NAM data interpretation be consulted for potential integration into mixtures screening/assessment to appropriately contextualize the applicability of results, and that they transparently communicate uncertainties associated with a given platform or assay output(s) in human health assessment.

4.3.1 Illustrative Example Application of Hazard Index to PFAS Mixtures: Screening-Level Hazard Index

As mentioned previously, in addition to PFOA and PFOS, final EPA human health assessments exist for PFBS and GenX chemicals. EPA has derived chronic oral RfDs for all four of these PFAS. Further, ATSDR has published a ToxProfile for several PFAS (ATSDR, 2021); beyond PFOA and PFOS, ATSDR has derived MRLs for PFNA and PFHxS (see Table 4-1). Many states and others (e.g., international entities) are addressing rapidly evolving PFAS issues under their respective purviews, including the development of toxicological assessment documents. Although there is overlap in the landscape of PFAS evaluated (or currently being evaluated) across federal, state, and international agencies, at the state/international level, there may be assessment values available for a broader array of PFAS; in the context of this framework, these will be collectively referred to as "PFAS 1" (Table 4-2). Several other PFAS have varying levels of human epidemiological and/or experimental animal toxicity information available, but do not currently (at the time of the drafting of this framework) have completed human health assessments or associated RfVs (e.g., EPA ORD is currently assessing PFBA, PFHxA, PFHxS, PFNA, and PFDA); any PFAS with available hazard and dose response data but no final RfV will be collectively referred to as "PFAS 2" (Table 4-2). Lastly, information coming from the tiered testing collaboration between EPA and NIEHS[10], or other sources of bioactivity data, and/or read-across may provide opportunities to calculate a value similar to an oral RfD when traditional human epidemiological and experimental animal study data are lacking; in the context of this framework, these PFAS will be collectively referred to as "PFAS 3" (Table 4-2). However, because there are no EPA-published HBWCs (e.g., Health Advisories, MCLGs) at this time for other PFAS with federal or state assessments/RfVs (e.g., PFBS (EPA), GenX chemicals (EPA), PFHxS (ATSDR), and PFNA (ATSDR)) or chemicals categorized under PFAS 1 or

[10] See: https://www.epa.gov/chemical-research/pfas-chemical-lists-and-tiered-testing-methods-descriptions#2

PFAS 2, these values would need to be calculated in order to develop component HQs and an overall PFAS mixture HI (Equation 4-1).

Table 4-2. Summary of Information Available for PFAS in the Hypothetical Screening HI Example

Chemical	Final U.S. Federal Assessment(s) with RfV Available	No Final U.S. Federal Assessment(s) with RfV; Final State or Other (e.g., International) Assessment(s) with RfV Available	No Final Assessment with RfV; Hazard and Dose-Response Information[a] Available	No Hazard and Dose-Response Information[a]; NAM Information Available	EPA Published HBWC (Health Advisory, MCLG)
PFOA, PFOS	X				X
EPA: PFBS, GenX chemicals; ATSDR: PFNA, PFHxS	X				
PFAS 1		X			
PFAS 2			X		
PFAS 3				X	

[a] Specifically refers to traditional human epidemiological and experimental animal study data.

The basic steps for calculating a *screening-level HI* for mixtures of PFAS with varying levels of available hazard and dose-response data are as follows:

Step 1. Identify Chronic Oral RfDs.

- **PFOA, PFOS, PFBS, GenX chemicals, PFHxS, and PFNA:** Review federal assessments containing oral RfDs or MRLs and select the appropriate values. Again, for screening-level HI, this will be the overall RfD (or MRL), regardless of underlying critical health effect. If an MRL is only available for an intermediate duration (akin to subchronic for EPA purposes), additional uncertainty may be considered for extrapolation to a corresponding chronic duration value.
- **PFAS 1:** No final U.S. federal assessments with RfV are available. Review state or other PFAS assessment documents for qualitative confidence in hazard identification and uncertainty(ies) in quantitative derivation of health/toxicity values for PFAS. Considerations should also include the level of rigor of peer-review associated with the published assessments/values.
- **PFAS 2:** No federal, state, or other assessments with RfV are available, but traditional hazard and dose-response (i.e., human epidemiological and/or experimental animal study) data are judged to support derivation. Thus, the user may choose to calculate a screening-level RfV using appropriate dose-response metrics (i.e., POD) and application of

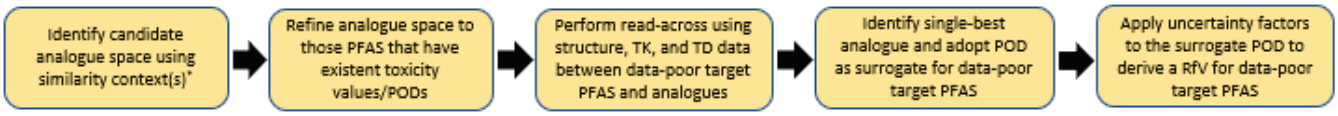
uncertainty factors. It is recommended that study hazard effect and dose-response data be systematically evaluated for suitability in supporting the derivation of screening-level RfVs using accepted EPA approaches and practice. Appropriate characterization and denoting of confidence and uncertainty(ies) in screening-level RfVs for PFAS in this category is imperative. Consultation with experts in the field and independent external peer review is recommended.

- **PFAS 3:** Because no final federal, state, or other RfD or MRL or traditional hazard and dose-response data are available, NAM data streams could be surveyed and leveraged for PFAS information that might facilitate development of a POD, and potentially, derivation of a NAM-based RfV using application of uncertainty factors consistent with the data scenario (Judson et al., 2011; Parish et al., 2020). It is recommended that data be systematically evaluated for suitability in supporting the derivation of screening-level RfVs using accepted EPA approaches and practice. Appropriate characterization and denoting of confidence and uncertainty(ies) in screening-level RfVs for PFAS in this category is imperative. Consultation with experts in the field of NAM data interpretation and risk assessment application and independent external peer review is recommended.

  Currently, the general process proposed for translating/integrating NAM into a human health assessment context is as follows for: (a) bioactivity (where "bioactivity" includes any/all cell-based assays/platforms, as well as HTTr); or (b) analogue-based read-across:

  (a) Bioactivity-based (e.g., ToxCast, Tox21; HTTr)

  (b) Read-across



*similarity contexts include structure/physicochemical, TK, and/or TD

Step 2. Identify or Calculate HBWCs.

- **PFOA/PFOS:** Review and select an HBWC for PFOA and PFOS from available sources (e.g., EPA, 2016c,d).
- **PFBS, GenX chemicals, PFHxS, PFNA, or species under PFAS 1, PFAS 2, or PFAS 3:** Calculate HBWCs using approach similar to that described in the 2016 Health Advisories for PFOA and PFOS (EPA, 2016c,d). Once all possible RfVs are assembled across PFAS mixture components, HBWCs are then derived where and when feasible.

Step 3. Select Exposure Estimates.

- Select appropriate exposure estimates from monitoring data.

Step 4. Calculate Screening-Level HI.

- Calculate individual component HQs for PFOA, PFOS, PFBS, GenX chemicals, PFHxS, PFNA, and/or species categorized under PFAS 1, 2, and 3, depending on the PFAS mixture of interest. In the drinking water context, HQs are first calculated as a ratio of the exposure concentration to the corresponding PFAS HBWC. The component PFAS HQs are then added together to determine the screening-level HI. HI values in exceedance of 1.0 indicates potential mixture hazard.

Application of the TOSHI is essentially identical to the steps for the screening-level HI. The critical nuance is that identification and assembly of human health/toxicity values are effect/endpoint specific. For some PFAS, this might entail the overall RfD or MRL; for other PFAS, this will involve TTDs (i.e., an RfD for a specific health effect). In the TOSHI, there is a greater likelihood that TTDs have not been derived for effects other than the critical effect that underpins the derivation of an overall RfD for a given PFAS. In those instances, TTDs could potentially be derived for other health effect domains but should be accomplished with transparent characterization of qualitative and quantitative uncertainties associated with hazard and dose-response data on a case-by-case basis.

4.3.1.1 Example 1: PFOA+PFOS

PFOA and PFOS are included in this illustrative example to demonstrate a screening-level HI approach for these chemicals specifically in the context of drinking water. For this example, EPA is relying on hypothetical exposure estimates and the 2016 EPA drinking water Health Advisories (Table 4-3) for PFOA and PFOS (EPA, 2016c,d). As stated previously, EPA finalized drinking water Health Advisories of 70 ng/L (ppt) for PFOA and PFOS, for the individual chemicals and when present as a mixture based on an assumption of dose additivity, because the RfDs were based on similar developmental effects and numerically identical (EPA, 2016c,d). [Note: EPA's draft updated toxicity assessments and MCLG documents for PFOA and PFOS are currently undergoing SAB review, and thus, these values are subject to change. These HI calculations would need to be updated following finalization of updated RfDs].

Table 4-3. Summary of Input Parameters Used to Calculate the 2016 EPA Health Advisories for PFOA and PFOS

Chemical	RfD; Critical Effect (Developmental)	Drinking Water Intake (DWI) Rate/Body Weight (L/kg-d)	Relative Source Contribution (RSC)[a]	2016 EPA Health Advisory (ng/L)[b]
PFOA	2×10^{-5} mg/kg/d; Reduced ossification of the proximal phalanges and accelerated puberty in mice (EPA, 2016c)	0.054[b]	0.2	70
PFOS	2×10^{-5} mg/kg/d; Decreased pup body weight in rats (EPA, 2016d)	0.054 [b]	0.2	70

[a] RSC is the portion of an exposure for the target (sub)population estimated to equal the RfD that is attributed to drinking water; the remainder of the exposure equal to the RfD is allocated to other potential sources (see EPA, 2016c,d for details regarding PFOA/PFOS RSC = 0.2).
[b] Health Advisory = (RfD/(DWI/bw)) x RSC.
[c] 0.054 L/kg/d is the 90th percentile consumers only estimate of combined direct and indirect community water ingestion for lactating women (see EPA, 2016c,d). This value has been updated with the publication of the 2019 edition of Chapter 3 of EPA's Exposure Factors Handbook (EPA, 2019a)

For illustration purposes, example screening-level HQs are calculated using hypothetical low (Table 4-4) and higher (Table 4-5) exposure estimates for drinking water and the 2016 EPA Health Advisories for PFOA and PFOS.

Table 4-4. Example Screening-Level HQs and HI for PFOA and PFOS at Low Water Concentrations

Chemical	Hypothetical Exposure Estimate (ng/L)[a]	2016 EPA Health Advisory (ng/L)	Example HQ[b]
PFOA	20	70	0.29
PFOS	20	70	0.29
SCREENING LEVEL HAZARD INDEX			**0.6**

[a] The hypothetical water exposure estimates provided represent a 5-fold increase over the minimum reporting level listed in UCMR 5 (https://www.epa.gov/dwucmr/fifth-unregulated-contaminant-monitoring-rule).
[b] HQ = Exposure Estimate/Example HBWC (in this case the 2016 EPA Health Advisory). HI = the sum of individual HQs.

Table 4-5. Example Screening-Level HQs and HI for PFOA and PFOS at Higher Water Concentrations

Chemical	Hypothetical Exposure Estimate (ng/L)	2016 EPA Health Advisory (ng/L)	Example HQ[b]
PFOA	400	70	5.7
PFOS	400	70	5.7
SCREENING LEVEL HAZARD INDEX			**11**

[a] The hypothetical water exposure estimates provided represent a 100-fold increase over the minimum reporting level listed in UCMR 5 (https://www.epa.gov/dwucmr/fifth-unregulated-contaminant-monitoring-rule).
[b] HQ = Exposure Estimate/Example HBWC (in this case the 2016 EPA Health Advisory). HI = the sum of individual HQs.

In the first hypothetical application of the screening-level HI approach, the HQs for PFOA and PFOS individually are below the threshold of 1.0, indicating hazard is below a level of concern for either PFAS in isolation. Further, when the PFOA HQ is summed with the PFOS HQ, the screening-level HI for the mixture is also less than 1.0 (Table 4-4). This indicates that the potential for health outcomes in exposed populations is not expected, at the low water concentrations indicated, for the mixture of [PFOA+PFOS].

In the second example, the HQs for PFOA and PFOS individually are above the threshold of 1.0, indicating a potential human health hazard associated with either PFAS in isolation. Further, when the PFOA HQ is summed with the PFOS HQ, the screening-level HI for the mixture is 11 (Table 4-5). This indicates that there is potential for health outcomes in exposed populations, at the higher water concentrations indicated, for the mixture of [PFOA+PFOS].

Please note that the magnitude of an HI, or an individual component HQ, should not be directly interpreted as a quantitative estimate of increased level of concern. For example, a mixture HI of 10.0 is not necessarily of 2-fold greater concern than a mixture HI of 5.0. The practical interpretation is that both mixtures would be of concern and should be prioritized for further evaluation. In the illustrative example of PFOA+PFOS only, further analysis would be needed to better understand the potential for health effects associated with PFOA and PFOS individually and their relative contributions to the combined toxicity associated with the mixture.

4.3.2 Advantages and Challenges of the Approach

The HI approach provides an indication of the combined toxicity associated with co-occurrence of PFAS in environmental media, such as drinking water. One advantage of the HI formula in risk communication is that interpretation of the results is relatively straightforward. The simplicity of the method is in taking a ratio of the exposure to hazard to indicate potential concern for a mixture of PFAS and providing an alert to specific PFAS that may be potential drivers in risk to human health (i.e., those PFAS where the HQs have greater contribution to a HI ≥1.0, relative to other PFAS members of the mixture).

Another advantage is that the "hazard" does not necessarily have to be the same for screening-level HI (e.g., all liver or all kidney effects). Specifically, the screening-level HI approach can be used where the individual HQ calculated for each mixture PFAS is based on the most well-characterized, and oftentimes most sensitive, toxic effect and corresponding non-cancer RfV

(e.g., oral RfD). As such, a screening-level HI will typically represent the most conservative indicator of mixture risk, as each component HQ is based on its health-protective RfV.

A disadvantage of this approach is that the HI is an indication of potential hazard, not an estimate of the concentration of the mixture in water that may result in adverse health outcomes after a specific period of exposure. Comparisons of HI estimates across different exposure scenarios can be misleading. Because the HI is based on DA, it implies that if two exposure scenarios involve the same chemicals and their HI values are the same, then with other factors being equal (e.g., exposure frequency and duration, similar endpoints, and similar receptor (exposed population) age), the two exposure scenarios could be judged to have the same potential for causing toxic effects. This interpretation has the strongest scientific foundation when there are only minor differences in the component exposures (thus, same exposure route, similar exposure duration for specific receptors, and roughly similar estimates of the individual HQs) between the two scenarios. Interpretation is more difficult when the underlying information is poor. For example, if the dominant chemical (highest HQ) has a highly uncertain exposure estimate, or its RfV was derived using a large UF, then the associated HI is also highly uncertain.

Another disadvantage of the application of this HI approach to specific media such as water is that it requires derivation of a health-based, media-specific concentration like a drinking water Health Advisory or MCLG, in addition to the underlying oral RfV (e.g., RfD). Development of these values requires significant expertise and resources often on a longer timeframe (i.e., years). In addition, while a formal hierarchy of preferred human health reference/toxicity values is not being proposed in this framework per se, there is a recognized gradation of confidence across the range of possible PFAS values. Specifically, it would clearly be preferable to use RfVs obtained from assessment sources that use transparent systematic approaches and standardized protocols and have been vetted in rigorous peer-review processes. The level of confidence or certainty in such values would be greater than RfVs deriving from questionable toxicity data sources, entails non-transparent decision-making, and/or is associated with higher levels of qualitative and quantitative uncertainty.

What might be perceived as a challenge for PFAS human health assessment in general could be an opportunity to advance risk assessment science and practice. Specifically, in the case of NAM, dose-response metrics obtained from read-across and/or bioactivity-based assays/platforms may inappropriately be assigned some level of a priori uncertainty simply because of lack of confidence by end-users in interpretation and risk assessment application of such data and outputs. As mentioned previously in this framework, NAM may represent the only opportunity to integrate a data-poor PFAS into mixtures assessment. The end-user of this framework, in consultation with experts/practitioners in NAM development and application, would be advised to leverage NAM when and where possible, but always characterizing and transparently communicating qualitative and quantitative uncertainty(ies) along the continuum from data generation and fit-for-purpose application (Parish et al., 2020) to screening-level RfV and subsequent HQ and HI calculations. The disadvantage to not using NAM data and approaches when applicable to a given PFAS mixture is that data-poor PFAS would not be accounted for in the HI, thus potentially underestimating mixture hazard.

In summary, in scenarios where a diverse amalgamation of different types of RfVs (i.e., deriving from different assessment sources and/or data types) are used in the calculation of HQs and HIs,

the respective confidence and qualitative uncertainty characterizations for each PFAS need to be transparently communicated in overall mixture hazard interpretations.

4.3.3 Target Organ Specific Hazard Index

In a TOSHI, toxicity values are aggregated by the "same" target organ endpoint/effect, and HQ (and HI) values are developed for each effect domain independently (e.g., liver-specific HI, thyroid-specific HI). The disadvantage of a TOSHI is that it can only be performed for those PFAS for which a health effect specific RfD (e.g., TTD) is calculated. For example, for some PFAS a given health effect might be poorly characterized or not studied at all, or, as a function of dose may be one of the less(er) potent effects in the profile of toxicity for that particular PFAS. Another limitation is that so many PFAS species lack human epidemiological or experimental animal hazard and dose-response information across a broad(er) effect range thus limiting derivation of TTD values. As with the screening-level HI, a TOSHI approach might benefit from consideration of NAM data and approaches that can inform organ/tissue-specific dose-response.

4.4 Relative Potency Factors

4.4.1 Basic Principle, Data Requirements to Calculate Relative Potency Factors and Corresponding Index Chemical Equivalent Concentrations

RPF approaches comprise the second basic dose-addition method used most commonly by EPA in mixtures assessment. There are two key types of the RPF approach: (1) the general RPF approach that has been applied to pesticides, disinfection by-products, and a few other chemical groups and (2) the TEF approach that was originally developed for mixtures of dioxins and DLCs. The TEF approach is considered a special case of the RPF approach wherein mixture components are known to act via an identical MOA (e.g., dioxins and DLCs and AhR activation).

For chemicals demonstrated to act via a similar MOA, or in the case of this framework, those shown to induce the same/similar health effect (see Section 3 for discussion and justification), an RPF represents the relative difference in potency between a mixture IC and other members of the mixture. The IC does not necessarily have to be the most potent member of a given mixture. Rather, an IC is typically selected because it has the highest quality or most robust toxicological database and is considered to be most representative of the type of toxicity caused by the mixture components (EPA, 1986, 2000). Further, the IC must have dose-response data for the dose range of interest; chemicals with steep slopes that cause effect and/or induce significant toxicity at all doses tested are not ideal for IC selection. In the RPF approach, the assumption under dose additivity is that the toxicity of each mixture component chemical induces effects via a similar pathway of biological perturbation and can operationally be considered a fixed concentration or dilution of the IC (EPA, 2000). Mathematically, when using response-specific doses, the RPF is the ratio of the IC to that of each individual mixture component chemical (j) at a common point on the corresponding dose-response curves (e.g., human equivalent NOAELs, BMDs, or ED_X). Ideally, the dose-response functions used to calculate RPFs across mixture components would be approximately the same in exposure duration and study design (e.g., sex, species, life stage). Further, considering the known differences in TK characteristics across PFAS (e.g., internal plasma half-life) between rodents, non-human primates, and humans, it is advisable to convert experimental animal dose-response data to human equivalents where possible before calculating RPFs. Lastly, of the options for dose-response metrics to use in the calculation of RPFs across

mixture PFAS, BMDs (i.e., the central tendency estimate) would be optimal. BMDs incorporate the totality of a given dose-response and facilitate identification of a dose at a pre-defined benchmark response level (e.g., 0.5SD or 1SD over control; 10% change in some effect/endpoint). BMD modeling would optimize comparison of "same" as a function of dose across mixture PFAS for a given health effect or endpoint. It is recognized that dose-response data for chemicals is sometimes not amenable to BMD modeling. Human equivalent NOAELs or ED_X values are perfectly suitable alternatives. No matter which dose-response metric is used, the RPF for the IC is always one. The potency ratio can be calculated for each mixture component chemical (j) as the ratio of the effect doses as shown in Equation 4-2:

$$RPF_j = \frac{ED10_{IC}}{ED10_j} \qquad (4\text{-}2)$$

where IC refers to the index chemical.

For example, if mixture component chemical 2 is twice as potent as the IC, its NOAEL, BMD_X, or ED_X will be half as large and the calculated RPF would be a 2. Conversely, if mixture component chemical 2 is half as potent as the IC, its NOAEL, BMD_X, or ED_X will be twice as large and the RPF would be 0.5. In practice, EPA determines a single RPF for the response range or dose range of interest. When data are available, RPFs can potentially be determined for more than one health effect domain and/or exposure scenario (e.g., developmental versus thyroid toxicity, shorter-term versus chronic exposure, oral versus inhalation exposure). As illustrated in the RPF examples in the next section, that flexibility or scenario specificity is an advantage of the general RPF approach. Once RPFs are calculated for each mixture component chemical using a common metric (e.g., human equivalent NOAEL, BMD_X, ED_X) in Equation 4-2, IC equivalent concentrations (ICEC) are then calculated by multiplying each respective RPF_j by the corresponding component chemical's concentration (d_j), as shown in Equation 4-3:

$$ICEC_{MIX} = \sum_{j=1}^{n} d_j * RPF_j \qquad (4\text{-}3)$$

The total mixture ICEC ($ICEC_{MIX}$) is then obtained by taking the sum of the component chemical ICECs (including that of the IC) (Equation 4-4). A numerical estimate of risk for non-cancer health effects associated with exposure to the mixture of concern is then obtained by mapping the $ICEC_{MIX}$ onto the dose-response function for the IC. For example, if the IC's dose-response model is denoted f(d), then the RPF-based response to the mixture is estimated as:

$$y_{MIX} = f(ICEC_{MIX}) \qquad (4\text{-}4)$$

where the ICEC is derived from Equation 4-3. In the context of this PFAS mixture framework, there are important modifications or adaptations of this approach to note that include: (1) use of Index Chemical Effect Concentrations (ICECs), which are water-specific, correlates to index chemical equivalent doses (ICEDs) (EPA, 2000) and (2) using effect-specific HBWCs for the IC (e.g., 70 ppt for PFOS-induced developmental effects (decreased body weight in offspring)) as a benchmark point to compare a mixture ICEC to rather than directly mapping the mixture ICEC onto the IC dose-response. This serves the purpose of providing the end-user a basic indication of "yes," there is potential effect-specific risk associated with the mixture (e.g., $ICEC_{MIX} \geq$ IC HBWC), or "no," there is no anticipated effect-specific risk (e.g., $ICEC_{MIX} \leq$ IC HBWC), as well

as magnitude of health effect concern and identification of potential component chemical drivers of an ICEC.

EPA's supplementary guidance (EPA, 2000) states: "The common mode-of-action assumption can be met using a surrogate of toxicological similarity, but for specific conditions (endpoint, route, duration)." This suggests that although the common MOA metric for application of RPFs is optimal, there is flexibility in the level of biological organization at which "similarity" can be determined among mixture components. To date, EPA has developed RPFs for only a few chemical groups, largely pesticides (organophosphorous pesticides, triazines, N-methyl carbamates, chloroacetanilides, and pyrethrins/pyrethroids), which in each case were based on MOA-level information (EPA, 2018). However, considering that PFAS are an emerging chemical class of concern, MOA data are limited or not available for many PFAS. As such, in the interim, when using the RPF approach, it is advisable to focus the biological level of organization for component-based evaluation of potential mixtures additivity for PFAS on similarity in toxicity endpoint/effect. This is the approach taken in the illustrative RPF examples below and is consistent with previous NAS recommendations pertaining to the evaluation of chemicals that cause common adverse health outcomes presumably through diverse biological pathways (NAS, 2008).

4.4.2 Illustrative Example Application of Relative Potency Factors and Index Chemical Equivalent Concentrations to PFAS Mixtures

The examples below demonstrate the application of RPF and ICEC calculations for PFOA, PFOS, PFBS, and GenX chemicals as these have existing final EPA hazard assessments and contain databases suitable for estimation of PODs for multiple health effect domains. Specifically, among the spectrum of adverse effects reported in these final EPA assessments, these PFAS have been shown to have dose-dependent effects on several common target organs/pathways including, but not limited to, developmental, thyroid, and liver effects (Table 4-6). These same three health outcome domains have also been reported as potential targets of other compounds within the broader class of PFAS (ATSDR, 2021; EFSA et al., 2020; ITRC, 2021). The approach here is to use a grouping construct that allows for combination of PFAS with shared, common apical effects (e.g., decreased pup body weight), as opposed to a stringent requirement of same MOA, to calculate RPFs across one or more health effect domains. Inclusion of multiple effects/domains among the constellation of PFAS effects allows for evaluation of the potential impact of differences in RPFs across PFAS in the mixture for those effects (e.g., the potency of PFOA relative to PFOS may be different for effects on the liver as compared to effects on the thyroid) (Mumtaz et al., 2021).

The intention is not necessarily to seek the most sensitive effects/domains; rather, it is to seek those that are shared among the PFAS in the mixture being assessed. However, for purposes of evaluating mixture risk using the RPF approach, it is critical to have an IC effect-specific HBWC so that the mixture ICEC can be compared to a benchmark point or dose. For PFAS, given the limited availability of hazard effect and dose-response data, if one seeks to include several PFAS (i.e., beyond those few congeners with robust toxicity databases) the approach may be limited to a single effect domain, or only those endpoints for which reasonable estimation of dose-response metrics (e.g., PODs, ED_X) for "same/similar" is possible.

Critically, this is an illustrative example only and does not represent final RPFs. In the present example, for each PFAS and each effect domain the lowest POD_{HED} was selected from studies included in final EPA human health assessments, with the exception of the thyroid effect endpoint for PFOA, which is based on preliminary data from the 2021 EPA ORD studies (Appendix A). It is possible that different endpoints may be appropriate for different combinations of PFAS and/or updated effect and dose-response data (i.e., BMDs) may become available. For the examples in this section, BMDs were not available across all PFAS and/or health effects; as such, human equivalent NOAELs (or LOAELs) were harvested from the respective completed human health assessments as the dose response metric for potency comparisons of health effect/endpoint (Table 4-6).

Table 4-6. Summary of Health Effects/Endpoints Selected for Illustrative RPF Examples

Chemical	Reference	Species/life stage-sex	POD_{HED}[a] (mg/kg/d)
DEVELOPMENTAL EFFECT: Decreased Pup Body Weight			
PFOA[b]	EPA, 2016a (Table 4-9); Wolf et al., 2007	Mouse; gestation days (GDs) 1–17	0.0109 (LOAEL)
PFOS[b]	EPA, 2016b (Table 4-9); Luebker et al., 2005c	Rat; 2 generation (84 days)	0.00051 (NOAEL)
PFBS	EPA, 2021a (Table 6); Feng et al., 2017	Mouse; neonatal female	0.21 (NOAEL)
GenX Chemicals	EPA, 2021b (Table 12); DuPont-18405-1037, 2010	Mouse; neonatal	0.07 (NOAEL)
THYROID EFFECT: Decreased Total T4 (TT4)			
PFOA[b]	ORD studies 2021 (see Appendix A)	Rat; PND2 neonatal male and female	0.24 (LOAEL)
PFOS[b]	EPA, 2016b (Section 3.4.1.5); Lau et al., 2003	Rat; neonatal male and female	0.24 (LOAEL)
PFBS	EPA, 2021a (Table 9); Feng et al., 2017	Mouse; PND 1 neonatal female	0.21 (NOAEL)
GenX Chemicals	EPA, 2021b (Table 12); Conley et al., 2021b	Rat; female maternal on post-pregnancy Day 2	7.0 (NOAEL)
LIVER EFFECT: Increase in Liver Weight[c]			
PFOA[b]	EPA, 2016a (Table 4-9); Perkins et al., 2004	Rat; male	0.0044 (NOAEL)
PFOS[b]	EPA, 2016b (Table 4-8); Seacat et al., 2003	Rat; male	0.0013 (NOAEL)
PFBS	EPA, 2021a (Table 6); 3M Company, 2001	Rat; male	72.0 (NOAEL)
GenX Chemicals	EPA, 2021b (Table 12); DuPont-18405-1037, 2010	Mouse; male and female	0.01 (NOAEL)

[a] Following EPA (2011) and EPA (2014) guidance, animal doses from selected studies were converted to HEDs through the application of a dosimetric adjustment factor (DAF), where HED = animal exposure dose × DAF.

[b] PFOA and PFOS health effects presented here, with the exception of the PFOA thyroid effect example, are drawn from EPA's 2016 health effects support documents (EPA, 2016a,b) and do not reflect the updated information currently under review by the

EPA SAB. The PFOA thyroid effect example relies on preliminary data from the 2021 EPA ORD studies (Appendix A) because this information was not available in the 2016 assessments.

[c] Includes absolute and/or relative liver weight(s).

4.4.2.1 Example 1: PFOA and PFOS Only

An example illustration of the application of the RPF approach to a mixture of PFOA and PFOS is provided below using information for PFOA and PFOS (Table 4-6) from the 2016 EPA health effects support documents (EPA, 2016a,b), preliminary thyroid effect data for PFOA (Appendix A), and hypothetical exposure estimates.

Step 1. Identify Mixture IC And Effect-Specific HBWCs.

As both PFOA and PFOS are well-characterized PFAS and share an identical Health Advisory, selection of either chemical as the IC would be reasonable regardless of toxicity effect/endpoint. For this example, we selected PFOS as the IC for developmental and thyroid effects; PFOA was selected as the IC for liver effects as it has a more well-informed database for this effect domain, compared to PFOS. The only currently available HBWC for PFOA and PFOS derives from the EPA 2016 Health Advisories, which are based on developmental effects (70 ppt). Effect-specific HBWCs are not available for thyroid or liver effects and would need to be calculated using an approach similar to that described in the EPA 2016 Health Advisory.

Step 2. Calculate RPFs And ICECs For Each Effect Domain.

The RPF for the IC is always 1. The developmental effect RPF in this example is calculated for an effect of decreased body weight in offspring. Both PFOA and PFOS were shown to decrease body weight in offspring (EPA, 2016a,b). However, while this effect was the basis for the 2016 RfD for PFOS, the PFOA RfD was based on a different developmental effect, i.e., reduced ossification of the proximal phalanges and accelerated puberty in mice, because that was the more sensitive effect. Offspring body weight decreases were selected as a common developmental effect for the purposes of this RPF illustration, and PFOS was selected as the IC. The developmental effect RPF for PFOA is calculated by dividing the PFOS POD_{HED} by the PFOA POD_{HED}, resulting in a RPF of 0.5. Each component RPF is multiplied by the corresponding chemical-specific estimated water exposure concentration to derive a PFOS ICEC (e.g., Table 4-7). The example developmental RPF Mixture Total PFOS ICECs (Table 4-7 and 4-8) are compared to the EPA 2016 Health Advisory for PFOS (70 ppt), which is based on the developmental effect of decreased body weight in offspring (EPA, 2016d). Effect-specific HBWCs are not available for PFOA or PFOS for the thyroid or liver, and so the illustrative examples for thyroid and liver stop at the point of RPF calculation (Tables 4-9 and 4-10).

Table 4-7. Example Developmental Effect RPFs and ICECs for a Mixture of PFOA and PFOS at Low Water Exposure Concentrations

Mixture Component	POD_{HED} (mg/kg/d); Decreased Body Weight in Offspring	Example RPF	Hypothetical Exposure Estimate (ng/L)[a]	PFOS ICEC (ng/L)
PFOA	0.001 ($NOAEL_{HED}$)[b, c] (EPA, 2016a)	0.5	20	10
PFOS (IC)	0.00051 ($NOAEL_{HED}$)[b] (EPA, 2016b)	1	20	20
Mixture Total PFOS ICEC (ppt)				**30**

[a] The hypothetical water exposure estimates provided represent a 5-fold increase over the minimum reporting level listed in UCMR 5 (EPA, 2021g).
[b] PFOA and PFOS health effects presented here are drawn from EPA's 2016 health effects support documents (EPA, 2016a,b) and does not reflect the updated information currently under review by the EPA SAB.
[c] The POD for PFOA-induced effects on pup body weight is a LOAEL; as such, a LOAEL-to-NOAEL uncertainty factor (UF_L) of 10 was applied to convert the POD to a NOAEL.

The PFOA+PFOS mixture equivalent water concentration is then compared to the IC HBWC for the specified effect or hazard domain (e.g., for this example, decreased body weight in offspring). In this case, the mixture total PFOS ICEC of 30 ppt does not exceed the PFOS HBWC of 70 ppt, indicating no potential risk of body weight effects in birthed offspring associated with exposure to a mixture of PFOA+PFOS at the hypothetical water exposure estimates provided.

At higher hypothetical water exposure estimates (Table 4-8), the mixture total PFOS ICEC of 600 ppt exceeds the PFOS HBWC of 70 ppt by nearly an order of magnitude, indicating potential risk of body weight effects in offspring associated with exposure to a mixture of PFOA+PFOS.

Table 4-8. Example Developmental Effect RPFs and ICECs for a Mixture of PFOA and PFOS at Higher Water Exposure Concentrations

Mixture Component	POD_{HED} (mg/kg/d); Decreased Body Weight in Offspring	Example RPF	Hypothetical Exposure Estimate (ng/L)[a]	PFOS ICEC (ng/L)
PFOA	0.001 ($NOAEL_{HED}$)[b, c] (EPA, 2016a)	0.5	400	200
PFOS (IC)	0.00051 ($NOAEL_{HED}$)[b] (EPA, 2016b)	1	400	400
Mixture Total PFOS ICEC (ppt)				**600**

[a] The hypothetical water exposure estimates provided represent a 100-fold increase over the minimum reporting level listed in UCMR 5 (EPA, 2021g).
[b] PFOA and PFOS health effects presented here are drawn from EPA's 2016 health effects support documents (EPA, 2016a,b) and does not reflect the updated information currently under review by the EPA SAB.
[c] The POD for PFOA-induced effects on pup body weight is a LOAEL; as such, a UF_L of 10 was applied to convert the POD to a NOAEL.

For thyroid effects (decreased total T4), PFOS is identified as the IC. The POD_{HEDs} are identical indicating equipotency between PFOA and PFOS for effect on total T4; thus, both RPFs are 1 (Table 4-9). There is currently no thyroid effect HBWC available for PFOS so this would need to be calculated following the steps described in Section 4.3.1 and compared to the PFOS ICEC to determine the potential risk of the mixture.

Table 4-9. Example Thyroid Effect RPFs for a Mixture of PFOA and PFOS

Mixture Component	POD_{HED} (mg/kg/d); Decrease in Total T4	Example RPF
PFOA	0.024 ($NOAEL_{HED}$)[a, b] (EPA, 2016a)	1
PFOS (IC)	0.024 ($NOAEL_{HED}$)[a, b] (EPA, 2016b)	1

[a]The PODs for PFOA- and PFOS-induced decrease in total T4 are LOAELs; as such, a UF_L of 10 was applied to convert the PODs to corresponding NOAELs.
[b] PFOA and PFOS health effects presented here are drawn from EPA's 2016 health effects support documents (EPA, 2016a,b) and does not reflect the updated information currently under review by the EPA SAB.

For liver effects (increased liver weight(s)), PFOA is identified as the IC. Based on the identified POD_{HEDs}, PFOS is approximately 3-fold more potent than PFOA for effects on liver weight (Table 4-10). There is currently no liver effect HBWC available for PFOA so this would need to be calculated following the steps described in Section 4.3.1 and compared to the PFOA ICEC to determine the potential risk of the mixture.

Table 4-10. Example Liver Effect RPFs for a Mixture of PFOA and PFOS

Mixture Component	POD_{HED} (mg/kg/d); Increase in Liver Weight	Example RPF
PFOA (IC)	0.0044 ($NOAEL_{HED}$) [a] (EPA, 2016a)	1
PFOS	0.0013 ($NOAEL_{HED}$) [a] (EPA, 2016b)	3

[a] PFOA and PFOS health effects presented here are drawn from EPA's 2016 health effects support documents (EPA, 2016a,b) and does not reflect the updated information currently under review by the EPA SAB.

4.4.2.2 Example 2: PFOA, PFOS, PFBS, and GenX Chemicals

Example application of the RPF approach to a mixture of PFOA, PFOS, PFBS, and GenX chemicals is provided below using information from EPA's 2016 Health Effects Support Documents for PFOA and PFOS (EPA, 2016a,b), preliminary thyroid effect data for PFOA from the 2021 EPA ORD study (Appendix A), final PFBS toxicological assessment (EPA, 2021a), final GenX chemicals toxicological assessment (EPA, 2021b), and hypothetical low or high(er) exposure estimates. Illustrative RPFs and corresponding ICECs are shown for a developmental health effect only, as the only HBWC available for comparison to the mixture total ICEC is for this effect domain (Tables 4-11 and 4-12). RPFs are presented for thyroid (Table 4-13) and liver

(Table 4-14) effects for the same four-component mixture, however mixture ICECs are not derived as there are no effect-specific HBWCs available for comparison purposes.

Table 4-11. Example Developmental Effect RPFs and ICECs for a Mixture of PFOA, PFOS, PFBS, and GenX Chemicals at Low Water Exposure Concentrations

Mixture Component	POD_{HED} (mg/kg/d); Decreased Body Weight in Offspring	Example RPF	Hypothetical Exposure Estimate (ng/L)[a]	PFOS ICEC (ng/L)
PFOA	0.001 ($NOAEL_{HED}$)[b, c] (EPA, 2016a)	0.5	20	10
PFOS (IC)	0.00051 ($NOAEL_{HED}$)[b] (EPA, 2016b)	1	20	20
PFBS	0.21 ($NOAEL_{HED}$) (EPA, 2021a)	0.002	15	0.04
GenX chemicals	0.07 ($NOAEL_{HED}$) (EPA, 2021b)	0.007	25	0.2
Mixture Total PFOS ICEC (ppt)				**30**

[a] The hypothetical water exposure estimates provided represent a 5-fold increase over the minimum reporting level listed in UCMR 5 (EPA, 2021g).
[b] PFOA and PFOS health effects presented here are drawn from EPA's 2016 health effects support documents (EPA, 2016a,b) and does not reflect the updated information currently under review by the EPA SAB.
[c] The POD for PFOA-induced effects on pup body weight is a LOAEL; as such, a UF_L of 10 was applied to convert the POD to a NOAEL.

In this illustrative example, the addition of PFBS and GenX chemicals to the example mixture of PFOA+PFOS, at the hypothetical low exposure concentrations, did not meaningfully impact the overall PFOA ICEC for developmental effects. This is primarily a function of significantly lower potency of PFBS and GenX chemicals for effects on body weight in offspring, compared to PFOA and PFOS. In this case, the total mixture PFOS ICEC of 30 ppt does not exceed the PFOS HBWC of 70 ppt, indicating minor concern for risk of body weight effects in offspring associated with exposure to the four PFAS mixture at the hypothetical low water exposure estimates provided.

Similarly, the addition of PFBS and GenX chemicals to the example mixture of PFOA+PFOS, at the hypothetical higher water exposure concentrations (Table 4-12), did not meaningfully impact the overall PFOS ICEC for developmental effects. Again, this is primarily a function of significantly lower potency of PFBS and GenX chemicals for effects on body weight in offspring, compared to PFOA and PFOS. In this case, the total mixture PFOS ICEC of 603 ppt exceeds the PFOS HBWC of 70 ppt by nearly an order of magnitude, indicating the potential risk of body weight effects in offspring. Further, based on the PFOS ICECs, both PFOA and PFOS are significant drivers for the mixture risk to offspring (200 and 400 ng/L, respectively).

Table 4-12. Example Developmental Effect RPFs and ICECs for a Mixture of PFOA, PFOS, PFBS, and GenX Chemicals at Higher Water Exposure Concentrations

Mixture Component	POD_{HED} (mg/kg/d); Decreased Body Weight in Offspring	Example RPF	Hypothetical Exposure Estimate (ng/L)[a]	PFOS ICEC (ng/L)
PFOA	0.001 ($NOAEL_{HED}$)[b] (EPA, 2016a)	0.5	400	200
PFOS (IC)	0.00051 ($NOAEL_{HED}$); (EPA, 2016b)	1	400	400
PFBS	0.21 ($NOAEL_{HED}$); (EPA, 2021a)	0.002	300	0.7
GenX chemicals	0.07 ($NOAEL_{HED}$) (EPA, 2021b)	0.007	500	2.1
Mixture Total PFOS ICEC (ppt)				**603**

[a] The hypothetical water exposure estimates provided represent a 100-fold increase over the minimum reporting level listed in UCMR 5 (EPA, 2021e).
[b] The POD for PFOA-induced effects on pup body weight is a LOAEL; as such, a UF_L of 10 was applied to convert the POD to a NOAEL.

Based on the POD_{HED}s for thyroid effect (i.e., decreased total T4) across the four-component mixture, PFOA and PFOS are approximately 1-2 orders of magnitude more potent than PFBS or GenX chemicals, and are reflected in the corresponding RPFs (Table 4-13). As such, at approximately equivalent water concentrations, in mixture, PFOA and PFOS would contribute equipotently to an effect on total T4, whereas PFBS would contribute 1/10th of the mixture potency, and the contribution of GenX chemicals to thyroid effect would be virtually negligible.

Table 4-13. Example Thyroid Effect RPFs for a Mixture of PFOA, PFOS, PFBS, and GenX Chemicals

Mixture Component	POD_{HED} (mg/kg/d); Decrease in Total T4	Example RPF
PFOA	0.024 ($NOAEL_{HED}$)[a] (ORD studies 2021; see Appendix A)	1
PFOS (IC)	0.024 ($NOAEL_{HED}$)[a] (EPA, 2016b)	1
PFBS	0.21 ($NOAEL_{HED}$) (EPA, 2021a)	0.1
GenX chemicals	7.0 ($NOAEL_{HED}$) (EPA, 2021b)	0.003

[a] The PODs for PFOA- and PFOS-induced decrease in total T4 are LOAELs; as such, a UF_L of 10 was applied to convert the PODs to corresponding NOAELs.

Based on the POD_{HED}s for liver effect (i.e., increased liver weight) across the four-component mixture, GenX chemicals are approximately 4-fold more potent and PFOS is approximately 3-fold more potent than PFOA for this effect (Table 4-14). The contribution of PFBS is effectively

negligible in the mixture. At approximately equivalent water concentrations, GenX chemicals and PFOS might be significant drivers for mixture risk of liver weight effects.

Table 4-14. Example Liver Effect RPFs for a Mixture of PFOA, PFOS, PFBS, and GenX Chemicals

Mixture Component	POD_{HED} (mg/kg/d); Increase in Liver Weight	Example RPF
PFOA (IC)	0.0044 ($NOAEL_{HED}$) (EPA, 2016a)	1
PFOS	0.0013 ($NOAEL_{HED}$) (EPA, 2016b)	3
PFBS	7.2 ($NOAEL_{HED}$) [a] (EPA, 2021a)	0.0006
GenX chemicals	0.001 ($NOAEL_{HED}$)[a] (EPA, 2021b)	4

[a] The PODs for PFBS and GenX chemicals-induced effects on increase in liver weight are derived from subchronic studies; as such, a UF_S of 10 was applied to convert the PODs to corresponding "chronic duration" NOAELs.

As illustrated in the RPF examples above, PFAS can have different potencies across health effect domains. Due to differences in both TKs and TDs, PFAS may exhibit complex gradations of potency for different effects, and this will be reflected in the corresponding RPFs. Some PFAS may be exquisitely potent for some effects and yet virtually inactive in others, however expanding the number of PFAS and the toxicity endpoint profiles across the structural landscape will be key to illustrating such a diversity in relative potency. Further, another critical consideration illustrated in the RPF examples is the impact of component chemical concentrations. That is, in practical field application, PFAS concentrations in water, soil, or air may be drastically different dependent on a number of factors (e.g., different physicochemical and environmental fate and transport properties; proximity to PFAS manufacturing or use locales; water sources (well water vs. finished drinking water); waste handling). In application, transparent presentation and communication of hazard and dose-response data sources, RPFs, media concentrations, ICECs, and any associated uncertainties, across as many health effect domains as is practicable is ideal for RPF-based evaluation of PFAS mixtures. As mentioned previously, a limitation for PFAS is the availability of human health assessment grade toxicity data; Section 4.5 offers an alternative to the RPF approach in such a scenario.

4.4.3 Advantages and Challenges of the Relative Potency Factor Approach

A significant advantage of the RPF approach is that formal toxicity or RfV derivation is not necessary for the component chemicals. Rather, only effects/endpoints and associated dose-response metrics (e.g., NOAEL, BMD_X, ED_X) are needed to perform the exercise. While it would be ideal to conduct potency comparisons between mixture components for same effect/endpoint using same dose metrics from same study design/durations, calculation of RPFs across PFAS may in practical application entail (i.e., necessitate) use of effect data deriving from diverse study designs and exposure durations. As such, in some cases, there may be a need to selectively apply uncertainty factors in the RPF method, in particular, the LOAEL-to-NOAEL (UF_L) and/or subchronic-to-chronic duration (UF_S) factors. For example, in many of the

illustrative RPF calculations in this chapter, the effect data for PFBS, GenX chemicals, PFOA, and PFOS came from a mixture of reproductive/developmental study design in mice (e.g., GDs 1-20), less than lifetime repeat-dose (e.g., 28- or 90-days in rats), and/or 2-year bioassays in rats (e.g., PFOA, PFOS). For the expressed purpose of deriving RPFs, applying a UF_S of 10 to convert a subchronic $NOAEL_{HED}$ to a corresponding chronic $NOAEL_{HED}$, or, converting a $LOAEL_{HED}$ to a $NOAEL_{HED}$, provides the opportunity for a more 1:1 comparison of potency for a given effect (e.g., developmental body weight, increase in liver weight) between PFOA, PFOS, PFBS, and GenX chemicals. In practice, users of this framework document may find the need to apply UF(s) consistent with the examples summarized here. A critical facet to this is to be transparent about such POD adjustments (i.e., purpose/rationale) when applied.

RPFs were generally intended for use when mixture components are demonstrated to have similar/same MOA. This presents a problem as it pertains to practical application of RPF methodology in that a vast majority of environmental chemicals have limited-to-no MOA data available. This is particularly true of PFAS as an emerging chemical class of concern. EPA mixtures guidance does provide flexibility in use of data from different levels of biological organization in dose additive approaches such as RPF. As demonstrated in this framework document, this flexibility is an advantage in that there is greater probability of identifying effect/endpoint and associated dose-response data (e.g., effect-specific PODs) for mixture components than there is for MOA type data. However, as the data for PFAS evolve over time, the toxicity profiles including number of effect types and granularity of biological perturbations (e.g., potential KE data that inform proposed MOA(s)) may eventually support MOA-based evaluations.

Another advantage is that the RPF method facilitates calculation of an actual mixture toxicity dose or concentration estimate, as opposed to the HI which is considered an indicator of potential hazard/toxicity. Although a given mixture ICEC is traditionally mapped to the IC's effect-specific dose-response function to arrive at a corresponding "mixture response," an advantage of the RPF approach is that the mixture ICEC may alternatively be used to inform mixture risk in the context of the relationship to a media-specific health-based value (such as a HBWC).

A clear challenge, not uniquely associated with the RPF approach per se, is the use of potentially disparate hazard and dose-response data across mixture components. The implicit assumption for dose-response data selection in the calculation of RPFs in this tiered framework, is that the same dose-response data that underpinned the derivation of corresponding RfVs (overall RfDs or effect-specific TTDs) for use as input(s) for HQs and HIs (i.e., Tier 1) would also be leveraged in Tier 2 (be it for RPF and/or Mixture BMD approaches (see Section 4.5)). However, although ideal, this is not an expressed requirement of the framework. The user should be afforded the flexibility to make decisions regarding suitable dose-response selection for RPF calculations on a case-by-case basis. Key to this flexibility is transparent characterization and communication of literature searching strategy and review results, hazard data selection, dose-response evaluation (e.g., BMD preferred, effect levels such as NOAELs are acceptable), and qualitative and quantitative uncertainties or confidence in what could potentially be a diverse assembly of data/metrics to support RPF application(s).

An additional potential challenge, that actually may present an opportunity for advancing the science of mixtures risk assessment is the use of NAM data. The constantly evolving information coming from alternative toxicity testing assays and platforms may be of paramount importance

to human health assessment of environmental chemicals in general (not just for mixtures applications), however there are inherent challenges associated with application to hazard identification and dose-response assessment. In a PFAS mixtures assessment context, for some mixture component chemicals, NAM data (e.g., read-across or cell-based bioactivity (such as ToxCast and/or Tox21)) might be the only source(s) of evidence available to inform an RPF approach. The challenge might then be identifying and assembling "same" or "similar" effect/endpoint data compared to other PFAS in the mixture that have human epidemiological and/or experimental animal (i.e., apical (phenotypic) effect level) bioassay data. While the RPF approach affords flexibility in selection of "effect" data, a key requirement is that the "effect" on which RPFs are based be the same. For example, one mixture PFAS may have histopathological evidence of multi-focal liver necrosis from in vivo repeat-dose rat studies, whereas another PFAS may have evidence of cytochrome c release, mitochondrial damage, and cell death in in vitro rat hepatocyte cell culture studies only. While the NAM data clearly demonstrate hallmarks of cellular demise typically associated with necrotic (and apoptotic) cell death, pathologically consistent with cell death foci observed in whole rat liver, it may be difficult to make the case that the in vitro-based concentration-response data (converted to an administered equivalent dose) is suitable for traditional RPF calculation simply based on the optics of "same" effect. Further investigation is needed to investigate the qualitative and quantitative merits of applying hazard and dose-response data from across different levels of biological organization in a component-based mixtures assessment context.

4.5 Dose Addition Mixture BMD Approach

4.5.1 Basic Principle, Data Requirements for Mixture Benchmark Doses and Corresponding Mixture Health-Based Water Concentrations

Both the HI/Quotient and RPF steps described above require published or user-derived human health assessment values, such as oral RfDs or individual chemical HBWCs. For example, in the HI approach, these metrics serve as the denominator in determining if the exposure exceeds a level estimated to be acceptable for human intake. In some cases, a PFAS mixture may contain component chemicals that do not have corresponding human health assessment values (e.g., RfDs) for use in mixture-based risk calculation. Further, for the RPF approach, for a given effect domain being considered, there may not be a corresponding dose-response assessment value (e.g., POD_{HED}) for the common effect being used to derive relative potencies. In these cases, a third approach, described in EPA supplementary guidance (2000) (Section 4.2.6) and NAS (2008) (Appendix C), employs a DA model-based calculation of a mixture BMD based on a defined benchmark response (e.g., ED_{10}) for a PFAS mixture with a specific mixing-ratio of component chemicals. As previously discussed, DA has broadly been viewed as the most appropriate model for estimating combined effects of "toxicologically similar" compounds. The rationale for the use of DA as a default model for estimating combined effects of exposure to multiple PFAS is reviewed in Section 3.

DA modeling of a PFAS mixture requires empirical data-driven or reasonable estimation (e.g., read-across between structures) of effect-equivalent endpoints for all PFAS in the mixture. Importantly, the endpoint selected must be the same for all PFAS included in the calculation, for example BMD_{10}s for the same liver effect, or NOAELs for the same developmental effect. The model output will produce and equivalent metric (i.e., BMD_{10} or NOAEL) for the specific total mixture of PFAS being evaluated. Effect equivalent BMDs are more statistically robust and the

equation explanation and example below will reference BMD_X as the model components using Equation 4-5, where t_{add} is the total mixture dose in mg/kg/d, a_i are the fixed proportions of the component PFAS in the mixture, and BMD_i is i^{th} chemical BMD (e.g., a BMD_X). Similar to the RPF approach above, due to the potential for different effect domains to have variable potencies across PFAS within a given mixture, the DA model should be applied across more than one effect domain for which data are available for each of the PFAS in the mixture to identify the lowest mixture-specific endpoint, which indicates the most sensitive domain.

$$t_{add} = \left(\sum_{i=1}^{n} \frac{a_i}{BMD_i}\right)^{-1} \quad (4\text{-}5)$$

4.5.2 Illustrative Example Application of Mixture Benchmark Doses and Mixture Health-Based Water Concentrations

An example is described here and in Tables 4-15 and 4-16 for two hypothetical mixtures of four different PFAS. The two samples have the same four PFAS, which have existing dose response data on liver weight (i.e., liver endpoint), pup body weight (i.e., developmental endpoint), and reduced serum thyroid hormone concentrations (i.e., thyroid endpoint) in rodent models for each compound. The difference in the two samples are the specific concentrations of each of the four PFAS and therefore the mixing ratios and the overall total mixture PFAS concentration. Dose responses for each chemical and each endpoint are modeled and effect-appropriate BMDs calculated for each compound (e.g., ED_5, ED_{10}, ED_{20}). These values serve as the denominator values in Equation 4-5. The numerator values are the proportions of each component PFAS in the precise mixture. The total mixture BMD (t_{add}) is the inverse of the sum of the relative ratio divided by the BMD_X for each PFAS in the mixture. The total mixture BMD_X represents an equivalent BMD_X as each of the individual chemical effects that were used in the calculations (i.e., if the individual chemical data were ED_{10} values, the DA calculation derives an ED_{10} for the mixture of PFAS with that specific mixing ratio).

The total mixture BMD, which is in the same units as the component chemical BMDs (e.g., oral dose in a rodent study such as mg/kg/d), can then be adjusted based on user-defined extrapolation factors (e.g., dosimetric adjustment, RSC correction, UFs, and life stage-specific drinking water consumption rates) to derive a unique HBWC for the total PFAS mixture (as opposed to an IC-specific HBWC as in the RPF approach). The derived "mixture-HBWC" can then be compared to the actual (measured) mixture concentration and if the actual mixture concentration exceeds the mixture-HBWC there is risk of the specific effect from exposure to that mixture at the measured concentrations.

In practice, the lowest mixture-specific endpoint indicates the most sensitive domain for the mixture and this endpoint can then be used for the derivation of an equivalent mixture-HBWC and estimation of risk. In the present example, for Water Sample 1 (Table 4-15) the liver specific domain produced the lowest mixture BMD (i.e., 0.094 mg/kg/d), representing the most sensitive effect domain. This mixture dose could then be used to derive a mixture-HBWC for comparison to the actual mixture concentration of the sample (i.e., 470 ng/L). If the mixture-HBWC is greater than 470 ng/L, in this instance, then there is potential risk of liver effects in the exposed population. For Water Sample 2 (Table 4-16), the different mixing ratio resulted in the

developmental domain having the lowest mixture BMD (0.0068 mg/kg/d) and this would be the mixture BMD used to calculate the mixture-HBWC and for comparison to the measured water concentration (70 ng/L).

Table 4-15. Mixture BMD Approach: Hypothetical Water Sample 1

	Measured Water Concentration (ng/L)	Mixing Ratio (Proportion)	Thyroid BMD (mg/kg/d)	Liver BMD (mg/kg/d)	Developmental BMD (mg/kg/d)
PFAS 1	10	0.02	0.24	0.044	0.01
PFAS 2	10	0.02	0.24	0.013	0.0051
PFAS 3	50	0.11	2.1	720	2.1
PFAS 4	400	0.85	70	0.1	0.7
Mixture Total	470	1.0			
DA Mixture BMD Calculation			4.16	**0.094***	0.132

*The lowest mixture BMD is converted to a mixture-HBWC for comparison to the measured concentration (i.e., 470 ng/L).

Application of Equation 4-5 to the example water sample in Table 4-15 to derive the DA Mixture BMD. This example is for the liver domain as it was the lowest mixture BMD in this example.

$$t_{add} = \left(\sum_{i=1}^{4} \frac{a_i}{BMD_i}\right)^{-1} = \left(\frac{0.02}{0.044} + \frac{0.02}{0.013} + \frac{0.11}{720} + \frac{0.85}{0.1}\right)^{-1} = 0.094 \text{ mg/kg/d}$$

Table 4-16. Mixture BMD Approach: Hypothetical Water Sample 2

	Measured Water Concentration (ng/L)	Mixing Ratio (Proportion)	Thyroid BMD (mg/kg/d)	Liver BMD (mg/kg/d)	Developmental BMD (mg/kg/d)
PFAS 1	5	0.07	0.24	0.044	0.01
PFAS 2	50	0.71	0.24	0.013	0.0051
PFAS 3	10	0.14	2.1	720	2.1
PFAS 4	5	0.07	70	0.1	0.7
Mixture Total	70	1.0			
DA Mixture BMD Calculation			0.299	0.017	**0.0068***

*The lowest mixture BMD is converted to a mixture-HBWC for comparison to the measured concentration (i.e., 70 ng/L).

4.5.3 Advantages and Challenges of the Mixture Benchmark Dose Approach

There are several advantages to the Mixture BMD approach. First, there is no a priori requirement for having formal human health assessment values, such as oral RfDs or chemical-specific HBWCs, for any of the individual PFAS in the mixture. The only data needs are effect endpoints (i.e., BMDs or NOAELs) for each of the PFAS in the mixture for the common endpoint(s) being modeled. Another advantage is that it avoids any potential confusion that could arise from putting the mixture POD in the units of a single chemical (i.e., the IC from the RPF approach). Rather, the end result is a mixture POD that is specific for the assortment and ratios of PFAS in the mixture being evaluated. It is important to recognize that the DA model calculation of combined mixture effect (mixture BMD) is different for each PFAS mixture depending on: (1) the specific PFAS in the mixture, (2) the mixing ratio, and (3) the effect endpoint being modeled. For example, one could expect that a mixture of PFAS that has a greater amount of a more potent compound and a lower amount of a less potent compound would have a lower (i.e., more potent) mixture BMD than a similar assortment of compounds that has less of the more potent PFAS and more of the less potent PFAS. It is also advantageous that the Mixture BMD approach does not actually require or assume that the component PFAS in a given mixture have congruent dose response curves for each effect being evaluated (reviewed in NAS (2008)). Finally, it is ideal to have well resolved dose response curves for each component PFAS in a mixture to estimate equivalent BMDs (e.g., ED_{10}); however, DA modeling is also amenable to simple point estimates such as NOAELs, as long as they are toxicologically similar across component chemicals (i.e., for same endpoint, such as increased liver weight).

There are also several challenges with the Mixture BMD approach. Like the RPF approach, the user needs effect data for at least one common endpoint from the constellation of PFAS effects for all components of the mixture. For certain mixtures with less well-studied PFAS there may be limited or no available dose response data comparable to other PFAS in the mixture for calculating the mixture BMD. In that case, similar to the RPF approach, read-across or NAM data may be the only source of data for estimating an effect endpoint and with associated uncertainty. Another challenge is that the mixture BMD and subsequent mixture-HBWC is unique for each specific mixture based on PFAS assortment and ratios, which is conceptually different from the classical approach of a single value specific to a single chemical (e.g., an RfD for a specific compound). Further, it is assumed that the PFAS mixture composition is fairly constant, when in actuality, PFAS mixtures may change over time in environmental media. However, the calculation can readily be repeated for different mixing ratios and mixture concentrations once the effect endpoint values have been determined. Finally, for both the RPF and Mixture BMD approach, depending on data availability for the individual compounds, the effect domains modeled may potentially not be the overall most sensitive out of the total constellation of common PFAS effects (e.g., in reality liver effects are the most sensitive and would produce the lowest mixture BMD, but data are only available for the component PFAS to calculate mixture BMDs for developmental and thyroid effects).

References

3M Company. 2000. *Voluntary Use and Exposure Information Profile Perfluorooctanic Acid and Salts*. EPA Administrative Record AR226-0595. EPA, Washington, DC.

3M Company. 2001. *A 28-day oral (gavage) toxicity study of T-7485 in Sprague-Dawley rats.* Study Number 132-007. 3M Corporate Toxicology, St. Paul, MN.

Abbott, B.D., C.J. Wolf, J.E. Schmid, K.P. Das, R.D. Zehr, L. Helfant, S. Nakayama, A.B. Lindstrom, M.J. Strynar, and C. Lau. 2007. Perfluorooctanoic acid induced developmental toxicity in the mouse is dependent on expression of peroxisome proliferator activated receptor-alpha. *Toxicological Sciences* 98(2):571–581.

Abbott, B.D., C.J. Wolf, K.P. Das, R.D. Zehr, J.E. Schmid, A.B. Lindstrom, M.J. Strynar, and C. Lau. 2009. Developmental toxicity of perfluorooctane sulfonate (PFOS) is not dependent on expression of peroxisome proliferator activated receptor-alpha (PPAR alpha) in the mouse. *Reproductive Toxicology* 27(3-4):258–265.

Ahrens, L. 2011. Polyfluoroalkyl compounds in the aquatic environment: a review of their occurrence and fate. *Journal of Environmental Monitoring* 13(1):20–31.

Alaska DEC (Alaska Department of Environmental Conservation). 2019. *Technical Memorandum Action Levels for PFAS in Water and Guidance on Sampling Groundwater and Drinking Water.* AK DEC, Division of Spill Prevention and Response Contaminated Sites Program and Division of Environmental Health Drinking Water Program. https://dec.alaska.gov/media/15773/pfas-drinking-water-action-levels-technical-memorandum-10-2-19.pdf.

Altenburger, R., T. Backhaus, W. Boedeker, M. Faust, M. Scholze, and L.H. Grimme. 2000. Predictability of the toxicity of multiple chemical mixtures to *Vibrio fischeri*: mixtures composed of similarly acting chemicals. *Environmental Toxicology and Chemistry: An International Journal* 19(9):2341–2347.

Anderson, R.H., G.C. Long, R.C. Porter, and J.K. Anderson. 2016. Occurrence of select perfluoroalkyl substances at U.S. Air Force aqueous film-forming foam release sites other than fire-training areas: field-validation of critical fate and transport properties. *Chemosphere* 150:678–685.

ATSDR (Agency for Toxic Substances and Disease Registry). 2018. *Interaction Profile for Mixtures of Insecticides: Pyrethroids, Organophosphorus Compounds, and Carbamates.* U.S. Department of Health and Human Services, Public Health Service, ATSDR, Atlanta, GA.

ATSDR (Agency for Toxic Substances and Disease Registry). 2021. *Toxicological Profile for Perfluoroalkyls*. U.S. Department of Health and Human Services, Public Health Service, ATSDR, Atlanta, GA.

Australian Government Department of Health. 2019. *Health Based Guidance Values for PFAS.* https://www1.health.gov.au/internet/main/publishing.nsf/Content/2200FE086D480353CA2580C900817CDC/$File/HBGV-Factsheet-20190911.pdf.

Bailey, D.G. 2013. Grapefruit-medication interactions. *CMAJ* 185(6):507–508.

Beach, S.A., J.L. Newsted, K. Coady and J.P. Giesy. 2006. Ecotoxicological evaluation of perfluorooctanesulfonate (PFOS). *Reviews of Environmental Contamination and Toxicology* 186:133–174.

Beckett, K. J., Yamini, B., and Bursian, S. J. 2008. The effects of 3,3',4,4',5-pentachlorobiphenyl (PCB 126) on mink (Mustela vison) reproduction and kit survivability and growth. *Archives of Environmental Contamination and Toxicology* 54:123-129.

Behr, A.C., C. Plinsch, A. Braeuning, and T. Buhrke. 2020. Activation of human nuclear receptors by perfluoroalkylated substances (PFAS). *Toxicology In Vitro* 62:104700.

Benskin, J.P., D.C. Muir, B.F. Scott, C. Spencer, A.O. De Silva, H. Kylin, J.W. Martin, A. Morris, R. Lohmann, G. Tomy, B. Rosenberg, S. Taniyasu, and N. Yamashita. 2012. Perfluoroalkyl acids in the Atlantic and Canadian Arctic Oceans. *Environmental Science & Technology* 46(11):5815–5823.

Bil, W., M. Zeilmaker, S. Fragki, J. Lijzen, E. Vergruggen, and B. Bokkers. 2020. Risk assessment of per- and polyfluoroalkyl substance mixtures: a relative potency factor approach. *Environmental Toxicology and Chemistry* 40(4): 859-870. doi:10.1002/etc.4835.

Bishop, C.A., P. Ng, K.E. Petit, S.W. Kennedy, J.J. Stegeman, R.J. Norstrom, and R.J. Brooks. 1998. Environmental contamination and developmental abnormalities in eggs and hatchlings of the common snapping turtle (Chelydra serpentina serpentina) from the Great Lakes-St Lawrence River basin (1989-1991). *Environmental Pollution* 101(1):143-156.

Bjork, J.A., C. Lau, S.C. Chang, J.L. Butenhoff, and K.B. Wallace. 2008. Perfluorooctane sulfonate-induced changes in fetal rat liver gene expression. *Toxicology* 251(1-3):8–20.

Blake, B.E., H.A. Cope, S.M. Hall, R.D. Keys, B.W. Mahler, J. McCord, B. Scott, H.M. Stapleton, M.J. Strynar, S.A. Elmore and S.E. Fenton. 2020. Evaluation of maternal, embryo, and placental effects in CD-1 mice following gestational exposure to perfluorooctanoic acid (PFOA) or hexafluoropropylene oxide dimer acid (HFPO-DA or GenX). *Environmental Health Perspectives* 128(2):27006.

Bliss, C.I. 1939. The toxicity of poisons applied jointly. *Annals of Applied Biology* 26:585−615.

Boobis, A., R. Budinsky, S. Collie, K. Crofton, M. Embry, S. Felter, R. Hertzberg, D. Kopp, G. Mihlan, M. Mumtaz, P. Price, K. Solomon, L. Teuschler, R. Yang, and R. Zaleski. 2011. Critical analysis of literature on low-dose synergy for use in screening chemical mixtures for risk assessment. *Critical Reviews in Toxicology* 41(5):369–383.

Borg, D., B-E. Lund, N-G. Lindquist, and H. Hakansson. 2013. Cumulative health risk assessment of 17 perfluoroalkylated and polyfluoroalkylated substances (PFASs) in the Swedish population. *Environment International* 59:112-123.

Boulanger, B., J. Vargo, J. L. Schnoor, and K. C. Hornbuckle. 2004. Detection of perfluorooctane surfactants in Great Lakes water. *Environmental Science & Technology* 38(15):4064–4070.

Brendel S., É. Fetter, C. Staude, L. Vierke, and A. Biegel-Engler. 2018. Short-chain perfluoroalkyl acids: environmental concerns and a regulatory strategy under REACH. *Environmental Sciences Europe* 30(1):9.

Buck, R.C., J. Franklin, U. Berger, J.M. Conder, I.T. Cousins, P. de Voogt, A.A. Jensen, K. Kannan, S.A. Mabury, and S.P. van Leeuwen. 2011. Perfluoroalkyl and polyfluoroalkyl substances in the environment: terminology, classification, and origins. *Integrated Environmental Assessment and Management* 7(4):513–541.

Buck, R.C., S. H. Korzeniowski, E. Laganis, and F. Adamsky. 2021. Identification and classification of commercially relevant per- and poly-fluoroalkyl substances (PFAS). *Integrated Environmental Assessment and Management* 17(5):1045–1055.

Burkhard, L.P. 2021. Evaluation of published bioconcentration factor (BCF) and bioaccumulation factor (BAF) data for per-and polyfluoroalkyl substances across aquatic species. *Environmental Toxicology and Chemistry* 40(6):1530–1543.

Butenhoff, J.L., G.L. Kennedy, Jr., S.R. Frame, J.C. O'Connor and R.G. York. 2004. The reproductive toxicology of ammonium perfluorooctanoate (APFO) in the rat. *Toxicology* 196(1-2):95–116.

CAA (Clean Air Act). 1990. Clean Air Act Amendments of 1990. *Public Law,* November 15, 1990, 101–549.

Calafat, A.M., L. Wong, Z. Kuklenyik, J.A. Reidy, and L.L. Needham. 2007. Polyfluoroalkyl chemicals in the U.S. population: data from the National Health and Nutrition Examination Survey (NHANES) 2003–2004 and comparisons with NHANES 1999–2000. *Environmental Health Perspectives* 115(11):1596–1602.

Calafat, A.M., K. Kato, K. Hubbard, T. Jia, J.C. Botelho, and L.Wong. 2019. Legacy and alternative per and polyfluoroalkyl substances in the U.S. general population: paired serum-urine data from the 2013-2014 National Health and Nutrition Examination Survey. *Environment International* 131:105048.

CDPHE (Colorado Department of Public Health and Environment). 2020. *Regulation No. 42: Site-Specific Water Quality Classifications and Standards for Groundwater*. CDPHE, Water Quality Control Commission. https://www.sos.state.co.us/CCR/GenerateRulePdf.do?ruleVersionId=8815&fileName=5%20CCR%201002-42.

CERCLA (Comprehensive Environmental Response Compensation and Liability Act). 2002. Comprehensive Environmental response, compensation, and liability act of 1980 (Superfund). *Public Law,* December 31, 2002, 107–377.

CHAP (Chronic Hazard Avisory Panel). 2014. *Chronic Hazard Advisory Panel on Phthalates and Phthalate Alternatives*. Prepared for the U.S. Consumer Product Saftey Commission, Bethesda, MD, by CHAP, Bethesda, MD.

Clark, R. L., J.M. Antonello, S.J. Grossman, L.D. Wise, C. Anderson, W.J. Bagdon, S. Prahalada, J.S. MacDonald, and R.T. Robertson.1990. External genitalia abnormalities in male rats exposed in utero to finasteride, a 5 alpha-reductase inhibitor. *Teratology* 42:91-100.

Cochran, R.S. 2015. Evaluation of Perfluorinated Compounds in Sediment, Water, and Passive Samplers Collected from the Barksdale Air Force Base. MS Thesis. Texas Tech University, Lubbock, TX.

Conder, J.M., R.A. Hoke, W.D. Wolf, M.H. Russell, and R.C. Buck. 2008. Are PFCAs bioaccumulative? A critical review and comparison with regulatory criteria and persistent lipophilic compounds. *Environmental Science & Technology* 42(4):995–1003.

Conley, J. M., B.R. Hannas, J.R. Furr, V.S. Wilson, and L.E. Gray, Jr. 2016. A demonstration of the uncertainty in predicting the estrogenic activity of individual chemicals and mixtures from an in vitro estrogen receptor transcriptional activation assay (T47D-KBluc) to the in vivo uterotrophic assay using oral exposure. *Toxicological Sciences* 153(2):382-95.

Conley, J.M., C.S. Lambright, N. Evans, M. Cardon, J. Furr, V.S. Wilson, and L.E. Gray, Jr. 2018. Mixed "antiandrogenic" chemicals at low individual doses produce reproductive tract malformations in the male rat. *Toxicological Sciences* 164:166–178.

Conley, J.M., C. Lambright, N. Evans, E. Medlock Kakaley, D. Hill, A. Dixon, E. Hines, and L.E. Gray. 2021a. Dose additive developmental toxicity of a mixture of three PFAS (HFPO-DA, NBP2, PFOS) in the Sprague-Dawley rat. Abstract no. 2666. *The Toxicologist: Supplement to Toxicological Sciences* 180(1).

Conley, J.M., C.S. Lambright, N. Evans, J. McCord, M.J. Strynar, D Hill, E. Medlock-Kakaley, V.S. Wilson, and L.E. Gray, Jr. 2021b. Hexafluoropropylene oxide-dimer acid (HFPO-DA or GenX) alters maternal and fetal glucose and lipid metabolism and produces neonatal mortality, low birthweight, and hepatomegaly in the Sprague-Dawley rat. *Environment International* 146:106024.

Cousins, I.T., J.C. DeWitt, J. Gluge, G. Goldenman, D. Herzke, R. Lohmann, M. Miller, C.A. Ng, Martin, Scheringer, L. Vierke, and Z. Wang. 2020. Strategies for grouping per-and polyfluoroalkyl substances (PFAS) to protect human and environmental health. *Environmental Science: Processes and Impacts* 22:1444–1460.

Crofton, K.M., E.S. Craft, J.M. Hedge, C. Gennings, J.E. Simmons, R.A. Carchman, W.H. Carter, Jr., and M.J. DeVito. 2005. Thyroid-hormone–disrupting chemicals: evidence for dose-dependent additivity or synergism. *Environmental Health Perspectives* 113(11):1549–1554.

Crofton, K.M. 2008. Thyroid disrupting chemicals: mechanisms and mixtures. *International Journal of Andrology* 31(2):209–223.

CT DPH (Connecticut State Department of Public Health). 2017. *Perfluoroalkyl Substances (PFAS) in Drinking Water: Health Concerns*. CT DPH, Environmental & Occupational Health Assessment Program. https://www.ecos.org/wp-content/uploads/2018/06/CT-PFAS-in-drinking-water.pdf.

CT DPH (Connecticut State Department of Public Health). 2021. *Per- and polyfluoroalkyl Substances (PFAS) in Drinking Water: Health Concerns.* Accessed July 2021. https://portal.ct.gov/-/media/Departments-and-Agencies/DPH/dph/environmental_health/PFAS/Perfluoroalkyl-Substances-PFASs-in-DWHealth-Concerns-2021-03-25-update.pdf.

Danish Environmental Protection Agency. 2015. *Perfluoroalkylated Substances: PFOA, PFOS and PFOSA. Evaluation of Health Hazards and Proposal of a Health Based Quality Criterion for Drinking Water, Soil, and Groundwater*. Environmental Project Number 1665. Danish Ministry of the Environment, Environmental Protection Agency, Copenhagen, Denmark. https://www2.mst.dk/Udgiv/publications/2015/04/978-87-93283-01-5.pdf.

Das, K.P., B.E. Grey, M.B. Rosen, C.R. Wood, K.R. Tatum-Gibbs, R.D. Zehr, M.J. Strynar, A.B. Lindstrom, and C. Lau. 2015. Developmental toxicity of perfluorononanoic acid in mice. *Reproductive Toxicology* 51:133-144.

DE DNREC (Delaware Department of Natural Resources and Environmental Control). 2018. *PFAS in Delaware.* https://dnrec.alpha.delaware.gov/waste-hazardous/remediation/pfas/

De Silva, A.O., C. Spencer, B.F. Scott, S. Backus, and D.C. Muir. 2011. Detection of a cyclic perfluorinated acid, perfluoroethylcyclohexane sulfonate, in the Great Lakes of North America. *Environmental Science & Technology* 45(19):8060–8066.

De Silva, A.O., J.M. Armitage, T.A. Bruton, C. Dassuncao, W. Heiger-Bernays, X.C. Hu, A. Karrman, B. Kelly, C. Ng, A. Robuck, M. Sun, T.F. Webster, and E.M. Sunderland. 2020. PFAS exposure pathways for humans and wildlife, a synthesis of current knowledge and key gaps in understanding. *Environmental Toxicology and Chemistry* 40:631–657.

Dubois, K.P. 1961. Potentiation of the toxicity of organophosphorus compounds. *Advances in Pest Control Research* 4:117–151.

DuPont-18405-1037. 2010. *An Oral (Gavage) Reproduction/ Developmental Toxicity Screening Study of H-28548 in Mice.* EPA OPPTS 870.3550. OECD Test Guideline 421. WIL Research Laboratories, LLC, Ashland, OH.

EFSA (European Food Safety Authority), H.K. Knutsen, J. Alexander, L. Barregard, M. Bignami, B. Bruschweiler, S. Ceccatelli, B. Cottrill, M. Dinovi, L. Edler, B. Grasl-Kraupp, C. Hogstrand, L. R. Hoogenboom, C. S. Nebbia, I.P. Oswald, A. Petersen, M. Rose, A.C. Roudot, C. Vleminckx, G. Vollmer, H. Wallace, L. Bodin, J. P. Cravedi, T. I. Halldorsson, L. S. Haug, N. Johansson, H. van Loveren, P. Gergelova, K. Mackay, S. Levorato, M. van Manen, and T. Schwerdtle. 2018. Risk to human health related to the presence of perfluorooctane sulfonic acid and perfluorooctanoic acid in food. *EFSA Journal* 16(12):e05194.

EFSA (European Food Safety Authority), D. Schrenk, M. Bignami, L. Bodin, J.K. Chipman, J. Del Mazo, B. Grasl-Kraupp, C. Hogstrand, L.R. Hoogenboom, J.C. Leblanc, C.S. Nebbia, E. Nielsen, E. Ntzani, A. Petersen, S. Sand, C. Vleminckx, H. Wallace, L. Barregard, S. Ceccatelli, J.P. Cravedi, T.I. Halldorsson, L.S. Haug, N. Johansson, H.K. Knutsen, M. Rose, A.C. Roudot, H. Van Loveren, G. Vollmer, K. Mackay, F. Riolo, and T. Schwerdtle. 2020. Risk to human health related to the presence of perfluoroalkyl substances in food. *EFSA Journal* 18(9):e06223.

Egeghy, P.P., and M. Lorber. 2010. An assessment of the exposure of Americans to perfluorooctane sulfonate: a comparison of estimated intake with values inferred from NHANES data. *Journal of Exposure Science & Environmental Epidemiology* 21:150–168.

EPA (Environmental Protection Agency). 1986. *Guidelines for the Health Risk Assessment of Chemical Mixtures*. EPA/630/R-98/002. EPA, Risk Assessment Forum, Washington, DC. https://www.epa.gov/risk/guidelines-health-risk-assessment-chemical-mixtures.

EPA (Environmental Protection Agency). 1987. *Interim Procedures for Estimating Risks Associated with Exposures to Mixtures of Chlorinated Dibenzo-P-Dioxins and -Dibenzofurans (CDDs and CDFs)*. EPA/625/3-87/012. EPA, Risk Assessment Forum, Washington, DC

EPA (Environmental Protection Agency). 1989. *Interim Procedures for Estimating Risks Associated with Exposures to Mixtures of Chlorinated Dibenzo-P-Dioxins and -Dibenzofurans (CDDs and CDFs) and 1989 Update.* EPA/625/3-89/016. EPA, Risk Assessment Forum, Washington, DC.

EPA (Environmental Protection Agency). 1991. *Risk Assessment Guidance for Superfund, Vol 1: Human Health Evaluation Manual (Part B, Development of Risk-Based Preliminary Remediation Goals).* EPA/540/R-92/003. EPA, Emergency and Remedial Response, Washington, DC. https://www.epa.gov/risk/risk-assessment-guidance-superfund-rags-part-b.

EPA (Environmental Protection Agency). 2000. *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures*. EPA/630/R-00/002. EPA, Risk Assessment Forum, Washington, DC. https://cfpub.epa.gov/ncea/risk/recordisplay.cfm?deid=20533.

EPA (Environmental Protection Agency). 2003. *Chapter 9. Toxic Equivalency Factors (TEF) for Dioxin and Related Compounds in Part II: Exposure and Human Health Reassessment for 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD) and Related Compounds.* NAS review draft NCEA-I-0836. EPA, Office of Research and Development, National Center for Environmental Assessment, Washington, DC.

EPA (Environmental Protection Agency). 2006. *Organophosphorus Cumulative Risk Assessment (2006 Update).* EPA-HQ-OPP-2006-0618-0002. EPA, Office of Pesticide Programs, Washington, DC.

EPA (Environmental Protection Agency). 2007a. *Concepts, Methods and Data Sources for Cumulative Health Risk Assessment of Multiple Chemicals, Exposures and Effects: A Resource Document*. EPA/600/R-06/013F. EPA, Washington, DC. https://cfpub.epa.gov/ncea/risk/recordisplay.cfm?deid=190187.

EPA (Environmental Protection Agency). 2007b. *Revised N-Methyl Carbamate Cumulative Risk Assessment.* EPA, Office of Pesticide Programs, Washington, DC.

EPA (Environmental Protection Agency). 2008. *Framework for Application of the Toxicity Equivalence Methodology for Polychlorinated Dioxins, Furans, and Biphenyls in Ecological Risk Assessment.* EPA/100/R-08/004. EPA, Risk Assessment Forum, Washington, DC. https://www.epa.gov/risk/framework-application-toxicity-equivalence-methodology-polychlorinated-dioxins-furans-and.

EPA (Environmental Protection Agency). 2009. *Endocrine Disruptor Screening Program Test Guidelines OPPTS 890.1600: Uterotrophic Assay.* EPA/740/C-09/0010. EPA, Washington, DC.

EPA (Environmental Protection Agency). 2010. *Recommended Toxicity Equivalence Factors (TEFs) for Human Health Risk Assessments of 2,3,7,8- Tetrachlorodibenzo-p-dioxin and Dioxin-Like Compounds.* EPA/100/R-10/005. EPA, Risk Assessment Forum, Washington, DC. https://www.epa.gov/risk/documents-recommended-toxicity-equivalency-factors-human-health-risk-assessments-dioxin-and.

EPA (Environmental Protection Agency). 2011. *Recommended Use of Body Weight$^{3/4}$ as the Default Method in Derivation of the Oral Reference Dose*. EPA/100/R11/0001. EPA, Risk Assessment Forum, Washington, DC. https://www.epa.gov/risk/recommended-use-body-weight-34-default-method-derivation-oral-reference-dose.

EPA (Environmental Protection Agency). 2014.*Guidance for Applying Quantitative Data to Develop Data-Derived Extrapolation Factors for Interspecies and Intraspecies Extrapolation.* EPA/R-14/002F. EPA, Risk Assessment Forum, Washington, DC. https://www.epa.gov/risk/guidance-applying-quantitative-data-develop-data-derived-extrapolation-factors-interspecies.

EPA (Environmental Protection Agency). 2016a. *Health Effects Support Document for Perfluorooctanoic Acid (PFOA).* EPA/822/R-16/003. EPA, Washington, DC. https://www.epa.gov/sites/default/files/2016-05/documents/pfoa_hesd_final_508.pdf.

EPA (Environmental Protection Agency). 2016b. *Health Effects Support Document for Perfluorooctane Sulfonate (PFOS)*. EPA/822/R-16/002. EPA, Washington, DC. https://www.epa.gov/sites/default/files/2016-05/documents/pfos_hesd_final_508.pdf.

EPA (Environmental Protection Agency). 2016c. *Drinking Water Health Advisory for Perfluorooctanoic Acid (PFOA)*. EPA/822/R-16/005. EPA, Washington, DC. https://www.epa.gov/sites/default/files/2016-05/documents/pfoa_health_advisory_final_508.pdf.

EPA (Environmental Protection Agency). 2016d. *Drinking Water Health Advisory for Perfluorooctane Sulfonate (PFOS).* EPA/822/R-16/004. EPA, Washington, DC. https://www.epa.gov/sites/default/files/2016-05/documents/pfos_health_advisory_final_508.pdf.

EPA (Environmental Protection Agency). 2018. *Cumulative Assessment of Risk from Pesticides.* EPA, Washington, DC. https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/cumulative-assessment-risk-pesticides.

EPA (Environmental Protection Agency). 2019a. *Exposure Factors Handbook Chapter 3 (Update): Ingestion of Water and Other Select Liquids*. EPA/600/R-18/259F. EPA, Office of Research and Development, Washington, DC. https://cfpub.epa.gov/ncea/efp/recordisplay.cfm?deid=343661.

EPA (Environmental Protection Agency). 2019b. *Occurrence Data from the Third Unregulated Contaminant Monitoring Rule (UCMR 3)*. https://www.epa.gov/monitoring-unregulated-drinking-water-contaminants/occurrence-data-unregulated-contaminant#3.

EPA (Environmental Protection Agency). 2019c. Announcement of Preliminary Regulatory Determinations for Contaminants on the Fourth Drinking Water Contaminant Candidate List. *Federal Register*, March 10, 2020, 85(47):14098–14142.

EPA (Environmental Protection Agency). 2021a. *Human Health Toxicity Values for Perfluorobutane Sulfonic Acid (CASRN 375-73-5) and Related Compound Potassium Perfluorobutane Sulfonate (CASRN 29420-49-3).* EPA/600/R-20/345F. EPA, Office of Research and Development, Washington, DC. https://cfpub.epa.gov/ncea/risk/recordisplay.cfm?deid=350888.

EPA (Environmental Protection Agency). 2021b. *Human Health Toxicity Values for Hexafluoropropylene Oxide (HFPO) Dimer Acid and Its Ammonium Salt (CASRN 13252-13-6 and CASRN 62037-80-3). Also Known as "GenX Chemicals"*. EPA/822/R-21/010. EPA, Office of Water, Health and Ecological Criteria Division, Washington, DC. https://www.epa.gov/chemical-research/human-health-toxicity-assessments-genx-chemicals.

EPA (Environmental Protection Agency). 2021c. *Proposed Approaches to the Derivation of a Draft Maximum Contaminant Level Goal for Perfluorooctanoic Acid (PFOA) (CASRN 335-67-1) in Drinking Water*. EPA, Office of Water, Washington, DC.

EPA (Environmental Protection Agency). 2021d. *Proposed Approaches to the Derivation of a Draft Maximum Contaminant Level Goal for Perfluorooctane Sulfonic Acid (PFOS) (CASRN 1763-23-1) in Drinking Water*. EPA, Office of Water, Washington, DC.

EPA (Environmental Protection Agency). 2021e. *Analysis of Cardiovascular Disease Risk Reduction as a Result of Reduced PFOA and PFOS Exposure in Drinking Water*. EPA, Office of Water, Washington, DC.

EPA (Environmental Protection Agency). 2021f. *Assessing and Managing Chemicals under TSCA: Fact Sheet PFOA Stewardship Program*. https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/fact-sheet-20102015-pfoa-stewardship-program.

EPA (U.S. Environmental Protection Agency). 2021g. *Fifth Unregulated Contaminant Monitoring Rule*. https://www.epa.gov/dwucmr/fifth-unregulated-contaminant-monitoring-rule.

EU (European Union). 2020. *Commission Staff Working Document: Poly- and perfluoroalkyl substances (PFAS).* Accessed October 2021. https://ec.europa.eu/environment/pdf/chemicals/2020/10/SWD_PFAS.pdf

Feng, X., X. Cao, S. Zhao, X. Wang, X. Hua, L. Chen, and L. Chen. 2017. Exposure of pregnant mice to perfluorobutanesulfonate causes hypothyroxinemia and developmental abnormalities in female offspring. *Toxicological Studies* 155(2):409–419.

Finney, D.J. 1971. *Probit Analysis*. 3rd ed. Cambridge University Press, New York, NY.

Florida Health. 2020. *Per- and Polyfluoroalkyl Substances (PFAS).* http://www.floridahealth.gov/environmental-health/hazardous-waste-sites/contaminant-facts/_documents/doh-pfas-faq-update-03052020.pdf

FQPA (Food Quality Protection Act). 1996. Food Quality Protection Act of 1996. *Public Law* August 3, 1996, 104-170.

Furr, J. R., C.S. Lambright, V.S. Wilson, P.M. Foster, L.E. and Gray, Jr. 2014. A short-term in vivo screen using fetal testosterone production, a key event in the phthalate adverse outcome pathway, to predict disruption of sexual differentiation. *Toxicological Sciences* 140(2):403-24.

Gale, R. W., J.M. Bergeron, E.J. Willingham, and D. Crews. 2002. Turtle sex determination assay: mass balance and responses to 2,3,7,8-tetrachlorodibenzo-p-dioxin and 3,3',4,4',5-pentachlorobiphenyl. *Environmental Toxicology and Chemistry* 21:2477-82.

Gennings, C., W.H. Carter, Jr., E.W. Carney, G.D. Charles, B.B. Gollapudi, and R.A. Carchman. 2004. A novel flexible approach for evaluating fixed ratio mixtures of full and partial agonists. *Toxicological Sciences* 80(1):134–150.

Gray, L.E., J.R. Furr, J.M. Conley, C.S. Lambright, N. Evans, M.C. Cardon, V.S.Wilson, P.M. Foster, and P.C. Hartig. 2019. A conflicted tale of two novel AR antagonists in vitro and in vivo: pyrifluquinazon versus bisphenol C. *Toxicological Sciences* 168(2):632-643.

Gray, L.E., A. Hotchkiss, C.J. Wolf, J.R. Furr, M.G. Price, C.R. Lambright, L.G. Parks, V.S. Wilson, K.L. Bobseine, P.C. Hartig, and J.S. Ostby. 2001. Adverse effects of Antiandrogenic Pesticide and Toxic Substances on Reproductive Development in the Male. Presented at Society for the Study of Reproduction, July 28-August 1, 2001, Ottawa, Ontario, Canada.

Guelfo, J.L., and D.T. Adamson. 2018. Evaluation of a national data set for insights into sources, composition, and concentrations of per- and polyfluoroalkyl substances (PFASs) in U.S. drinking water. *Environmental Pollution* 236:505–513.

Gutleb, A. C., J. Appelman, M. Bronkhorst, J.H. van den Berg, and A.J. Murk. 2000. Effects of oral exposure to polychlorinated biphenyls (PCBs) on the development and metamorphosis of two amphibian species (*Xenopus laevis* and *Rana temporaria*). *Science of the Total Environment* 262(1-2):147–157.

Haas, U., M. Scholze, S. Christiansen, M. Dalgaard, A.M. Vinggaard, M. Axelstad, S.B. Metzdorff, and A. Kortenkamp. 2007. Combined exposure to anti-androgens exacerbates disruption of sexual differentiation in the rat. *Environmental Health Perspectives* 115(Suppl 1):122-128.

Hannas, B.R., C.S. Lambright, J. Furr, K.L. Howdeshell, V.S. Wilson, and L.E. Gray, Jr. 2011. Dose-response assessment of fetal testosterone production and gene expression levels in rat testes following in utero exposure to diethylhexyl phthalate, diisobutyl phthalate, diisoheptyl phthalate, and diisononyl phthalate. *Toxicological Sciences* 123(1):206-216.

Hannas, B.R., K.L. Howdeshell, J. Furr, and L.E. Gray, Jr. 2013. In utero phthalate effects in the female rat: a model for MRKH syndrome. *Toxicology Letters* 223:315–21.

Hansen, K. J., H. Johnson, J. Eldridge, J. Butenhoff and L. Dick. 2002. Quantitative characterization of trace levels of PFOS and PFOA in the Tennessee River. *Environmental Science & Technology* 36(8):1681–1685.

Hass, U., S. Christiansen, M. Axelstad, M. Scholze, and J. Boberg. 2017. Combined exposure to low doses of pesticides causes decreased birth weights in rats. *Reproductive Toxicology* 72:97–105.

Health Canada. 2018. *Guidelines for Canadian Drinking Water Quality: Guideline Technical Document – Perfluorooctane Sulfonate (PFOS)*. Health Canada, Ottawa, Ontario. https://www.canada.ca/content/dam/canada/health-canada/migration/healthy-canadians/publications/healthy-living-vie-saine/guidelines-canadian-drinking-water-quality-guideline-technical-document-perfluorooctane-sulfonate/PFOS%202018-1130%20ENG.pdf.

Hodgson, E. 2012. Metabolic interactions of environmental toxicants in humans. *Progress in Molecular Biology and Translational Science* 112:349–372.

Houck, K.A., G. Patlewicz, A.M. Richard, A.J. Williams, M.A. Shobair, M. Smeltz, M.S. Clifton, B. Wetmore, A. Medvedev, and S. Makarov. 2021. Bioactivity profiling of per- and polyfluoroalkyl substances (PFAS) identifies potential toxicity pathways related to molecular structure. *Toxicology* 457:152789.

Houde, M., A. Trevor, D. Bujas, J. Small, R. S. Wells, P. A. Fair, G. D. Bossart, K.R. Solomon, and D. Muir. 2006. Biomagnification of perfluoroalkyl compounds in the bottlenose dolphin (*Tursiops truncatus*) food web. *Environmental Science & Technology* 40(13): 4138–4144.

Howdeshell, K. L., V.S. Wilson, J. Furr, C.R. Lambright, C.V> rider, C.R. Blystone, A.K. Hotchkiss, and L.E. Gray, Jr. 2008. A mixture of five phthalate esters inhibits fetal testicular testosterone production in the sprague-dawley rat in a cumulative, dose-additive manner. *Toxicological Sciences* 105(1):153-65.

Howdeshell, K.L., C.V. Rider, V.S. Wilson, J.R. Furr, C.R. Lambright, and L.E. Gray. 2015. Dose addition models based on biologically relevant reductions in fetal testosterone accurately predict postnatal reproductive tract alterations by a phthalate mixture in rats. *Toxicological Sciences* 148(2):488–502.

Howdeshell, K.L., A.K. Hotchkiss, and L.E. Gray, Jr. 2017. Cumulative effects of antiandrogenic chemical mixtures and their relevance to human health risk assessment. *International Journal of Hygiene and Environmental Health* 220:179–188.

Illinois EPA (Illinois Environmental Protection Agency). 2019. *Title 35: Environmental Protection, Subtitle: Public Water Supplies, Chapter I: Pollution Control Board, Part 620 Groundwater Quality, Subpart A: General.* Accessed July 2021. https://www.ilga.gov/commission/jcar/admincode/035/03500620sections.html.

Illinois EPA (Illinois Environmental Protection Agency). 2021. *PFAS Statewide Health Advisory*. https://www2.illinois.gov/epa/topics/water-quality/pfas/Pages/pfas-healthadvisory.aspx

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

ITRC (Interstate Technology Regulatory Council). 2021. *PFAS – Per- and Polyfluoroalkyl Substances: 2.2. Chemistry, Terminology, and Acronyms*. ITRC, Washington, DC. Accessed May 2021. https://pfas-1.itrcweb.org/2-2-chemistry-terminology-and-acronyms/.

Jacob, C.C., R. Reimschuessel, L.S. Von Tungeln, G.R. Olson, A.R. Warbritton, D.G. Hattan, F.A. Beland, and G.G. da Costa. 2011. Dose-response assessment of nephrotoxicity from a 7-day combined exposure to melamine and cyanuric acid in F344 rats. *Toxicological Sciences* 119:391–397.

Jarvis, A.L., J.R. Justice, M.C. Elias, B. Schnitker, and K. Gallagher. 2021. Perfluorooctane sulfonate in US ambient surface waters: a review of occurrence in aquatic environments and comparison to global concentrations. *Environmental Toxicology and Chemistry* 40(9):2425–2442.

Johnson, K.J., S.S. Auerbach, and E. Costa. 2020. A rat liver transcriptomic point of departure predicts a prospective liver or non-liver apical point of departure. *Toxicological Sciences* 176(1)86–102.

Jonker, D, R.A. Woutersen, and V.J. Feron. 1996. Toxicity of mixtures of nephrotoxicants with similar or dissimilar mode of action. *Food and Chemical Toxicology* 34:1075–1082.

Judson, R.S., R.J. Kavlock, R.W. Setzer, E.A. Cohen Hubal, M.T. Martin, T.B Knudsen, K.A. Houck, R.S. Thomas, B.A. Wetmore, and D.J. Dix. 2011. Estimating toxicity-related biological pathway altering doses for high-throughput chemical risk assessment. *Chemical Research in Toxicology*. 24(4):451-462. doi:10.1021/tx100428e.

Kannan, K., L. Tao, E. Sinclair, S.D. Pastva, D.J. Judem and J.P. Giesy. 2005. Perfluorinated compounds in aquatic organisms at various trophic levels in a Great Lakes food chain. *Archives of Environmental Contamination and Toxicology* 48(4):559–566.

Konwick, B.J., G.T. Tomy, N. Ismail, J.T. Peterson, R.J. Fauver, D. Higginbotham, and A.T. Fisk. 2008. Concentrations and patterns of perfluoroalkyl acids in Georgia, USA surface waters near and distant to a major use source. *Environmental Toxicology and Chemistry: An International Journal* 27(10):2011–2018.

Kortenkamp, A., and U. Haas. 2009. *Expert Workshop on Combination Effects of Chemicals, 28-20 January 2009*. Workshop Report. Hornbaek, Denmark.

Kotlarz, N., J. McCord, D. Collier, C.S. Lea, M. Strynar, A.B. Lindstrom, A.A. Wilkie, J.Y. Islam, K. Matney, P. Tarte, M.E. Polera, K. Burdette, J. DeWitt, K. May, R.C. Smart, D. R.U. Knappe, and J.A. Hoppin. 2020. Measurement of novel, drinking water-associated pfas in blood from adults and children in Wilmington, North Carolina. *Environmental Health Perspectives* 128(7). doi:10.1289/EHP6837.

Lau, C., J.R. Thibodeaux, R. G. Hanson, J.M. Rogers, B. E. Grey, M.E. Stanton, J.L. Butenhoff, and L.A. Stevenson. 2003. Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. II: postnatal evaluation. *Toxicological Sciences* 74(2):382–392.

Lioy, P. J., R. Hauser, C. Gennings, H.M. Koch, P.E. Mirkes, B.A. Schwetz, and A. Kortenkamp. 2015. Assessment of phthalates/phthalate alternatives in children's toys and childcare articles: review of the report including conclusions and recommendation of the Chronic Hazard Advisory Panel of the Consumer Product Safety Commission. *Journal of Exposure Science and Environmental Epidemiology* 25:343–353.

Lorber, M., and P.P. Egeghy. 2011. Simple intake and pharmacokinetic modeling to characterize exposure of Americans to perfluorooctanoic acid, PFOA. *Environmental Science & Technology* 45(19):8006–8014.

Luebker, D.J., M.T. Case, R.G. York, J.A. Moore, K.J. Hansen and J.L. Butenhoff. 2005a. Two-generation reproduction and cross-foster studies of perfluorooctanesulfonate (PFOS) in rats. *Toxicology* 215(1-2):126–148.

Luebker, D.J., R.G. York, K.J. Hansen, J.A. Moore and J.L. Butenhoff. 2005b. Neonatal mortality from in utero exposure to perfluorooctanesulfonate (PFOS) in Sprague-Dawley rats: dose-response, and biochemical and pharamacokinetic parameters. *Toxicology* 215(1-2):149–169.

Luebker, D.J., M.T. Case, R.G. York, J.A. Moore, K.J. Hansen, and J.L. Butenhoff. 2005c. Two-generation reproduction and cross-foster studies of perfluorooctanesulfonate (PFOS) in rats. *Toxicology* 215:126–148.

Maine DEP (Maine Department of Environmental Protection). 2021. *Maine PFAS Screening Levels.* Accessed July 2021. https://www.maine.gov/dep/spills/topics/pfas/Maine-PFAS-Screening-Levels-Rev-6.28.21.pdf.

Marques, E.S., J. Agudelo, E.M. Kaye, S.M.S. Modaresi, M. Pfohl, J. Becanova, W. Wei, M. Polunas, M. Goedken, and A.L. Slitt. 2021. The role of maternal high fat diet on mouse pup metabolic endpoints following perinatal PFAS and PFAS mixture exposure. *Toxicology* 462:152921.

Martin, O., M. Scholze, S. Ermler, J. McPhie, S.K. Bopp, A. Kienzler, N. Parissis, and A. Kortenkamp. 2021. Ten years of research on synergisms and antagonisms in chemical mixtures: a systematic review and quantitative reappraisal of mixture studies. *Environment International* 146:106206.

Mass DEP (Massachusetts Department of Environmental Protection). 2019. *Technical Support Document, Per- and Polyfluoroalkyl Substances (PFAS): An Updated Subgroup Approach to Groundwater and Drinking Water Values.* Accessed July 2019. https://www.mass.gov/files/documents/2019/12/27/PFAS%20TSD%202019-12-26%20FINAL.pdf.

McCord, J., and M. Strynar. 2019. Identification of per-and polyfluoroalkyl substances in the cape fear river by high resolution mass spectrometry and nontargeted screening. *Environmental Science & Technology* 53(9):4717-4727.

McCord, J.P., M.J. Strynar, J.W. Washington, E.L. Bergman, and S.W. Goodrow. 2020. Emerging chlorinated polyfluorinated polyether compounds impacting the waters of southwestern New Jersey identified by use of nontargeted analysis. *Environmental Science & Technology Letters* 7(12): 903-908.

Metzdorff, S.B., M. Dalgaar, S. Christiansen, M. Axelstad, U. Hass, M.K. Kiersgaard, M. Scholze, A. Kortenkamp, and A.M. Vinggaard. 2007. Dysgenesis and histological changes of genitals and perturbations of gene expression in male rats after in utero exposure to antiandrogen mixtures. *Toxicological Sciences* 98:87–98.

MN DOH (Minnesota Department of Health). 2021. *Human Health-Based Water Guidance Table*. Accessed August 27, 2021. https://www.health.state.mn.us/communities/environment/risk/guidance/gw/table.html.

Monosson, E. 2000. Reproductive and developmental effects of PCBs in fish: a synthesis of laboratory and field studies. *Reviews in Toxicology* 3(1):25–75.

Moser, V.C., M. Casey, A. Hamm, W.H. Carter, J.E. Simmons, and C. Gennings. 2005. Neurotoxicological and statistical analyses of a mixture of five organophosphorus pesticides using a ray design. *Toxicological Sciences* 86(1):101–115.

Moser, V.C., J.E. Simmons, and C. Gennings. 2006. Neurotoxicological interactions of a five-pesticide mixture in preweanling rats. *Toxicological Sciences* 92:235–245.

Moser, V.C., S. Padilla, J.E. Simmons, L.T. Haber, and R.C. Hertzberg. 2012. Impact of chemical proportions on the acute neurotoxicity of a mixture of seven carbamates in preweanling and adult rats. *Toxicological Sciences* 129(1):126–134.

MT DEQ (Montana Department of Environmental Quality). 2020. *PFAS and Your Health*. https://deq.mt.gov/files/DEQAdmin/PFAS/July%202021%20PFAS%20Fact%20Sheet.pdf.

Mumtaz, M., M.C. Buser, and H.R Pohl. 2021. Per- and polyfluoroalkyl mixtures toxicity assessment "proof-of-concept" illustration for the hazard index approach. *Journal of Toxicology and Environmental Health, Part A* 84(13):553-567. doi:10.1080/15287394.2021.1901251.

Murphy, S. D., and K.P. Dubois. 1957. Quantitative measurement of inhibition of the enzymatic detoxification of malathion by EPN (ethyl p-nitrophenyl thionobenzenephosphonate). *Proceedings of the Society for Experimental Biology and Medicine* 96:813–818.

Mwanza, J. C., D.F. Lyke, R.C. Hertzberg, L. Haber, M. Kohrman-Vincent, R. Li, Y. Pan, R.H. Lyles, J.E. Simmons, D.K. Macmillan, R.D. Zehr, A.E. Swank, and D.W. Herr. 2012. Cholinesterase inhibition and depression of the photic after discharge of flash evoked potentials following acute or repeated exposures to a mixture of carbaryl and propoxur. *Neurotoxicology* 33(3):332–346.

Nakayama, S., M.J. Strynar, L. Helfant, P. Egeghy, X. Ye, and A.B. Lindstrom. 2007. Perfluorinated compounds in the Cape Fear drainage basin in North Carolina. *Environmental Science & Technology* 41(15):5271–5276.

NAS (National Academy of Science). 2008. *Phthalates and Cumulative Risk Assessment: The Task Ahead*. NAS, National Research Council, Committee on the Health Risks of Phthalates, National Academies Press, Washington, DC.

NATO (North Atlantic Treaty Organization). 1988. *Pilot Study on International Information Exchange on Dioxins and Related Compounds, Waste Disposal Sites Contaminated with Dioxins and Related Compounds.* NATO Report 175. NATO, Brussels, Belgium.

NC DEQ (North Carolina Department of Environmental Quality). 2021. *Managing Emerging Compounds in Water*. NC DEQ, Raleigh, NC. https://deq.nc.gov/news/key-issues/emerging-compounds/managing-emerging-compounds-water#groundwater-and-surface-water-quality-standards-actions

NHDES (New Hampshire Department of Environmental Services). 2021. *Status Report on the Occurrence of Per- and Polyfluoroalkyl Substances (PFAS) Contamination in New Hampshire*. NHDES, Concord, NH. https://www4.des.state.nh.us/nh-pfas-investigation/wp-content/uploads/Statewide-PFAS-Occurrence-Status-Report.June_.2021.pdf

NTP (National Toxicology Program). 2016. *NTP Monograph: Immunotoxicity Associated with Exposure to Perfluorooctanoic Acid (PFOA) or Perfluorooctane Sulfonate (PFOS).* U.S. Department of Health and Human Services, NTP, Research Triangle Park, NC.

NTP (National Toxicology Program). 2019a. *NTP Technical Report on the Toxicity Studies of Perfluoroalkyl Carboxylates (Perfluorohexanoic Acid, Perfluorooctanoic Acid, Perfluorononanoic Acid, and Perfluorodecanoic Acid) Administered by Gavage to Sprague-Dawley (Hsd: Sprague-Dawley SD) Rats.* NTP Toxicity Report 97. U.S. Department of Health and Human Services, NTP, Research Triangle Park, NC.

NTP (National Toxicology Program). 2019b. *NTP Technical Report on the Toxicity Studies of Perfluoroalkyl Sulfonates (Perfluorobutane Sulfonic Acid, Perfluorohexane Sulfonate Potassium Salt, and Perfluorooctane Sulfonic Acid) Administered by Gavage to Sprague-Dawley (Hsd: Sprague-Dawley SD) Rats.* NTP Toxicity Report 96. U.S. Department of Health and Human Services, NTP, Research Triangle Park, NC.

OECD (Organisation for Economic Co-operation and Development). 2018. *Toward a New Comprehensive Global Database of Per- and Polyfluoroalkyl Substances (PFASs): Summary Report on Updating the OECD 2007 List of Per and Polyfluoroalkyl Substances (PFASs).* Series on Risk Management No. 39. OECD, Paris, France. https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV-JM-MONO(2018)7&doclanguage=en.

OECD (Organisation for Economic Co-operation and Development). 2021. *Reconciling Terminology of the Universe of Per- and Polyfluoroalkyl Substances: Recommendations and Practical Guidance.* Series on Risk Management No. 61. OECD, Paris, France. https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/CBC/MONO(2021)25&docLanguage=en.

Ohio EPA (Environmental Protection Agency). *Per- and Polyfluoroalkyl Substances (PFAS)*. https://epa.ohio.gov/pfas#184345314-public-drinking-water.

Padilla, P. 2006. Cumulative Effects of Organophosphorus or Carbamate Pesticides: Chapter 41 in *Toxicology of Organophosphate and Carbamate Compounds*, pp. 607–615. EPA, Research Triangle Park, NC.

Parish, S.T., M. Aschner, W. Casey, M. Corvaro, M.R. Embry, S. Fitzpatrick, D. Kidd, N.C. Kleinstreuer, B.S. Lima, R.S. Settivari, D.C. Wolf, D. Yamazaki, and A. Boobis. 2020. An evaluation framework for new approach methodologies (NAMs) for human health safety assessment. *Regulatory Toxicology and Pharmacology* 112:104592. doi:10.1016/j.yrtph.2020.104592.

Paul-Friedman, K., M. Gagne, L. Loo, P. Karamertzanis, T. Netzeva, T. Sobanski, J.A. Franzosa, A.M. Richard, R.R. Lougee, A. Gissi, J.J. Lee, M. Angrish, J.L. Dorne, S. Foster, K. Raffaele, T. Bahadori, M.R. Gwinn, J. Labert, M. Whelan, M. Rasenberg, T. Barton-Maclaren, and R.S. Thomas. 2020. Utility of in vitro bioactivity as a lower bound estimate of in vivo adverse effect levels and in risk-based prioritization. *Toxicological Sciences* 173(1):202–225.

Perkins, R., J. Butenhoff, G. Kennedy, and M. Palazzolo. 2004. 13-Week dietary toxicity study of ammonium perfluorooctanoate (APFO) in male rats. *Drug & Chemical Toxicology* 27:361–378.

Podder, A., A.H.M.A. Sadmani, D. Reinhart, N.B. Chang, and R. Goel. 2021. Per and poly-fluoroalkyl substances (PFAS) as a contaminant of emerging concern in surface water: a transboundary review of their occurrences and toxicity effects. *Journal of Hazardous Materials* 419:126361.

Poothong, S., E. Papadopoulou, J.A. Padilla-Sanchez, C. Thomsen, and L.S. Haug. 2020. Multiple pathways of human exposure to poly- and perfluoroalkyl substances (PFASS): from external exposure to human blood. *Environment International* 134:105244.

Remucal, C. K. 2019. Spatial and temporal variability of perfluoroalkyl substances in the Laurentian Great Lakes. *Environmental Sciences: Processes and Impacts* 21(11):1816–1834.

Restum, J. C., J.P. Giesy, J.A. Render, E.B. Shipp, D.A Vergrugge, and R.J. Aulerich. 1998. Multigenerational study of the effects of consumption of PCB-contaminated carp from Saginaw Bay, Lake Huron, on mink. 1. Effects on mink reproduction, kit growth and survival, and selected biological parameters. *Journal of Toxicological and Environmental Health, Part A* 54(5):343-75.

RIDEM (Rhode Island Department of Environmental Management). 2017. *Management Determination of a Groundwater Quality Standard for: Perfluorooctanoic Acid (PFOA) and Perfluorooctane Sulfonate (PFOS)*. http://www.dem.ri.gov/programs/benviron/water/quality/pdf/pfoa.pdf.

Rider, C.V., J. Furr, V.S. Wilson, and L.E. Gray, Jr. 2008. A mixture of seven antiandrogens induces reproductive malformations in rats. *International Journal of Andrology* 31(2):249–262.

Rider, C.V., V.S. Wilson, K.L. Howdeshell, A.K. Hotchkiss, J.R. Furr, C.R. Lambright, and L.E. Gray, Jr. 2009. Cumulative effects of in utero administration of mixtures of "antiandrogens" on male rat reproductive development. *Toxicologic Pathology* 37(1):100–113.

Rider, C.V., J. Furr, V.S. Wilson, and L.E. Gray, Jr. 2010. Cumulative effects of in utero administration of mixtures of reproductive toxicants that disrupt common target tissues via diverse mechanisms of toxicity. *International Journal of Andrology* 33(2):443–462.

RIVM. 2018. *Mixture exposure to PFAS: A Relative Potency Factor Approach*. RIVM Report 2018-0070. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. https://www.rivm.nl/bibliotheek/rapporten/2018-0070.pdf.

Rosen, M. B., J.R. Thibodeaux, C.R. Wood, R.D. Zehr, J.E. Schmid, and C. Lau. 2007. Gene expression profiling in the lung and liver of PFOA-exposed mouse fetuses. *Toxicology* 239(1-2):15–33.

Rosen, M. B., K.P. Das, J. Rooney, B. Abbott, C. Lau, and J.C. Corton. 2017. PPARalpha-independent transcriptional targets of perfluoroalkyl acids revealed by transcript profiling. *Toxicology* 387:95–107.

Roth, K., Z. Yang, M. Agarwal, W. Liu, Z. Peng, Z. Long, J. Birbeck, J. Westrick, W. Liu, and M.C. Petriello. 2021. Exposure to a mixture of legacy, alternative, and replacement per- and polyfluoroalkyl substances (PFAS) results in sex-dependent modulation of cholesterol metabolism and liver injury. *Environment International* 157:106843.

Rotroff, D.M., B.A. Wetmore, D.J. Dix, S.S. Ferguson, H.J. Clewell, K.A. Houck, E.L. Lecluyse, M.E. Anderson, R.S. Judson, C.M. Smith, M.A. Sochaski, R.J. Kavlock, F. Boellmann, M.T. Martin, D. Reif, J.F. Wambaugh, and R.S. Thomas. 2010. Incorporating human dosimetry and exposure into high-throughput in vitro toxicity screening. *Toxicological Sciences* 117:348–358.

Ruyle, B.J., C.P. Thackray, J.P. McCord, M.J. Strynar, K.A. Mauge-Lewis, S.E. Fenton, and E.M. Sunderland. 2021. Reconstructing the composition of per- and polyfluoroalkyl substances in contemporary aqueous film-forming foams. *Environmental Science & Technology Letters* 8(1):59-65. doi:10.1021/acs.estlett.0c00798.

Safe, S.H. 1994. Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. *Critical Reviews in Toxicology* 24(2):87–149.

SARA (Superfund Amendments and Reauthorization Act). 2002. Superfund Amendments and Reauthorization Act of 1986. *Public Law* December 31, 2002, 107–377.

SDWA (Safe Drinking Water Act). (1996). Safe Drinking Water Act Amendments of 1996. *Public Law* August 6, 1996, 104–182.

Seacat, A.M., P.J. Thomford, K.J. Hansen, L.A. Clemen, S.R. Eldridge, C.R. Elcombe, and J.L. Butenhoff. 2003. Sub-chronic dietary toxicity of potassium perfluorooctanesulfonate in rats. *Toxicology* 183:117–131.

Sedlak, M.D., J.P. Benskin, A. Wong, R. Grace, and D.J. Greig. 2017. Per- and polyfluoroalkyl substances (PFASs) in San Francisco Bay wildlife: temporal trends, exposure pathways, and notable presence of precursor compounds. *Chemosphere* 185:1217–1226.

Silva, E., N. Rajapakse, and A. Kortenkamp. 2002. Something from "nothing"—eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environmental Science & Technology* 36:1751–1756.

Simcik, M. F., and K.J. Dorweiler. 2005. Ratio of perfluorochemical concentrations as a tracer of atmospheric deposition to surface waters. *Environmental Science & Technology* 39(22):8678–8683.

Sinclair, E., and K. Kannan. 2006. Mass loading and fate of perfluoroalkyl surfactants in wastewater treatment plants. *Environmental Science & Technology* 40(5):1408–1414.

Svendsgaard, D.J., and R.C. Hertzberg. 1994. Statistical methods for the toxicological evaluation of the additivity assumption as used in the EPA chemical mixture risk assessment guidelines. *Toxicology of Chemical Mixtures: Case Studies, Mechanisms, and Novel Approaches* 23:599–642.

Swedish Food Agency. 2021. *PFAS in Drinking Water and Self-Caught Fish – Risk Management*. https://www.livsmedelsverket.se/en/production-control-and-trade/drinking-water-production-and-control/t.

Takacs, M.L., and B.D. Abbott. 2007. Activation of mouse and human peroxisome proliferator-activated receptors (alpha, beta/delta, gamma) by perfluorooctanoic acid and perfluorooctane sulfonate. *Toxicological Sciences* 95(1):108–117.

Thibodeaux, J.R., R.G. Hanson, J.M. Rogers, B.E. Grey, B.D. Barbee, J.H. Richards, J.L. Butenhoff, L.A. Stevenson, and C. Lau. 2003. Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. I: maternal and prenatal evaluations. *Toxicological Sciences* 74(2):369–381.

Thomas R.S., H.J. Clewell 3rd, B.C. Allen, S.C. Wesselkamper, N.C.Y. Wang, J.C. Lambert, J.K. Hess-Wilson, Q.J. Zhao, and M.E. Anderson. 2011. Application of transcriptional benchmark dose values in quantitative cancer and noncancer risk assessment. *Toxicological Sciences* 120:194–205.

Thomas R.S., S.C. Wesselkamper, N.C. Wang, Q.J. Zhao, D.D. Petersen, J.C. Lambert, I. Cote, L. Yang, E. Healy, M.B. Black, H.J. Clewell 3rd, B.C. Allen, and M.E. Andersen. 2013. Temporal concordance between apical and transcriptional points of departure for chemical risk assessment. *Toxicological Sciences* 134(1):180–194.

Tinwell, H., and J. Ashby. 2004. Sensitivity of the immature rat uterotrophic assay to mixtures of estrogens. *Environmental Health Perspectives* 112:575–582.

Van den Berg, M., L.S. Birnbaum, M. Denison, M.D. Vito, W. Farland, M. Feeley, H. Fiedler, H. Hakansson, A. Hanberg, L. Haws, M. Rose, S. Safe, D. Schrenk, C. Tohyama, A. Tritscher, J. Tuomisto, M. Tysklind, N. Walker, and R.E. Peterson. 2006. The 2005 World Health Organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. *Toxicological Sciences* 93(2):223-41.

Vanden Heuvel, J.P., J.T. Thompson, S.R. Frame and P.J. Gillies. 2006. Differential activation of nuclear receptors by perfluorinated fatty acid analogs and natural fatty acids: a comparison of human, mouse, and rat peroxisome proliferator-activated receptor-alpha, -beta, and -gamma, liver X receptor-beta, and retinoid X receptor-alpha. *Toxicological Sciences* 92(2):476–489.

VT DEC (Vermont Department of Environmental Conservation). 2021. *PFAS & Drinking Water Information Page*. VT DEC, Drinking Water & Groundwater Protection Division, Montpelier, VT. Accessed July 2021. https://dec.vermont.gov/water/drinking-water/pfas.

Walker, N.J., P.W. Crockett, A. Nyska, A.E. Brix, M.P. Jokinen, D.M. Sells, J.R. Hailey, M. Easterling, J.K. Haseman, M. Yin, J.R. Bucher, and C.J. Portier. 2005. Dose-additive carcinogenicity of a defined mixture of "dioxin-like compounds". *Environmental Health Perspectives* 113(1):43–48.

Wambaugh, J.F., B.A. Wetmore, R. Pearce, C. Strope, R. Goldsmith, J.P. Sluka, A. Sedykh, A. Tropsha, S. Bosgra, I. Shah, R. Judson, R.S. Thomas, and R.W. Setzer. 2015. Toxicokinetic triage for environmental chemicals. *Toxicological Sciences* 147(1):55–67.

Wang, N.C.Y., Q.J. Zhao, S.C. Wesselkamper, J.C. Lambert, D. Petersen, and J.K. Hess-Wilson. 2012. Application of computational toxicological approaches in human health risk assessment. I. A tiered surrogate approach. *Regulatory Toxicology and Pharmacology* 63(1):10-19. doi:10.1016/j.yrtph.2012.02.006.

Wang, Z., J.C. DeWitt, C.P. Higgins, and I.T. Cousins. 2017. A never-ending story of per- and polyfluoroalkyl substances (PFASs)? *Environmental Science & Technology* 51(5):2508–2518.

Wetmore, B.A., J.F. Wambaugh, S.S. Ferguson, M.A. Sochaski, D.M. Rotroff, K. Freeman, H.J. Clewell 3rd, D.J. Dix, M.E. Andersen, K.A. Houck, B. Allen, R.S. Judson, R. Singh, R.J. Kavlock, A.M. Richard, and R.S. Thomas. 2012. Integration of dosimetry, exposure, and high-throughput screening data in chemical toxicity assessment. *Toxicological Sciences* 125(1):157–74.

Wetmore, B.A., B. Allen, H.J. Clewell 3rd, T. Parker, J.F. Wambaugh, L.M. Almond, M.A. Sochaski, and R.S. Thomas. 2014. Incorporating population variability and susceptible subpopulations into dosimetry for high-throughput toxicity testing. *Toxicological Sciences* 142(1):210–224.

WHO (World Health Organization). 2008. *Melamine and Cyanuric acid: Toxicity, Preliminary Risk Assessment and Guidance on Levels in Food*. WHO, Geneva, Switzerland. https://web.archive.org/web/20080927021953/http://www.who.int/foodsafety/fs_management/infosan_events/en/index1.html.

WI DHS (Wisconsin Department of Health Services). 2019a. *Perfluorooctanoic acid (PFOA) Scientific Support Document*. WI DHS, Madison, WI. https://dnr.wi.gov/topic/Contaminants/documents/pfas/PFOAScientificSupport.pdf.

WI DHS (Wisconsin Department of Health Services). 2019b. *Perfluorooctane Sulfonic acid (PFOS) Scientific Support Document*. WI DHS, Madison, WI. https://dnr.wi.gov/topic/Contaminants/documents/pfas/PFOSScientificSupport.pdf.

Wolansky, M.J., C. Gennings, M.J. DeVito, and K.M. Crofton. 2009. Evidence for dose-additive effects of pyrethroids on motor activity in rats. *Environmental Health Perspectives* 117:1563–70.

Wolf, C.J., S.E. Fenton, J.E. Schmid, A.M. Calafat, Z. Kuklenyik, X.A. Bryant, J. Thibodeaux, K.P. Das, S.S. White, C.S. Lau, and B.D. Abbott. 2007. Developmental toxicity of perfluorooctanoic acid in the CD-1 mouse after cross-foster and restricted gestational exposure. *Toxicological Sciences* 95:462–473.

Wolf, C.J., C.V. Rider, C. Lau, and B.D. Abbott. 2014. Evaluating the additivity of perfluoroalkyl acids in binary combinations on peroxisome proliferator-activated receptor-alpha activation. *Toxicology* 316:43–54.

Wu, S., K. Blackburn, J. Amburgey, J. Jaworska, and T. Federle. 2010. A framework for using structural, reactivity, metabolic and physicochemical similarity to evaluate the suitability of analogs for SAR-based toxicological assessments. *Regulatory Toxicology and Pharmacology* 56(1):67–81.

Yamazoe, Y., T. Yamada, and K. Nagata. 2020. Prediction and characterization of CYP3A4-mediated metabolisms of azole fungicides: an application of the fused-grid template* system. *Food Safety (Tokyo)* 8:34–51.

Yasunaga, R., J. Ikuta, Y. Murata, K. Inoue, H. Koga, T. Masaki, and H. Tamura. 2013. Ligand-independent androgen receptor antagonism caused by the newly developed pesticide pyrifluquinazon (PFQ). *Reproductive Toxicology* 35(1):1-6.

Yu, C.H., C.D. Riker, S. Lu, and Z. Fan. 2020. Biomonitoring of emerging contaminants, perfluoroalkyl and polyfluoroalkyl substances (PFAS), in New Jersey adults in 2016–2018. *International Journal of Hygiene and Environmental Health* 223(1):34–44.

Zareitalabad, P., J. Siemens, M. Hamer, and W. Amelung. 2013. Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) in surface waters, sediments, soils and wastewater - a review on concentrations and distribution coefficients. *Chemosphere* 91(6):725–732.

APPENDIX A: EPA Internal Report, Preliminary Results from a USEPA ORD Study: Cumulative Developmental Toxicity of Combined Exposure to Ammonium Perfluorooctanoic Acid (PFOA, CASRN: 3825-26-1) and Potassium Perfluorooctane Sulfonate (PFOS, CASRN: 2795-39-3) in the Sprague-Dawley Rat

INTERNAL REPORT

August 26, 2021

Cumulative Developmental Toxicity of Combined Exposure to Ammonium Perfluorooctanoic Acid (PFOA, CASRN: 3825-26-1) and Potassium Perfluorooctane Sulfonate (PFOS, CASRN: 2795-39-3) in the Sprague-Dawley Rat:

Preliminary Results from a USEPA ORD Study

Justin M. Conley, Christy Lambright, Nicola Evans,
Elizabeth Medlock Kakaley, L. Earl Gray, Jr.

U.S. Environmental Protection Agency
Office of Research and Development
Center for Public Health and Environmental Assessment
Public Health and Integrated Toxicology Division
Reproductive and Developmental Toxicology Branch

A.1 Executive Summary

Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) are the two most commonly detected and studied PFAS found in environmental media, however experimental evidence of their combined toxicity is lacking in the literature. As part of our on-going research program and at the request of the USEPA Office of Water we performed a series of oral exposure experiments in pregnant Sprague-Dawley rats beginning with single chemical dose response evaluations of PFOA and PFOS, followed by a binary mixture study in which we repeated a dose response of PFOA but combined with a fixed dose of PFOS added to each PFOA dose. Preliminary results identified numerous significant adverse effects from each chemical individually and the mixture including reduced maternal gestational weight gain, reduced pup body weight, reduced pup viability, and increased maternal and pup liver weights. As a clear demonstration of cumulative mixture effects, individual exposures to 62.5 mg/kg PFOA and 2 mg/kg PFOS produced 12% and 8% post-implantation loss (PIL; a measure of fetal and pup mortality), respectively, while a combination of the two (62.5 mg/kg PFOA+2 mg/kg PFOS) produced 65% PIL. Further, by combining PFOS with PFOA, the dose response curves for PFOA across multiple effects such as pup bodyweight and maternal and pup liver weights were significantly shifted towards effects at lower doses meaning that because of the additive effect of PFOS, PFOA produced a similar effect but at a lower dose than PFOA alone. Study is on-going with multiple analyses still to be conducted on samples collected during the studies, but preliminary results clearly support additive effects of combined exposure to PFOA and PFOS during pregnancy in the Sprague-Dawley rat.

A.2 Background

The U.S. Environmental Protection Agency announced on March 3, 2021 the final decision to regulate perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) under the Safe Drinking Water Act. EPA is now working to establish a National Primary Drinking Water Regulation (NPDWR) and has up to two years from the final decision to propose health-based Maximum Contaminant Level Goals (MCLGs) and NPDWR. The current PFOA and PFOS Drinking Water Health Advisories are 70 ng/L each or 70 ng/L combined concentration of PFOA and PFOS because the reference doses were determined to both be based on critical effects that were developmental and thus should be considered cumulative. The Health Advisories were set at levels that EPA concluded would “not result in adverse developmental effects to fetuses during pregnancy or breastfed infants, who are the groups most sensitive to the potential harmful effects of PFOA and PFOS” (81 FR 33250).

The USEPA Office of Water Office of Science and Technology is currently updating and revising the existing Health Effects Support Documents (HESDs) for PFOA and PFOS. As part of this process, OST scientists identified a literature database deficiency pertaining to cumulative mixture effects associated with combined exposures to two or more PFAS, including a lack of in vivo studies directly investigating co-exposure to PFOA and PFOS. On April 14, 2021 OST formally requested additional research and assistance from ORD scientists to support the SDWA regulatory effort for PFOA and PFOS. Specifically, OST requested that ORD prioritize *in vivo* studies regarding the combined toxicity of PFOA and PFOS (and potentially other PFAS) by characterizing the maternal and fetal/postnatal effects of combined exposure to PFOA and PFOS during pregnancy in a rodent model. The study design was expected to assess a hypothesis of dose additivity to inform a combined toxicity approach for the PFOA and PFOS NPDWR.

Our research group in ORD/CPHEA/PHITD/RDTB has nearly two decades of laboratory research experience investigating the mixture-based developmental and reproductive effects of co-exposure to multiple compounds that perturb the androgen signaling pathway, notably multiple phthalates and pesticides. Further, our group has been studying the developmental toxicity of several emerging PFAS (e.g., hexafluoropropylene oxide dimer acid (HFPO-DA or GenX), Nafion byproduct 2 (NBP2), and perfluoromethoxyacetic acid (PFMOAA)) for the past three years and has already conducted a mixture-based experiment on the combined toxicity of HFPO-DA, NBP2, and PFOS (manuscript in preparation).

Here, at the request of OW/OST, we designed and conducted a series of three *in vivo* experiments designed to robustly assess the combined toxicity of PFOA and PFOS on multiple endpoints associated with the spectrum of PFAS adverse effects, specifically poor birth outcomes, liver effects, and thyroid effects from exposure during pregnancy. The in-life phases of the studies were conducted May-July 2021 and have been completed. This report describes the methods, study design, and preliminary findings on apical effects and evidence of combined toxicity. The studies are on-going with multiple sample analyses in-process (e.g., histopathology, clinical chemistry, tissue gene expression, thyroid hormone concentrations, analytical chemical determinations of test chemical in serum and tissues). Once complete, the studies will be published in one or more peer-reviewed journal articles (expected by FY23).

A.3 Methods

Dosing solutions were prepared in high performance liquid chromatography-grade water (Honeywell - Burdick&Jackson, CASRN: 7732-18-5, Cat. No. 356-4, Lot: BC128) containing 0.5% (by volume) Tween-80 (Sigma-Aldrich, CASRN: 9005-65-6, Cat. No.: P1754, Lot: BCCB5237). Pentadecafluorooctanoic acid ammonium salt (PFOA, CASRN: 3825-26-1, Cat. No. 77262, Lot: BCBW7054, Purity: 100%) and Heptadecafluorooctanesulfonic acid potassium salt (PFOS, CASRN: 2795-39-3, Cat. No. 77282, Lot: BCBX5798, Purity: >98%) were purchased from Sigma-Aldrich. Dosing was administered once daily via oral gavage at 2.5 mL/kg body weight from gestation day (GD) 8 to postnatal day (PND) 2 across the range of doses specified in Table 1. Dosing solutions are reported as nominal and not corrected for the conjugate cation.

Table 1. Study dosing design for in vivo studies of PFOA, PFOS, and combined PFOA+PFOS

Study 1 – PFOA dose response							
PFOA (mg/kg/d)	0	10	30	62.5	125	250	--
Study 2 – PFOS dose response							
PFOS (mg/kg/d)	0	0.1	0.3	1	2	5	--
Study 3 – Mixture study – PFOA dose response with PFOS fixed							
PFOA (mg/kg/d)	0	3	10	30	40	62.5	80
PFOS (mg/kg/d)	0	2	2	2	2	2	2

Time-mated Sprague-Dawley rats (Crl:CD(SD), 78 days old) were purchased from Charles River Laboratories (Raleigh, NC, USA) and shipped to USEPA (Research Triangle Park, NC, USA) on

GD 2 (GD0 = bred date; GD1 = plug positive date). Dams and their offspring were housed individually in clear polycarbonate cages (20 x 25 x 47 cm) with heat-treated, laboratory-grade pine shavings (Northeast Products, Warrensburg, NY) and fed NIH07 Rat Chow and filtered (5 μm) municipal tap water (Durham, NC) ad libitum. Dams were weight-ranked and stratified then randomly assigned to treatment groups to produce similar mean weights and variances given the range of dam body weights. This study was conducted in accordance with a protocol approved by the USEPA Center for Public Health and Environmental Assessment Institutional Animal Care and Use Committee (ACUP 22-03-001). Animals were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and maintained at 20-22°C, 45-55% humidity, and a 12:12 h photoperiod (lights off 18:00 EDST).

Three blocks of 30 dams each were used to assess the neonatal effects of *in utero* and short-term postnatal exposure to PFOA, PFOS and combined PFOA+PFOS (as described in Table 1). Dams gave birth naturally and were checked for parturition hourly beginning at 6AM on the morning of GD22 (i.e., PND0) until all dams had delivered. Hourly checks on maternal and neonatal pup health continued until 5PM on PND1 and dead pups were removed and those that were moribund were removed and euthanized via decapitation. As dams delivered, we recorded the time the first pup was observed in a cage and then the assumed time of completion of delivery based on normal maternal behaviors (e.g., retrieving pups to the nest, licking/grooming of pups, hovering over nest to stimulate nursing). Upon assumed completion of delivery, all pups were removed, counted and whole litter weight was recorded. All pups were returned to their nest except for two randomly selected pups, which were euthanized via decapitation and pooled trunk blood was collected. One pup had the thoracic and abdominal cavities exposed and then the whole carcass was fixed in 10% formalin. Subsequently, livers from formalin fixed pup carcasses were removed and shipped to Experimental Pathology Laboratories, Inc. (Durham, NC) where they will be embedded, sectioned, stained with hematoxylin and eosin (H&E), and evaluated by a Diplomate of the American College of Veterinary Pathology. For the second euthanized pup we removed liver tissue for RNA extraction and gene expression by homogenizing in TRI reagent and a second liver sample was weighed and snap frozen in liquid nitrogen for glycogen assay.

Dams were weighed and dosed on the morning of PND2 and time of dosing was recorded. Dams were euthanized via decapitation and necropsied 2 – 6 hr after dosing and time of euthanasia was recorded. Euthanasia order was stratified such that the timing of necropsy was equally distributed across dose groups. Maternal trunk blood was collected for serum isolation, liver weight was recorded and subsamples of maternal liver were collected for RNA extraction, chemical determination, and fixation in 10% formalin for histopathology. Kidneys were weighed, section longitudinally and fixed in 10% formalin for histopathology. The uterus was removed and implantation sites were scored. All pups were sexed and weighed then all pups were euthanized via decapitation and trunk blood was collected for serum isolation, liver weight was recorded for 1 male and 1 female pup per litter, both livers were fixed in 10% formalin for histopathology (for Study 3 only). Maternal serum (PND2) will be analyzed for clinical chemistry parameters, thyroid hormone concentrations (free and total T3 and T4), test chemical determination via LC-MS, and multiplex metabolomics assays (Biocrates). Neonatal serum will be analyzed for clinical chemistry parameters and thyroid hormone concentrations (total T3 and T4).

All values are reported as mean ± standard error of the mean (SEM) and all statistical comparisons were conducted at α=0.05 significance level analysis of variance (ANOVA),

followed by pairwise comparison at α=0.05 to determine differences of treatment compared to control for significant genes. Data were log10-transformed and treatment effects were identified by ANOVA using the PROC GLM statement in SAS (v.9.4, SAS Institute, Cary, NC, USA) and pairwise comparison versus control was performed using the least squares means (LSMEANS) statement using the PDIFF option. For all fetal and neonatal data, litter means were used as the statistical unit to account for the nested effects of individuals within litters. GraphPad Prism was used to generate all figures and to conduct sigmoidal dose-response curve analyses.

A.4 Preliminary Results

The individual dose response studies for PFOA and PFOS both produced statistically significant reduced maternal gestational weight gain, increased maternal and pup relative liver weight, increased post-implantation loss (i.e., reduced pup survival), reduced pup body weight, reduced pup liver glycogen content, and reduced maternal and pup serum total T3 and T4 concentrations. In the PFOA only experiment overt maternal toxicity occurred in some or all of the top two dose groups (125 and 250 mg/kg/d) and those animals were removed from the study.

The mixture experiment (Study 3) was based on dose response data from the individual chemical experiments with PFOA and PFOS (Studies 1 and 2, respectively). The design of PFOS fixed at 2 mg/kg/d combined with increasing doses of PFOA 3-80 mg/kg/d provided the greatest difference in dose addition and response addition model predictions for some endpoints. This design allows for direct comparison of the magnitude of effect at several single doses of PFOA (10, 30, and 62.5 mg/kg/d) and a single dose of PFOS (2 mg/kg/d) with the mixed doses (e.g., 62.5 mg/kg/d PFOA+2 mg/kg/d PFOS) across multiple endpoints. We also predicted that a fixed dose of PFOS mixed with increasing doses of PFOA would significantly shift the PFOA dose response curves towards effects at lower doses as compared to PFOA alone. Overall, this study design allows for multiple complementary approaches for statistically evaluating whether the two chemicals produced cumulative mixture adverse developmental effects.

Mixture toxicity was clearly demonstrated for the effect of post-implantation loss (PIL) (Figure 1). PFOA at 62.5 mg/kg/d alone produced 12.2±7.2% PIL, while PFOS at 2 mg/kg/d alone produced 8.2±5.9% PIL. In contrast, the mixture of 62.5 mg/kg PFOA plus 2 mg/kg PFOS produced 65.6±15.0% PIL, which was significantly greater than PFOA or PFOS alone at the same oral doses (Figure 1A). The full PIL dose response curves for PFOA plus 2 mg/kg/d PFOS and PFOA alone resulted in an ED50 parameter that was significantly lower, by a factor of 2.2-fold (Figure 1B), for PFOA combined with PFOS (58.4 mg/kg) compared to PFOA alone (129.7 mg/kg/d).

As described above, the mixture study was designed in part based on the hypothesis that combined exposure to PFOA with a fixed amount of PFOS would shift the PFOA dose response curves towards effects at lower doses compared to PFOA alone. This was clearly demonstrated by the effects on pup bodyweight (Figure 2A), pup liver weight (Figure 2B), and maternal liver weight (Figure 2C). Measurements were normalized to vehicle controls and plotted as percent (%) of control then fit with linear or nonlinear models providing the best fit of the observed data. For all three endpoints the co-exposure of 2 mg/kg/d PFOS with varying doses of PFOA significantly shifted the data and best fit lines towards lower x-axis doses resulting in similar dose response trend lines but at significantly lower oral doses of PFOA due to the combined effects with PFOS.

A.5 Conclusion

These preliminary results provide robust evidence of combined toxicity of PFOA and PFOS on multiple developmental endpoints. Studies 1 and 2 (PFOA alone and PFOS alone) demonstrated that both PFOA and PFOS independently produce numerous similar developmental adverse outcomes including increased post-implantation loss, reduced pup bodyweight, and increased pup and maternal liver weight. Study 3 demonstrated that co-exposure to PFOA and PFOS produced cumulative mixture effects that are at least dose additive for most endpoints and support the combined toxicity of these compounds.

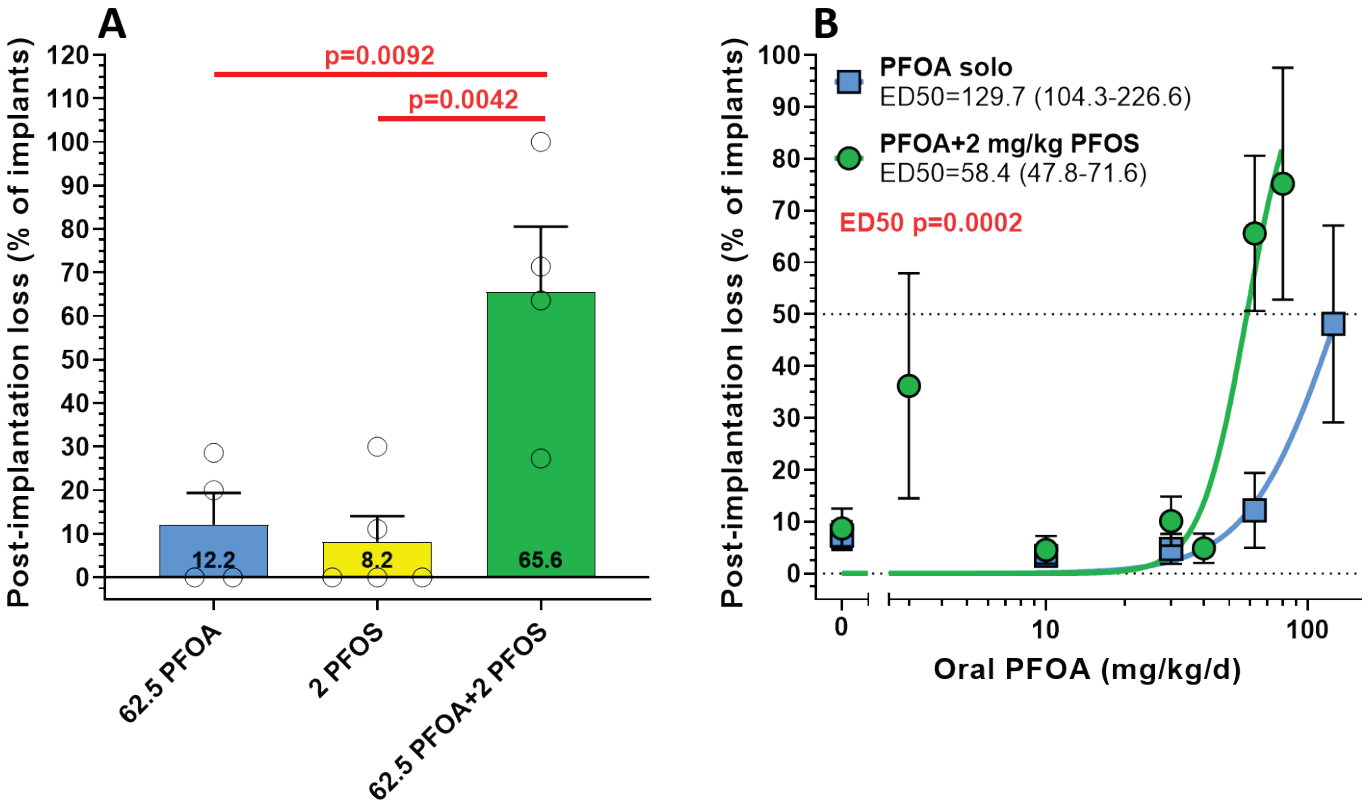


Figure 1. **(A) Post-implantation loss from GD8-PND2 oral maternal exposure to PFOA alone (62.5 mg/kg/d) or PFOS alone (2 mg/kg/d) compared to the mixture of 62.5 mg/kg/d PFOA plus 2 mg/kg/d PFOS. The mixture exposure produced a response that was significantly greater than PFOA alone (p=0.0092) or PFOS alone (p=0.0042). (B) Post-implantation loss dose response curves for PFOA alone (blue points and line) compared to PFOA combined with 2 mg/kg PFOS (green points and line). The dose response effective dose 50% (ED50) value for PFOA alone (129.7 mg/kg/d, 95% CI 104.3-226.6 mg/kg/d) was significantly greater (p=0.0002) than PFOA combined with PFOS (58.4 mg/kg/d, 95% CI 47.8-71.6 mg/kg/d), representing greater potency of PFOA when combined with PFOS.**

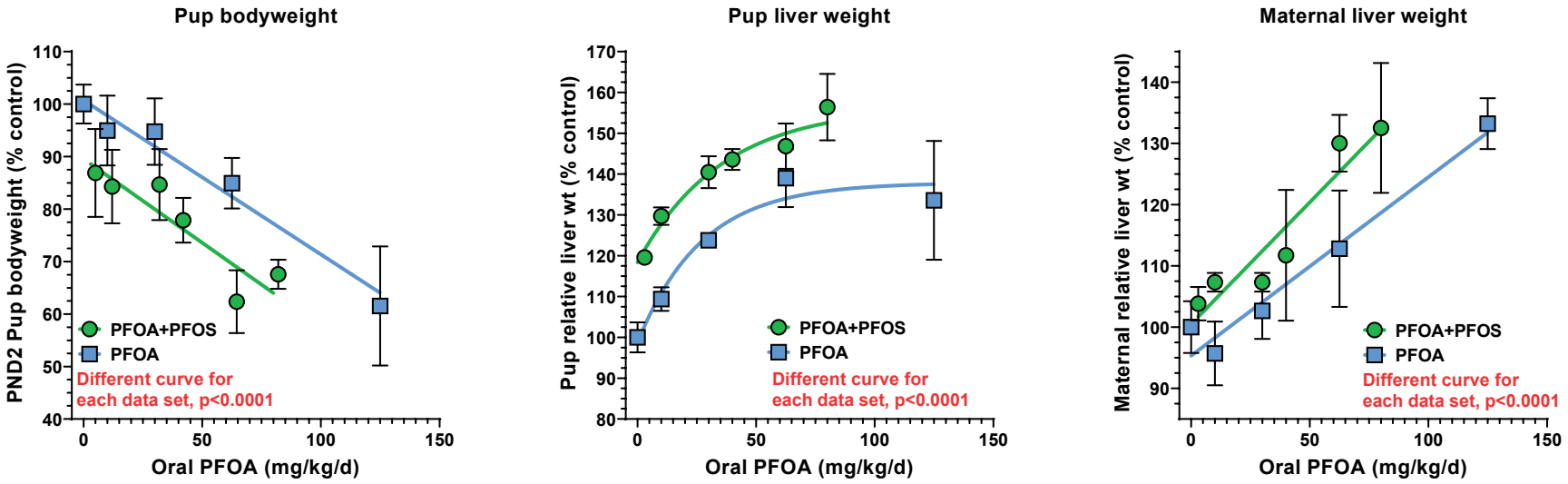


Figure 2. **Comparisons of best-fit regressions between PFOA only exposure (blue points and lines) and PFOA combined with 2 mg/kg PFOS (green points and lines) for PND2 pup bodyweight (A), PND2 pup relative liver weight (B), and PND2 maternal relative liver weight (C) normalized to respective controls. For all endpoints the best fit regressions were highly significantly different (p<0.0001) with the combined exposure shifted towards effects at lower doses than when PFOA exposure occurred alone, demonstrating combined toxicity of PFOA and PFOS for these endpoints.**