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

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

Prepared by:

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Notices

This document has been reviewed in accordance with U.S. Environmental Protection Agency (EPA) policy and approved for publication.

This document provides a 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. The extent of the utility of this document for a particular programmatic application will need to be assessed on a case-by-case basis within each specific decision context under applicable statutory and regulatory authority. The framework included in this document does not supersede previously published EPA guidance on mixtures (e.g., EPA, 1986, 2000b) or EPA approaches used to assess cumulative risks of contaminants including chemical mixtures under various environmental statutes (e.g., Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA); Food Quality Protection Act (FQPA); Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA)). Based upon evolving availability of human health risk assessment relevant information and increasing confidence in New Approach Methods, 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.

Dedication

This document is dedicated to the memory of Dr. Jane Ellen Simmons and Mr. Jeffrey Swartout. Jane Ellen and Jeff were both dedicated civil servants in EPA's Office of Research and Development for more than 30 years where they conducted rigorous chemical mixtures research and championed cumulative risk assessment approaches for exposure to multiple stressors. Their contributions to the field live on in this framework document.

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List of Abbreviations and Acronyms

6:2 FTS	6:2 fluorotelomer sulfonic acid
AED	Administered Equivalent Dose
AFFF	aqueous film forming foam
AhR	aryl hydrocarbon receptor
AIC	Akaike Information Criteria
AOF	adsorbable organofluorine
AOP	adverse outcome pathway
AR	androgen receptor
ATSDR	Agency for Toxic Substances and Disease Registry
AUC	area under the concentration vs. time curve
BBP	butyl benzyl phthalate
BMD	benchmark dose
BMDL	lower statistical bound on a BMD
BMR	benchmark response
C-F	carbon-fluoride bond
CAA	Clean Air Act
CAR	constitutive androstane receptor
CCL	Contaminant Candidate List
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
CPSC CHAP	Consumer Product Safety Commission Chronic Hazard Advisory Panel
DA	dose addition
DAF	dosimetric adjustment factor
DBP	di-n-butyl phthalate
DEHP	di(2-ehtylhexyl)phthalate
DIBP	diisobutyl phthalate
DLC	dioxin-like chemical
DWI	drinking water intake
DWI-BW	body weight-based drinking water intake
E	duration-relevant exposure
EC_X	effect concentration
ED_X	effective dose in x percent of test animals
EFSA	European Food Safety Authority
EOF	extractable organofluorine
EPA	U.S. Environmental Protection Agency
EU	European Union
FT4	free serum thyroxine
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
HepaRG	epoxide hydrolase endpoint in liver
HFPO	hexafluoropropylene oxide
HI	hazard index
HQ	hazard quotient
IA	integrated addition
IC	index chemical

ICEC	Index Chemical Equivalent Concentration
$ICEC_{MIX}$	total mixture Index Chemical Equivalent Concentration
$ICEC_{NAM}$	New Approach Methodology (NAM)-based 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
M-BMD	mixture benchmark dose
MAC	maximum acceptable concentration
MCL	Maximum Contaminant Level
MCLG	Maximum Contaminant Level Goal
mg/kg/day	milligrams per kilogram per day
MIE	molecular initiating event
MOA	mode of action
MRL	minimal 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
NIEHS	National Institute of Environmental Health Sciences
NOAEL	no observed adverse effect level
NRC	National Research Council
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
PECO	Population, Exposure, Comparator, and Outcome
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
PND	post-natal day
POD	point-of-departure
POD_{HED}	human-equivalent point-of-departure
PPARα	peroxisome proliferator activated receptor alpha
PPARγ	peroxisome proliferator activated receptor gamma
PPRTV	Provisional Peer-Reviewed Toxicity Value
ppt	parts per trillion
PWS	public water system
RA	response addition
RfD	reference dose
RfV	reference value
RPF	relative potency factor
RPF_{NAM}	New Approach Methodology (NAM)-based RPF
RSC	relative source contribution
rTK	reverse toxicokinetic
SAB	Science Advisory Board
SARA	Superfund Amendments and Reauthorization Act
SDWA	Safe Drinking Water Act
T3	triiodothyronine
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
$TOSHI_{DEV}$	target organ specific hazard index for developmental effects
TSCA	Toxic Substances Control Act
TTD	target-organ toxicity dose
UCMR	Unregulated Contaminant Monitoring Rule
UF	uncertainty factor
UF_A	interspecies uncertainty factor
UF_D	database uncertainty factor
UF_H	human interindividual variability uncertainty factor
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 releasing for public review the *Framework for Estimating Noncancer Health Risks Associated with Mixtures of Per- and Polyfluoroalkyl Substances (PFAS)* ("PFAS Mixtures Framework"). This document is designed to communicate and illustrate the practical application of existing EPA chemical mixtures approaches and methods to two or more PFAS co-occurring in environmental media, using drinking water examples. In November 2021, EPA released a draft version of this document for Science Advisory Board (SAB) review. EPA has considered the SAB comments and revised the document accordingly.

In a mixtures risk assessment context, while it would be optimal to leverage whole mixture hazard and dose-response data, such data are extremely rare, particularly at component-chemical proportions and concentrations consistent with environmentally occurring mixtures. As such, mixtures risk assessment commonly relies upon integration of available toxicity information for the individual component chemicals that co-occur in environmental media. This PFAS Mixtures Framework document describes flexible, data-driven approaches that facilitate practical component chemical-based mixtures evaluation of two or more PFAS, based on an assumption of dose additivity. Studies with PFAS and other classes of chemicals support the health protective assumption that a mixture of chemicals with similar apical effects should be assumed to act in a dose additive manner unless data demonstrate otherwise. Descriptions of dose additivity-based approaches such as the Hazard Index (HI), Relative Potency Factor (RPF), and Mixture Benchmark Dose (M-BMD) 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, 2000b) exist for such a purpose. EPA's 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.

It is not the intent of the framework to ignore potential carcinogenic effects associated with PFAS exposure. Rather, at present, few PFAS have information available to evaluate potential carcinogenic effects via any route of exposure. Should such information become available for an increasing number of PFAS in the future, EPA would consider approaches for addressing joint carcinogenic effects. EPA's National PFAS Testing Strategy (EPA, 2021f) is underway to develop and issue test orders that may help inform human health hazard data needs, including potential for carcinogenicity.

PFAS are a large and diverse structural family of compounds used in myriad 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, or their precursors or degradants, are environmentally persistent, bioaccumulative, and have long half-lives in humans, particularly the longer-chain perfluorocarboxylic acid (PFCA) and perfluorosulfonic acid (PFSA) species such as PFOA and PFOS, respectively. PFCAs/PFSAs with shorter carbon chain

length, such as perfluorobutanesulfonic acid (PFBS) and hexafluoropropylene oxide (HFPO) dimer acid and HFPO dimer acid ammonium salt (also known as GenX Chemicals[1]), were subsequently developed and integrated into various consumer products and industrial applications because they have the desired industrial properties and characteristics associated with this class of compounds but are more quickly eliminated from the human body than PFOA and PFOS. The range of PFAS encountered in environmental media is often a diverse milieu of linear, branched, cyclic, and/or aromatic parent species, metabolites, and/or abiotic degradants, leading to significant potential for PFAS mixture exposures in aquatic, terrestrial, and human populations.

As of February 2023, final EPA human health assessments are available for PFBS (EPA, 2021a), HFPO-DA (EPA, 2021b), and perfluorobutanoic acid (PFBA; EPA 2022e). EPA is in the process of updating the 2016 health assessments for PFOA and PFOS (EPA, 2023b,c,d,e), and they will be publicly reviewed as part of the development of a National Primary Drinking Water Regulation for PFAS. In addition, EPA's Integrated Risk Information System (IRIS) program is developing four additional PFAS human health assessments for 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 PFAS that EPA has not yet formally assessed. However, beyond PFOA and PFOS, ATSDR derived quantitative minimal risk levels (MRLs) for 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. In response to the critical need, EPA and the National Institute of Environmental Health Sciences (NIEHS) are actively engaged in research and testing to help address data gaps for a broad landscape of PFAS (approximately 150 structures at the time of the drafting of this document). Until results from ongoing research and testing efforts are available, the evaluation of potential toxicity/risk associated with mixtures of PFAS is primarily limited to existing hazard and dose-response data under the purview of human health assessments by federal, state, and/or international entities.

To facilitate the use of potentially disparate sources of PFAS information in a mixture context, the application of the component-based methods presented in this framework document is demonstrated using a hypothetical mixture of five PFAS as follows:
(1) **PFAS 1** = comprehensively studied, most potent for effect(s), and has formal noncancer human health assessment value(s) (i.e., reference dose or RfD) and a health-based water concentration (HBWC) available; (2) **PFAS 2** = well-studied, second most potent for effect(s) among PFAS 1–3, and has formal noncancer human health assessment value(s) and HBWC available; (3) **PFAS 3** = studied, least potent for effect(s) among PFAS 1–3, and has formal noncancer human health assessment value(s) and HBWC available; (4) **PFAS 4** = experimental animal toxicity data available but no formal human health assessment and no HBWC; and (5) **PFAS 5** = data-poor. The hypothetical PFAS mixture is purposefully designed to demonstrate how this framework allows for flexible integration of information derived from

[1]EPA notes that the chemical HFPO-DA is used in a processing aid technology developed by DuPont to make fluoropolymers without using PFOA. The chemicals associated with this process are commonly known as GenX Chemicals and the term is often used interchangeably for HFPO-DA along with its ammonium salt.

health assessment data sources (e.g., federal, state, international), available human epidemiological and/or experimental animal hazard and dose-response data (that have not yet been formally evaluated in an assessment product), 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.

1.0 Introduction and Background

1.1 Purpose

PFAS are an urgent public health and environmental issue facing communities across the United States. In April 2021, Administrator Michael Regan established EPA's Council on PFAS, and charged the Council to develop a bold, strategic, whole-of-EPA strategy to protect public health and the environment from the impacts of PFAS. In October 2021, EPA released the PFAS Strategic Roadmap[2] ('The Roadmap') which lays out EPA's approach to tackling PFAS and sets timelines by which the agency plans to take concrete actions to deliver results for the American people. The Roadmap is built on a number of key principles, including considering the lifecycle of PFAS, getting upstream of the problem, holding polluters accountable, ensuring science-based decision-making, and prioritizing protection of disadvantaged communities.

Recognizing that PFAS tend to occur in mixtures in environmental media (see Section 1.4), EPA has developed this data-driven framework for estimating the noncancer human health risks associated with oral exposures to mixtures of PFAS. The approaches presented in this document are based on longstanding EPA guidelines and guidance related to human health risk assessment for mixtures (EPA, 1986, 2000b). 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 noncancer 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 (instead see the EPA National PFAS Testing Strategy for categorization efforts; EPA, 2021f). Rather, the framework is designed for practical application of EPA chemical mixtures approaches and methods to gain insight on the potential joint toxicity associated with exposure to mixtures of PFAS. The 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 risks associated with PFAS mixtures.

The framework and example calculations presented here are intended to demonstrate data-driven application of EPA component-based mixture methods based on gradations of data availability and completeness, anticipated to occur in real-world scenarios for PFAS. While the examples provided are focused on drinking water, the approaches described in this framework could also

[2] https://www.epa.gov/pfas/pfas-strategic-roadmap-epas-commitments-action-2021-2024

be applied to other environmental media with oral[3] exposure routes (e.g., soil, fish/shellfish, food). Due to the constantly evolving science related to PFAS, the approaches presented herein have the flexibility to consider information as it becomes available, including forthcoming EPA human health assessments, assessments from other sources (e.g., federal, state, international), available hazard and dose-response data in the public domain, information from high(er) throughput bioassays and other NAMs including data submitted to the Agency through the Toxic Substances Control Act (TSCA) Section 4 authority for developing and issuing PFAS test orders.

Experimental evidence supports dose additive effects from combined exposure to multiple PFAS. Dose additivity, described in detail in Section 3.0, means that each of the component chemicals in the mixture behaves as a concentration or dilution of every other chemical in the mixture differing only in relative potency for toxicity. Several alkyl acid species (PFAAs) of PFAS tested to date have been shown to elicit common adverse effects on several biological systems including thyroid hormone levels, lipid synthesis and metabolism, as well as on development, and immune and liver function (ATSDR, 2021; EFSA, 2018, 2020; USEPA, 2022c).

The 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, including public comment, EPA may change certain aspects of this document in the future.

1.2 EPA Science Advisory Board (SAB) Review

In November 2021, EPA released the Draft Framework for Estimating Noncancer Health Risks Associated with Mixtures of PFAS ("Draft PFAS Mixtures Framework"; EPA, 2021e) for EPA SAB review. The SAB held public meetings on December 16, 2021; January 4, 6, and 7, 2022; and July 20, 2022, to discuss the Draft PFAS Mixtures Framework and three other technical documents supporting EPA's development of a National Primary Drinking Water Regulation for PFAS under the Safe Drinking Water Act (SDWA). EPA sought SAB comment on whether the framework and illustrative examples provided in the draft document were scientifically supported, clearly described, and informative for assessing potential health risk(s) associated with exposure to mixtures of PFAS. EPA asked specific charge questions on the assumption of dose additivity and three component-based approaches: HI, RPF, and M-BMD. A draft of the written SAB recommendations was published on April 1, 2022, and EPA received the final report from the SAB on August 22, 2022 (EPA SAB, 2022).

EPA received a generally favorable review from SAB (EPA SAB, 2022) for its development of component-based mixture assessment approaches that rely on a health protective assumption of dose additivity based on a common health outcome, instead of a common mode of action (MOA), to evaluate risk from PFAS mixtures in drinking water and other environmental media. EPA has responded to the SAB's consensus advice in the development of this final *Framework for Estimating Noncancer Health Risks Associated with Mixtures of Per- and Polyfluoroalkyl*

[3] In general, the component-based approaches presented in this document may also be applicable in assessing health risks associated with inhalation exposures of PFAS mixtures. However, the dosimetry differences across categories of (volatile/semi-volatile) PFAS gases/vapors would need to be considered in such an assessment. Data regarding the volatilization and toxicity of inhaled PFAS are generally limited.

Substances (PFAS). The SAB's overarching consensus recommendations and EPA's responses are summarized below. To view EPA's complete responses to SAB comments on the Draft PFAS Mixtures Framework please see EPA (2023a).

- "The SAB supports dose additivity based on a common outcome, instead of a common mode of action as a health protective default assumption and does not propose another default approach. However, EPA should more thoroughly and clearly present the uncertainties associated with this approach along with information supporting this approach." (EPA SAB, 2022)
    - EPA has added text in the Section, "Dose Additivity for PFAS" to address the SAB's comments related to uncertainties associated with assuming dose addition as the default assumption for assessment of PFAS mixtures. EPA has added further discussion on deviations from dose additivity, such as synergy or antagonism, but available evidence suggests that dose addition should be considered as the default model.
- "The SAB expressed concern regarding the requirement for "external peer review" of toxicity values developed by states and recommends that this phrase in the draft framework be broadened to recommend the need for scientific input and review in general." (EPA SAB, 2022)
    - In response to this point of clarification, EPA has removed the text related to external peer review. The text now reads, "If de novo derivation of toxicity values is necessary, it is recommended that experts in hazard identification and dose response assessment be consulted for scientific input and review, and the associated uncertainties (e.g., data gaps) be transparently characterized."
- "EPA should consider using a menu-based framework to support selection of fit-for-purpose approaches, rather than a tiered approach as described in the draft Mixtures document. Tiered approaches that require increasingly complex information before reaching a final decision point can be extremely challenging for data-poor chemicals such as PFAS.
    - Based on this and other SAB comments, EPA has eliminated the tiered approach and restructured the framework as a data-driven, flexible approach to facilitate PFAS mixture assessment in various decision contexts (e.g., at a contaminated site, water system, etc.) (see Section 4.2 and Figure 4-1). With "fit-for-purpose assessment" in mind, EPA has included discussion of key steps in the framework, including problem formulation and scoping, assembling information, evaluating data objectives, considering the data landscape to select component-based approach(es), and performing component-based mixture assessment approach(es) (see Section 4.2.1).
- "EPA should provide clarification regarding the conceptual similarities and differences between the target-organ-specific hazard index (TOSHI) approach, the relative potency factor (RPF) approach, and the mixture benchmark dose (BMD) approach, since all are based on health effect-specific values (i.e., Reference Values (RfVs) or RPFs) for the individual PFAS in the PFAS mixture. More discussion and comparison of approaches, as well as when they converge, is needed. For instance, given the mathematical correspondence between the RPF and mixture BMD approaches, EPA should consider revising the discussion of these two approaches to present them as essentially the same

(or highlighting any essential differences), and perhaps also merging them into a single section." (EPA SAB, 2022)
    - EPA has added a new section (Section 8.0) that describes similarities and differences among the different component-based mixtures assessment approaches. In addition, EPA has made the revision to use the same hypothetical example mixture of five PFAS (ranging from data poor to well-studied) for all of the illustrative examples so that the user can better understand similarities/differences among the approaches.
- "For both the RPF and mixture BMD approach, EPA's approach would be strengthened by using PODs from animal studies that are based on human equivalent doses (HEDs) rather than administered doses. The SAB found it difficult to envision situations in which the mixture BMD was advantageous; therefore, EPA should provide additional information on how the proposed Mixtures BMD approach will be applied in practice." (EPA SAB, 2022)
    - Text has been added in several places to indicate that it is optimal to calculate and use HEDs rather than oral administered dose in test animals where and when possible. This includes additional text that walks the reader through EPA's logic flow for cross-species scaling (see new Subsection 5.2.1). Regarding the Mixture BMD (M-BMD), text has been added to better articulate when this specific approach is more appropriate (e.g., component chemical data that indicate common health outcome but with non-congruent dose-response functions). Further, Subsection 7.3 has been revised to reiterate the conditions that warrant consideration of this specific component-based mixtures approach (as opposed to the RPF method).

1.3 Background on PFAS

PFAS are a large group of structurally diverse anthropogenic chemicals that include PFOA, PFOS, and thousands of other fully or partially fluorinated chemicals. Based on three related structural definitions associated with EPA's identification of PFAS to be included in the fifth Contaminant Candidate List (CCL; see below), the universe of environmentally relevant PFAS, including parent chemicals, metabolites, and degradants, is approximately 12,000 compounds.[4] 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, Buck et al. (2021) proposed that the number of PFAS currently used in commercial products at the time of the drafting of this document is approximately 250 substances.

PFAS have been manufactured and used in a wide variety of industries around the world, including in the United States since the 1940s. In general, PFAAs studied to date 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; Evich et al., 2022). Conversely, the larger PFAS universe is more structurally and physicochemically diverse and includes categories of substances that may be more or less stable, persistent, and/or bioaccumulative compared to PFAAs studied thus far (see National PFAS Testing Strategy;

[4] See EPA List of PFAS Substances (Version 4) available at: https://comptox.epa.gov/dashboard/chemical-lists/PFASMASTER

EPA, 2021f). The chemical structures and physical-chemical properties of some 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 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 subclasses 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, 2021c). 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). Several U.S. federal, state, and industry stakeholders as well as European entities have posited various definitions of what constitutes a PFAS. OECD, an international organization comprised of 38 countries, recently published a practical guidance regarding the terminology of PFAS (OECD, 2021). The OECD-led "Reconciling Terminology of the Universe of Per- and Polyfluoroalkyl Substances: Recommendations and Practical Guidance" workgroup provided an updated definition of PFAS, originally posited in part by Buck et al. (2011), as follows: "PFASs are defined as fluorinated substances that contain at least one fully fluorinated methyl or methylene carbon atom (without any H/Cl/Br/I atom attached to it), i.e. with a few noted exceptions, any chemical with at least a perfluorinated methyl group (–CF3) or a perfluorinated methylene group (–CF2–) is a PFAS". It is not within the scope of this framework to compare and contrast the various definitions, or the nuances associated with defining or scoping PFAS; rather the reader of this document is referred to OECD (2021) for review. However, for the purpose of development of EPA's CCL 5, the structural definition of PFAS includes chemicals that contain at least one of the following three structures:

- R-(CF_2)-CF(R′)R″, where both the CF_2 and CF moieties are saturated carbons, and none of the R groups can be hydrogen (TSCA draft definition);
- R-CF_2OCF_2-R′, where both the CF_2 and CF moieties are saturated carbons, and none of the R groups can be hydrogen; and
- $CF_3C(CF_3)$R′R ″, where both the CF_2 and CF moieties are saturated carbons, and none of the R groups can be hydrogen.

It should also be noted that what defines or constitutes a PFAS may change or evolve over time and 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 acids	Compounds in which all carbon-hydrogen bonds, except those on the functional group, are replaced with carbon-fluorine bonds	Perfluoroalkyl carboxylic and sulfonic acids (e.g., PFOA, PFOS), perfluoroalkyl phosphonic and phosphinic acids, perfluoroalkylether carboxylic and sulfonic acids
Polyfluoroalkyl acids	Compounds in which all carbon-hydrogen bonds on at least one carbon (but not all) are replaced with carbon-fluorine bonds	polyfluoroalkyl carboxylic acids, polyfluoroalkylether carboxylic and sulfonic acids
PFAS Polymers	**Structural Elements**	**Example PFAS Families**
Fluoropolymers	Carbon-only polymer backbone with fluorines directly attached	polytetrafluoroethylene, polyvinylidene fluoride, fluorinated ethylene propylene, perfluoroalkoxyl polymer
Polymeric perfluoropolyethers	Carbon and oxygen polymer backbone with fluorines directly attached to carbon	F-(CmF2mO-)nCF3, where the CmF2mO represents -CF2O, -CF2CF2O, and/or -CF(CF3)CF2O distributed randomly along polymer backbone
Side-chain fluorinated polymers	Non-fluorinated polymer backbone with fluorinated side chains with variable composition	n:1 or n:2 fluorotelomer-based acrylates, urethanes, oxetanes, or silicones; perfluoroalkanoyl fluorides; perfluoroalkane sulfonyl fluorides

Notes:
[a] Amalgamation of information from Figure 9 (OECD, 2021) and 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 (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 vs. 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. Groups, Structural Traits and Examples of Perfluoroalkyl Acids (PFAA), including Perfluoroalkylether Acids (PFEAAs)[a]

Group	Functional Group	Examples
Perfluoroalkyl carboxylic acids (PFCAs)	-COOH	Perfluorooctanoic acid (PFOA), C7F15COOHb
Perfluoroalkane sulfonic acids (PFSAs)	-SO3H	Perfluorooctane sulfonic acid (PFOS), C8F17SO3H
Perfluoroalkyl phosphonic acids (PFPAs)	-PO3H2	Perfluorooctyl phosphonic acid (C8-PFPA)
Perfluoroalkyl phosphinic acids (PFPIAs)	-PO2H	Bis(perfluorooctyl) phosphinic acid (C8/C8-PFPIA)
Perfluoroalkylether carboxylates (PFECAs)	-OC2F4OCF2COOH	Perfluoro-2-methyl-3-oxahexanoic acid (GenX), 4,8-Dioxa-3H-perfluorononanoic acid (ADONA)
Perfluoroalkylether sulfonic acids (PFESAs)	-OCF2CF2SO3H	Nafion byproduct 2
Perfluoroalkyl dicarboxylic acids (PFdiCAs)	HOOC-CnF2n-COOH	Perfluoro-1,10-decanedicarboxylic acid, Perfluorosebacic acid
Perfluoroalkane disulfonic acids (PFdiSAs)	HO3S-CnF2n-SO3H	
Perfluoroalkane sulfinic acids (PFSIAs)	-SO2H	Perfluorooctane sulfinic acid

Notes:
[a] Modified from Figure 9 in OECD, 2021
[b] The anionic form is most prevalent in water matrices.

Table 1-3. Characterization System 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

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
	Short-chain PFSAs			Long-chain PFSAs				

Notes: 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.
For brevity, Table 1-3 only includes PFAAs of 3–10 carbons; the long-chain class of PFCAs and PFSAs can be expanded considerably.
[a] Modification of Table 2-2 (ITRC, 2022)

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, and the human body. Importantly, the protonated and anionic forms may have different physicochemical and environmental fate and transport properties. It should also be noted that the structural diversity of PFAS is far greater than the PFCAs and PFSAs indicated in Table 1-3. There are branched, cyclic, aromatic, and multi-component (e.g., polymers) structures that have been or are currently classified as PFAS. However, in general, the linear PFCAs and PFSAs have been the most well-studied PFAS to date and indeed have been the primary focus of formal human health risk assessment activities in the federal and state sectors.

1.4 Occurrence of PFAS Mixtures

Improved analytical monitoring and detection methods have enabled detection of the co-occurrence of multiple PFAS in drinking water, ambient surface waters, aquatic organisms, biosolids (sewage sludge), and other environmental media.[5] PFOA and PFOS have historically been target analytes, but recent monitoring studies have begun to focus on additional PFAS via advanced analytical instruments/methods and non-targeted analysis (De Silva et al., 2020; McCord and Strynar, 2019; McCord et al., 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 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)

[5] For a more detailed discussion of the occurrence of PFOA, PFOS, and other PFAS in potential human exposure sources see the relative source contribution (RSC) sections in EPA (2022a,b,c,d).

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 Adamson, 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.[6] 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, 2021d). Additionally, these results show that PFAS are very likely to co-occur as mixtures in the environment. For water systems, these data suggest that systems with high concentrations of one PFAS compound are more likely to have higher concentrations of other PFAS and that there is notable co-occurrence at elevated concentrations (Cadwallader et al., 2022).

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; McCord and Strynar, 2019). 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 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

[6] 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).

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, in the Laurentian Great Lakes region, and targeted studies of impacted (e.g., industrial) sites. 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). A study in New Jersey found co-occurrence of PFAS in ambient water, sediment, and fish at sites with historic and current industrial activities (Goodrow, et al., 2020). Fish tissue concentrations of PFOS were generally higher than other PFAS and high enough in nearly all fish species to trigger fish consumption advisories.

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).

1.5 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, 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, including PFNA and longer-chain PFCAs, by 95% on a global basis by no later than 2010 and eliminate these substances in products by 2015 (EPA, 2021c). 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 HFPO-DA. 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 (Kotlarz et al., 2020; Yu et al., 2020; Table 17-6 in ITRC 2022; ATSDR, 2022). 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.

1.6 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, 2016a,b) because the RfDs were based on 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 October 2022, Alaska, Colorado, Florida, Montana, 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 developed values below 70 ppt and/or included additional PFAS (beyond PFOA and PFOS) in their combined toxicity approach based on similarity in chemical structure and/or toxicity (Table 1-4).Wisconsin has established a maximum concentration of 20 ppt for combined PFOA and PFOS (WI DHS, 2019a,b), 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).

In June 2022, EPA issued draft interim updated health advisories for PFOA and PFOS and final health advisories for HFPO-DA and PFBS (EPA, 2022a,b,c,d). EPA's interim updated health advisories for PFOA and PFOS are 0.004 ng/L and 0.02 ng/L, respectively, and the final health advisories for HFPO-DA and PFBS are 10 ng/L and 2,000 ng/L, respectively. Each of the health advisory documents provides an example of how to use the HI approach to assess the potential noncancer risk of a mixture of PFOA, PFOS, HFPO-DA, and PFBS (EPA, 2022a,b,c,d) consistent with the approach presented in this framework document.

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 mixture 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, not including PFOSA, 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 framework (Borg et al., 2013; RIVM, 2018). Although not specifically related to drinking water, the European Food Safety Authority (EFSA) has also taken PFAS mixture toxicity into consideration in their development of a Tolerable Weekly Intake for the sum of PFOA, PFNA, PFHxS, and PFOS (4.4 ng/kg/week) (EFSA, 2020).

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, 2022a,b,c,d; EPA, 2016a,b;)	2022 (supersedes 2016 advisory)	0.004, PFOA (Interim) 0.02, PFOS (Interim) 10, HFPO-DA 2000, PFBS	HI example for PFOA, PFOS, HFPO-DA and PFBS	Interim Updated Drinking Water Health Advisory for PFOA and PFOS based on human epidemiological data. HI example assumes dose additive toxicity of PFOA, PFOS, HFPO-DA, and PFBS.
	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 2016 Health Advisory.
Colorado (USA) (CDPHE, 2020)	2020	70	PFOA and PFOS	Application of EPA 2016 Health Advisory.
Delaware (USA) (DE DHHS, 2021)	2018	17[c]	PFOA and PFOS	Based on the sum of approximately 50% of each individual MCL[d]
Florida (USA) (Florida Health, 2020)	2019	70	PFOA and PFOS	Application of EPA 2016 Health Advisory.
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 2016 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 2016 Health Advisory but includes an additional uncertainty factor.

Entity	Date	Conc (ng/L)	Sum of PFAS	Background
Montana (USA) (MT DEQ, 2020)	2019	70	PFOA and PFOS	Application of EPA 2016 Health Advisory
Ohio (USA) (Ohio EPA, 2019)	2019	70	PFOA and PFOS	Application of EPA 2016 Health Advisory
Rhode Island (USA) (RIDEM, 2017)	2019	70	PFOA and PFOS	Application of EPA 2016 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.

Entity	Date	Conc (ng/L)	Sum of PFAS	Background
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, HI approach is applied.

Notes:
[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, Illinois, Michigan, Minnesota, New Jersey, New York, Pennsylvania, Texas, and Washington. Some states have applied EPA's 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] Proposed level based on the Delaware PFOA and PFOS MCL Implementation Plan
[d] Based on a PFOA Maximum Contaminant Level (MCL) of 21 ppt and PFOS MCL of 14 ppt.

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

This document describes a framework of options with different levels of data requirements and objectives for estimating the 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, 2000b). 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, 2000b); 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 (CAA) Amendments of 1990 (CAA, 1990); the Safe Drinking Water Act (SDWA) 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, 2000b) were developed, in part, to be responsive to these laws. When developing assessment 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 framework does not supersede previously published EPA guidance on mixtures or longstanding EPA approaches used to assess health risks of contaminants including chemical mixtures under various environmental statutes (e.g., Federal Insecticide, Fungicide, and

Rodenticide Act (FIFRA); FQPA; Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA)).

The objective of this document is to provide a flexible, data-driven framework that facilitates practical component-based mixtures evaluation of two or more PFAS under an assumption of dose additivity. All approaches presented involve integrating dose-response metrics that have been scaled based on the potency of each PFAS in the mixture. Three approaches are presented:

1) The HI approach is likely the most health protective approach because it is based on the most sensitive health outcome (lowest RfD) for each chemical and provides a risk indicator for exposure to a PFAS mixture of concern (Section 5),

2) The RPF approach provides a mixture toxicity estimate by scaling the potency of component chemicals, for a common health effect, relative to a well-characterized member of the mixture, referred to as the index chemical (IC) (Section 6);

3) The M-BMD approach uses a DA model-based equation (similar to the Berenbaum equation; Section 4.2.6 in EPA, 2000b) to calculate a BMD (e.g., BMD_{10HED}) for the mixture (Section 7).

The HI facilitates estimation of potential joint toxicity associated with co-occurrence of chemicals in environmental media (e.g., water, soil) (EPA, 2000b). 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 MOA[7] or have a conserved toxicological target (i.e., share a common apical endpoint/effect); and (2) the dose-response functions for the effect of concern are congruent (similar shape and slope) over the exposure ranges most relevant to the decision context (EPA, 2000b). The RPF method is illustrated in Section 6 using common target organs/pathways including liver, thyroid, and developmental effects. A MOA for a given toxic effect is a detailed description of the source to outcome pathway, including the key molecular/cellular or organellar events, leading to a defined health effect or syndrome of effects (e.g., "developmental" can be a collection of related outcomes). In general, a health effect or outcome is the terminus of one or more operant MOA(s). In addition to the HI and RPF methods, the assumption of similarity in MOA or toxicological target is also inherent when applying the M-BMD approach; however, in contrast to the RPF method there is no necessity or assumption of congruent dose response functions (i.e., same/similar shape or slope) across chemicals. This approach provides more accurate predictions of a mixture effect even if the slopes of the dose response curves differ among the chemicals (Section 7). 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 for many PFAS. As such, this framework focuses the biological level of organization for evaluation of potential dose additivity on ***similarity of toxicological endpoint/effect/adverse outcome*** rather than similarity in MOA, which is consistent with EPA mixtures guidance (EPA, 2000b) and expert opinion from the National Academy of Sciences, National Research Council (NRC, 2008).

[7] Mode of action is a sequence of key events and processes, starting with interaction of an agent and a cell, proceeding through operational and anatomical changes, and resulting in a noncancer effect or cancer formation.

Recognizing the evolving and dynamic nature of PFAS science, the component-based mixtures assessment approaches described herein are flexible to allow for consideration of new or evolving dose-response data and toxicity assessments as they become available. Additionally, because publicly available traditional (e.g., *in vivo* mammalian) toxicity studies are limited to only a small fraction of the ~12,000 PFAS, this framework also provides suggestions for practical integration of validated NAMs such as toxicogenomics (e.g., *in vitro* cell bioactivity) and *in silico* platforms (e.g., structure-activity, read-across) into the HI, RPF, and M-BMD approaches. The illustrative examples in Sections 5, 6, and 7 are intended to demonstrate the application of dose-additive-based component-chemical mixture approaches using hypothetical human health relevant toxicity and 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, 2000b). In those guidance documents, EPA proposed a 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

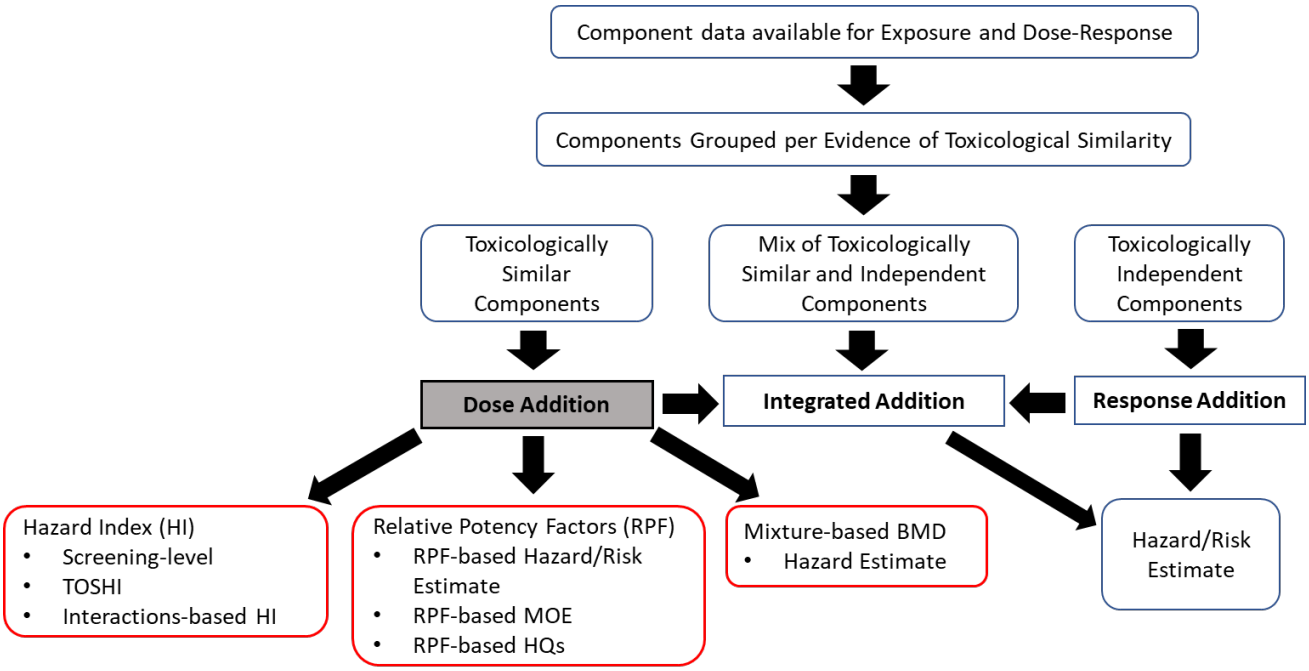
Component-based methods that EPA has developed for evaluating potential additivity of dose, response, or both are shown in Figure 2-1. Based primarily on similarity in toxicity endpoint/health effect of PFAS, this framework document focuses on the use of dose-additive, component-based methods (left side of Figure 2-1; shaded box), specifically the HI (Section 5), RPF (Section 6), and M-BMD (Section 7) approaches. The methods involve different assumptions and data requirements and objectives for evaluating the joint toxicity of component chemicals in a "mixture." Each of the methods are introduced and detailed in Sections 5–7 and include demonstration of application using a hypothetical five component PFAS mixture. Specifically, to facilitate the use of potentially disparate sources and types of PFAS information in a mixture context, the data-driven application of the component-based methods presented in this framework document is demonstrated using a hypothetical mixture of five PFAS as follows:

> **PFAS 1** = comprehensively studied, most potent for effect(s), and has formal noncancer human health assessment value(s) and an HBWC available;
>
> **PFAS 2** = well-studied, second most potent for effect(s) among PFAS 1–3, and has formal noncancer human health assessment value(s) and HBWC available;
>
> **PFAS 3** = studied, least potent for effect(s) among PFAS 1–3, and has formal noncancer human health assessment value(s) and HBWC available;
>
> **PFAS 4** = experimental animal toxicity data available but no formal human health assessment and no HBWC; and
>
> **PFAS 5** = data-poor.

This hypothetical PFAS mixture is purposefully designed to demonstrate how the framework allows for flexible integration of information derived from diverse data types and sources. Opportunities for integrating PFAS into 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/or *in silico* platforms.

An important property of DA-based methods is that they can aid in the indication or estimation 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; i.e., 'something from nothing'). In dose additivity models such as the RPF approach, the sum of the scaled IC[8] equivalent doses/concentrations for each component can exceed the equivalent threshold dose of the mixture and result in a detectable response, which has been supported experimentally (Jonker et al., 1996; Silva et al., 2002).

[8] 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.



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.

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 (Van Der Ven et al., 2022; NRC, 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 component-based evaluation of PFAS mixtures 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. Section 3.2 discusses the evidence demonstrating that mixtures of chemicals disrupting common pathways typically produce dose additive alterations. In *in vivo* studies that rigorously tested accuracy for DA, Integrated Addition (IA) and RA model predictions for mixtures with components that disrupted common pathways, DA models provided predictions that were better than or equal to IA and RA predictions of the observed mixture effects (Section 3.2). Consistent with the conclusions of the National Academy of Sciences (NAS) (NRC, 2008), Boobis et al. (2011) and Martin et al. (2021) found that published studies in the literature (Section 3.2) support the assumption of DA as the default model for estimating mixture effects, even when the mixtures included chemicals with diverse MOAs (but common targets of toxic action). Further, these two large systematic reviews of the literature on chemical mixtures found little evidence for deviations from dose additivity, such as synergy or antagonism (Boobis et al. 2011; Martin et al., 2021). For example, Martin et al. (2021), following a review of more than 1,200 mixture studies (selected from > 10,000 reports), concluded that there was little evidence for synergy or antagonism among chemicals in mixtures and that DA should be considered as the default model. 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 data demonstrate otherwise. Further, experimental data demonstrate that PFOS, PFOA, and other PFAS disrupt signaling of multiple biological pathways resulting in common adverse effects on several biological systems and functions including thyroid hormone functioning, lipid synthesis and metabolism, developmental toxicity, and immune and liver function, and 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 mixture effects on common apical endpoints that generally are well predicted using DA models.

The general applicability of DA models is based on reviews of studies specifically designed to evaluate how well different mixture models predict the way chemicals in a mixture interact to produce effects. Studies evaluating mixture models and effects typically include an evaluation of individual chemical dose response curves and apply this information to different statistical models. In general, systematic reviews have noted that many mixture studies do not include

information conducive for evaluating the utility of different mixture models. The data from several studies (reviewed in Section 3.2) 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 *in vivo* database to evaluate mixture models, it should be assumed that any mixture acts in a DA manner if the individual chemicals produce common effects. This approach was fully endorsed by NRC (2008) and Martin et al. (2021).

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

3.2.1 Dioxin-Like Chemicals and Aryl Hydrocarbon Receptor Pathway Toxic Equivalence Factors (TEFs)

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). It should be noted that the TEF approach is a specialized application under the RPF umbrella but is only applicable when all mixture components induce an effect via an identical MIE/MOA (e.g., AhR agonism). DLCs such as PCBs, PCDFs, and PCDDs have been identified as AhR agonists. As such, 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 2,3,7,8-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 joint 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 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); and
- TEFs and TEQs based on AhR agonism are not necessarily predictive of chemical potency for effects mediated by other receptors or pathways.

EPA's TEFs have undergone several revisions (Van den Berg et al., 2006). In 2010, EPA published recommended TEFs for human health risk assessment of 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; and
- 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 effective dose (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/Pyrethrins – Central Nervous System and Behavior

Pyrethrins and pyrethroids share the ability to interact with voltage-gated sodium channels ultimately leading to neurotoxicity. Wolansky et al. (2009) administered a mixture of 11 pyrethroid pesticides to adult male rats 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.

EPA has determined that naturally occurring pyrethrins and synthetic pyrethroid pesticides form a common mechanism group. This common mechanism grouping is based on 1) shared structural characteristics, 2) shared ability to interact with voltage-gated sodium channels (VGSC), resulting in disruption of membrane excitability in the nervous system, and 3) ultimately neurotoxicity characterized by two different toxicity syndromes. In 2011, after establishing a common mechanism grouping for the pyrethroids and pyrethrins, EPA conducted a cumulative risk assessment using a RPF approach (EPA, 2011a).

3.2.3 Organophosphates – 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 non-additivity 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 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 1999, EPA determined that the OPs form a common mechanism group based on their shared ability to bind to and phosphorylate the enzyme acetylcholinesterase, leading to accumulation of acetylcholine and ultimately cholinergic neurotoxicity (EPA, 1999). As such, cumulative risk to OPs has been assessed using AChE inhibition as the source of dose response data. The most recent OP cumulative assessment was conducted in 2006 employing a RPF approach (EPA, 2006).

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 Estrogen agonists - Mixture Effects on the Female Reproductive Tract

Scientists have examined the effects of mixtures of estrogenic chemicals in the female rat using a 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 vs. 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, Conley et al. (2016) determined that DA was the most appropriate model for mixtures of these estrogenic compounds.

3.2.5 Phthalates in utero - Mixture Effects on the Female Reproductive Tract

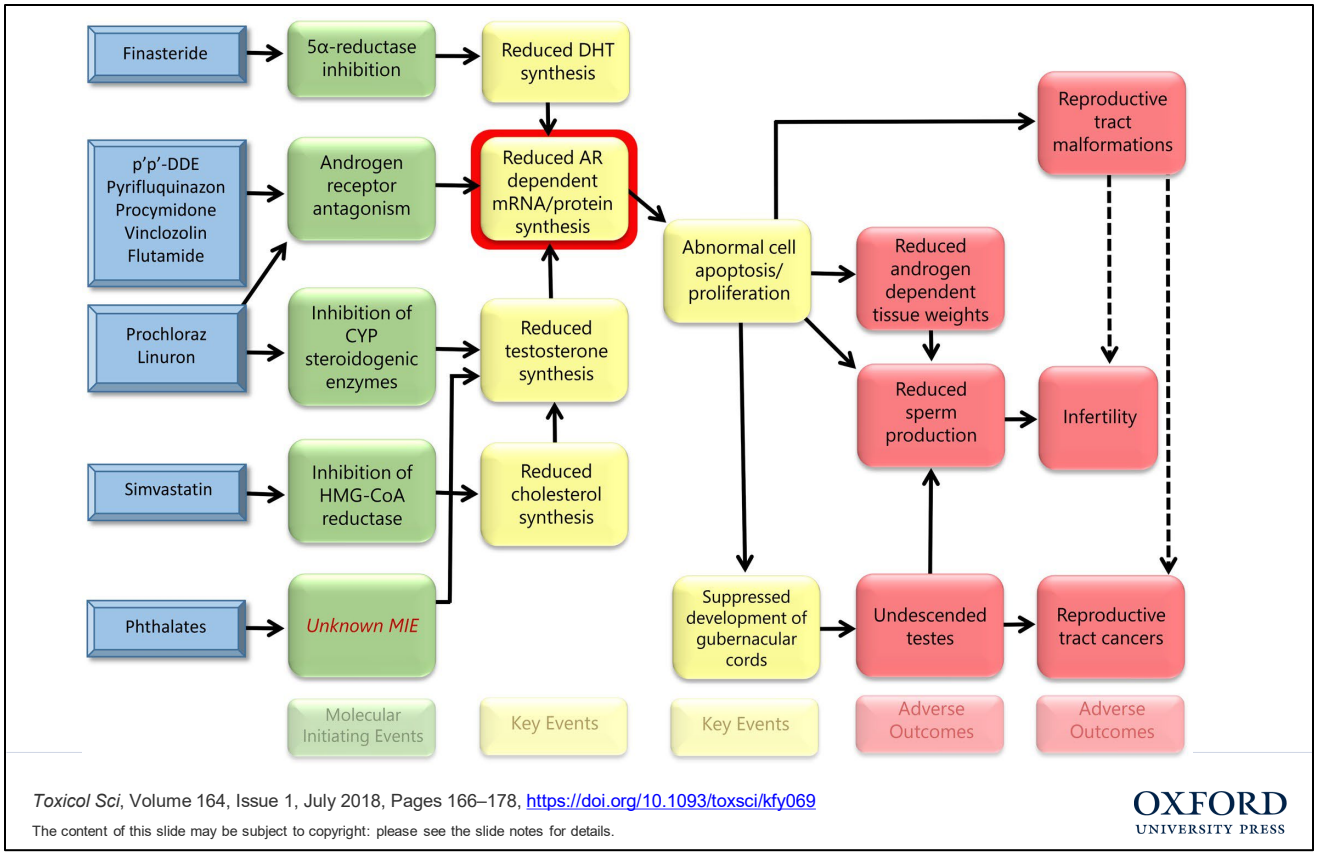
Hannas et al. (2013) reported that administration of a mixture of five phthalates (> 520 mg total phthalate) to pregnant rats from gestational days (GDs) 8 to 13 induced reproductive tract malformations in female rat offspring. These malformations included complete to partial uterine agenesis and agenesis 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 milligrams per kilogram per day (mg/kg/day)) and diethylhexyl (750 mg/kg/day) 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 Antiandrogens - Male Reproductive Tract Development

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 NRC (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 of 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 appear 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 mixture 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.

Recently, Gray et al. (2022) demonstrated that the *in utero* effects of a PFAS pesticide, pyrifluquinazon (contains a heptafluoroisopropyl side chain; see https://comptox.epa.gov/dashboard/chemical/details/DTXSID6058057) combined with the di-ortho phthalate ester dibutyl phthalate produced dose-additive effects that were more accurately predicted with DA models that did not require parallel slopes than with RA models for multiple male reproductive abnormalities.

All of these endocrine active 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 androgen-dependent tissues. Each of the identified chemicals/classes reduce the number of androgen receptor (AR) dimers, AR/AR, activated by an androgen agonist. AR antagonists, like vinclozolin or procymidone, accomplish abrogation of androgen-dependent signaling by blocking androgens from binding to ARs, and the PFAS pesticide 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 (DBP), di(2-ethylhexyl)phthalate (DEHP), dipentyl phthalate (DPeP), butyl benzyl phthalate (BBP), and diisobutyl phthalate (DIBP) reduce the levels of androgens available to respondent cell populations (Hannas et al., 2011; Howdeshell et al., 2008; Furr et al., 2014). In contrast, chemicals like finasteride inhibit the enzyme in tissues that converts testosterone to dihydrotestosterone (a more active androgen that has higher affinity for the AR) (Clark et al., 1990). The result of these related androgen disrupting AOPs is that 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 KEs disrupted in androgen signaling pathways by chemicals such as those identified in Figure 3-1, at the cellular-molecular level, explains why one should expect the mixtures to behave in a DA manner.

In summary, an examination of the literature on the effects of mixtures on male reproductive tract development demonstrates that common effects can be adequately modeled by DA, the chemicals acted in a DA manner even when including chemicals with different MIEs, and IA and RA models can underestimate the hazard of a mixture of chemicals acting on a common KE and 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) vs. DA or less-than-DA (sometimes referred to as antagonistic) interactions was recently reassessed by Martin et al. (2021). The authors conducted a systematic literature 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, greater-than-additive (i.e., synergism) and less-than-additive (i.e., antagonism) interactions may occur with co-exposure to chemicals that affect different target organs or different, unrelated AOPs. There are several examples of chemical interactions that deviate from DA in which one chemical has the capability to alter the metabolism of the other chemical(s). For example, 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, 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 vs. 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.

In addition to metabolic activity leading to synergistic or antagonistic interactions among chemical mixtures, there are other examples of deviations from DA that do not include chemicals that disrupt common KEs, AOPs, AOP networks, or target organs. For example, although melamine and its derivatives, including cyanuric acid, individually present low toxicity, together the compounds can lead to the formation of cyanurate crystals in nephrons, causing kidney effects and kidney failure in mammals. Such impacts have been observed in cats and dogs from adulteration of pet food (Jacob et al., 2011), as well as infants and young children in China from contaminated infant formula and related dairy products (WHO, 2008).

3.4 PFAS Dose Additivity

Per- and polyfluoroalkyl acids (PFAA), such as PFOA and PFOS, with linear or branched alkyl or alkyl ether chains and sulfonic or carboxylic acid functional groups, as well as PFAA precursors, while not necessarily toxicologically identical, do 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 common KEs and adverse outcomes, support the use of dose additive models for estimating mixture-based risks, even in instances where chemicals with disparate MIEs were included. Thus, in the absence of detailed characterization of 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 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 PFAAs including ether-linked structures. Below is a brief overview of similarities and differences in MIEs, KEs, and adverse outcomes that have been reported for those PFAS studied to-date and experimental evidence which supports dose additive effects from combined exposure to multiple PFAS. This overview highlights study 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 PFAS toxicity endpoints in experimental animal studies and observational human studies can be found elsewhere (ATSDR, 2021; EFSA et al., 2018, 2020).

Mechanistically, *in vitro* and *in vivo* studies have demonstrated the activation of multiple nuclear receptors from exposure to a range of PFAS indicating several potential MIEs for PFAS-relevant AOPs. The most commonly reported MIE associated with many PFAS is activation of peroxisome proliferator activated receptor alpha (PPARα) based on *in vitro* binding and transcriptional activation assays (Behr et al., 2020; Evans et al., 2022; Ishibashi et al., 2019; Nielsen et al., 2022; Takacs and Abbott, 2007; Vanden Heuvel et al., 2006; Wolf et al., 2012), *in vitro* upregulation of PPARα target genes (Bjork et al., 2011), and *in vivo* tissue-specific upregulation of PPARα target genes (Bjork et al., 2008; Rosen et al., 2007). All PFAA carboxylates and sulfonates included in the NTP 28-day studies displayed upregulation of the PPARα target genes Acox1 and Cyp4a1 in male and female rat livers. PPARα is a highly conserved transcription factor that regulates pleiotropic effects on mammalian energy homeostasis and lipid metabolism, among others. Similarly, multiple PFAS have been shown to activate peroxisome proliferator activated receptor gamma (PPARγ) *in vitro* (Evans et al., 2022; Houck et al., 2021; Vanden Heuvel et al., 2006) and upregulate PPARγ target genes *in vitro* (Marques et al., 2022) and *in vivo* (Rosen et al. 2017). PPARγ is also a highly conserved transcription factor that regulates multiple physiological processes including adipogenesis and glucose metabolism. Further, *in vivo* studies of tissue-specific gene expression patterns have also demonstrated activation of constitutive androstane receptor (CAR) for both PFOA and PFOS, among other PFAS, due to upregulation of CAR-dependent genes (Rosen et al., 2017). Similar to the PPARα target genes above, all PFAA studied by NTP displayed upregulation of the CAR-inducible genes Cyp2b1 and Cyp2b2 in adult male and female rat livers (NTP, 2019a,b). Additional *in vitro* data indicate potential involvement from several other nuclear receptors including estrogen receptor alpha (Evans et al., 2022; Houck et al., 2021, Kjeldsen and Bonefeld-Jorgensen, 2013), pregnane X receptor (Bjork et al., 2011; Houck et al. 2021), farnesoid X receptor (Bjork et al., 2011), and liver X receptor (Bjork et al. 2011, Houck et al. 2021). Multiple PFAAs, including PFOA and PFOS, activate multiple nuclear receptors and gene transcription pathways, which is a primary basis for positing shared or overlapping AOPs across PFAAs.

In addition to the cell and/or tissue specific gene expression changes described above, multiple KEs downstream of the above potential MIEs are also shared between PFOA, PFOS, and other PFAAs. In both rodent and non-human primate studies, serum lipids (cholesterol, triglycerides) are consistently reduced and markers of liver injury or 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, and PFOS (NTP, 2019a,b). Further, circulating thyroid hormone concentrations are reduced from oral PFAA exposure with all compounds tested in the NTP 28-day studies being associated with decreased serum total and free thyroxine (T4) (NTP, 2019a,b). Ether linked PFAAs have also been shown to reduce circulating thyroid hormone concentrations (Conley et al. 2019, 2022). 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. Characterization of PFAS relevant KEs is currently an area of high research activity as additional pathways associated with the reported MIEs across various life stages and species continue to be investigated.

Similar adverse outcomes at the organ and whole animal levels have been described for PFAAs including PFOA and PFOS. Developmental exposure studies with PFOA, PFOS, PFNA, HFPO-DA and Nafion byproduct 2 (NBP2) in rats and/or mice have reported consistent effects on pups including reduced offspring survival/viability and reduced offspring body weight (Abbott et al., 2007, 2009; Blake et al., 2020; Butenhoff et al., 2004; Conley et al., 2021b, 2022a; Das et al., 2015; Lau et al., 2003, 2006; Luebker et al., 2005a,b; Thibodeaux et al., 2003). PFAS studied by NTP (2019a,b) have been observed to increase rat liver weights and produce 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 conserved across PFAS such as PFOA, PFOS and other PFAAs, and plausibly associated with the common molecular mechanisms and KEs. However, it is important to recognize that while there are same/similar qualitative effect profiles across many PFAS (e.g., liver injury; decreased thyroid hormones) there are quantitative oral potency differences in reported effects, and not all effects appear to be shared across all PFAS, or even across all PFAAs, at the dose levels reported in published studies. For example, PFHxS did not affect serum ALT in male or female rats at any dose in the NTP 28-day studies, while all other PFAAs tested increased serum ALT levels. The specific molecular mechanisms or precise modes of action for a given adverse outcome may be disparate across some PFAS. Studies utilizing transgenic mice with PPARα deletion have demonstrated that some effects, such as survival of neonates following *in utero* PFOA exposure, were dependent on PPARα involvement, while this effect appeared independent of PPARα for PFOS (Abbott et al. 2007, 2009). It is important to note in those studies that fetal mortality (as opposed to neonatal mortality) was independent of PPARα genotype for PFOA. The relevance of rodent PPARα-based effects has been debated in the toxicology literature for decades, largely as it relates to hepatocarcinogenesis, yet the pharmacological utility of PPARα modulation is widely accepted and exploited in the development of therapeutics. Further, studies of PPARα knockout mice have also demonstrated that many liver effects are PPARα independent for PFOA and PFOS (Abbott et al., 2007, 2009). Although there is potential for disparate MIEs in PFAS related AOPs and there is a lack of mechanistic characterization for most PFAS-mediated effects, it is a reasonable health-protective assumption that health effects shared across PFAS in a given mixture will be dose additive.

Limited work has been conducted on combined exposure to PFAS in experimental systems either *in vitro* or *in vivo*. A few *in vitro* studies have directly assessed the mixture-based effects of combined PFAS exposures by comparing observed experimental data with model-based predictions. For example, Wolf et al. (2014) evaluated *in vitro* PPARα activation and observed joint toxicity of combined exposure to binary combinations of PFOA and PFOS, PFNA, PFHxA, and PFHxS that were consistent with dose additivity in the lower dose ranges, but the authors reported slightly greater than additive effects at higher mixture doses. In contrast, Carr et al. (2013) reported slightly less than additive responses for *in vitro* PPARα activation of binary mixtures of PFAAs including PFOA, PFNA, PFOS, and PFHxS. Further, Ojo et al. (2020)

reported both synergistic and antagonistic effects compared to a concentration addition model for binary, ternary, and multi-component mixtures of PFAAs for cytotoxicity in HepG2 cells. Most recently, Nielsen et al. (2022) demonstrated the utility of generalized concentration addition for PPARα activation of PFAA mixtures in an *in vitro* system with variable efficacy across compounds; however, there are no studies indicating this model variation on simple concentration addition is applicable for prediction of *in vivo* mixture effects. Importantly, as described above, systematic reviews of chemical mixture studies across various compound classes indicate that departures from dose additivity are uncommon and rarely exceed minor deviations (~2-fold) from predictions based on additivity (Martin et al. 2021). Similarly, recent PFAS mixture studies in zebrafish reported interactions for combinations of PFOA and PFOS, but departures from additive models were also minor (Ding et al., 2013). Menger et al. (2020) reported zebrafish behavioral effects from a PFAS mixture that were less than individual PFAS, however evaluation of chemical dose response and comparison to mixture models was not conducted. Regarding zebrafish PFAS effects, it is notable that fish PPARγ has relatively low sequence homology to that of mammalian PPARγ (Zhao et al., 2015) and the potent PPAR*γ* agonist rosiglitazone activates this rat, mouse and man receptor *in vitro* but not in three species of fish or the clawed frog (Medvedev et al., 2020). The interactions described in the literature thus far for combined *in vitro* exposure to PFAAs demonstrate results that are either consistent with or have relatively minor deviations from predictions based on concentration additive models.

Mammalian *in vivo* toxicity studies evaluating exposure to multiple PFAS are more limited but recent studies indicate that exposure to a mixture of PFOA, PFOS, and PFHxS in mice (Marques et al., 2021), a mixture of PFOA, PFOS, PFNA, PFHxS, and HFPO-DA in mice (Roth et al., 2021), and a mixture of PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFBS, PFHxS, and PFOS in rabbits produced numerous significant health effects compared to control animals, which were consistent with the spectrum of individual PFAS effects described above (e.g., liver injury; thyroid hormone alterations). However, these studies did not include individual PFAS dose response data or conduct any mixture model-based analyses, so it is not possible to ascertain if the mixtures behaved in a DA or RA manner, or if interactions occurred.

Currently, developmental toxicity studies of PFAS mixtures in rats are ongoing at EPA ORD. One study recently investigated *in vivo* effects in maternal rats and offspring from combined exposure to PFOA and PFOS during gestation and early lactation (Conley et al., 2022b). The study included a series of experiments designed to characterize dose response curves across multiple endpoints for PFOA and PFOS individually, followed by a mixture study of the two chemicals combined. The mixture experiment was designed to test for shifts in the PFOA dose response curves from combined exposure to a fixed dose of PFOS, compare DA and RA model predictions, and conduct an RPF analysis as clear demonstrations of mixture effects. Exposure to binary combinations of PFOA and PFOS significantly shifted the PFOA dose response curves left towards elicitation of effects at lower doses compared to PFOA only exposure. This clearly indicated mixture effects for a range of endpoints including decreased pup survival, maternal and pup bodyweight, pup serum T3 and glucose, and increased maternal kidney weight, maternal and pup liver weight, and pup bile acids, BUN, and bilirubin. Maternal kidneys and maternal and pup livers in the mixture study also displayed a range of treatment related histopathological lesions. For nearly all endpoints amenable to mixture model analyses, the DA equation produced equivalent or better estimates of observed data than RA. Similarly, for nearly all maternal and

neonatal endpoints modeled, the RPF approach produced accurate estimates of dose additive mixture effects. Only maternal bodyweight at term and gestational weight gain demonstrated departures from additivity and these effects were less than additive. This work is ongoing with multiple KE analyses still to be conducted on samples collected during the studies. However, results thus far support the hypothesis of joint toxicity on shared endpoints from PFOA and PFOS co-exposure, and dose additivity as a reasonable assumption for predicting mixture effects of co-occurring PFAS. Further, Conley et al. (2021a) presented preliminary data on an unpublished mixture study of PFOS, HFPO dimer acid, and 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 mixture effects. The results discussed above provide robust evidence of combined toxicity of PFOA, PFOS, and other PFAS on multiple maternal and developmental endpoints and the greater accuracy of DA for predicting mixture effects *in vivo* than RA.

In summary, systematic identification and assembly of PFAS data reported in the literature support an assumption of similarity in toxicity profiles for several health effect domains (for review see Carlson et al., 2022). Importantly, study results reported in this section across multiple chemical classes, biological effects, and study designs clearly support a dose-additive mixture assessment approach. For example, 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. Further, the National Academies of Sciences, Engineering, and Medicine (NASEM, 2022) recently recommended clinicians apply an additive approach for evaluating patient levels of PFAS currently measured in NHANES. EPA will continue to review how mixtures of PFAS and other chemicals interact. Dose additivity is proposed as the "default" model and other models will be evaluated when data empirically support or demonstrate significant deviations from dose additivity.

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 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 environmental 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 human exposure to mixtures of PFAS (see Sections 5–7).

EPA's *Supplemental Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (EPA, 2000b) 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 components' 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. However, as with a whole mixture of concern, information pertaining to a sufficiently similar mixture is rare. The whole mixture options should be investigated prior to moving to a component-based mixtures approach.

4.2 Data-Driven Component-Based Mixtures Approaches 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 derives from exposure-response studies of individual chemicals, component-based mixtures risk assessment is particularly relevant (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, 2000b), the currently available evidence on PFAS, and several other classes of environmental chemicals, supports an assumption of dose additivity (see Section 3). The HI and RPF are two component-based mixture approaches based on dose additivity, that are well validated, supported by peer-reviewed guidance, and actively used by EPA. These two approaches are discussed below and include illustrative examples that are based on a hypothetical five component mixture of PFAS (see Sections 5 and 6). An alternative "M-BMD" approach, generally based on the Berenbaum equation (see Section 4.2.6 in EPA mixtures guidance (2000b)), is also a dose additive approach that is described and illustrated (see Section 7). The primary difference in the RPF and M-BMD approaches is that RPF assumes component chemical dose response slopes are congruent, while the M-BMD approach is more applicable for mixture component chemicals with dissimilar slopes. 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, 2022; Mumtaz et al., 2021), lending confidence to the direction of this framework document in guiding formal component-based assessment of PFAS mixtures. The M-BMD approach was described and supported in both EPA's mixtures guidance (2000) and by the National Research Council (NRC) (NRC, 2008), and laboratory studies have provided empirical evidence (Gray et al., 2022 and example in Section 7).

4.2.1 Conceptual Framework of the Approach

A pragmatic data-driven approach to the application of component-based evaluation of mixtures of PFAS with variable hazard and dose-response databases is presented in Figure 4-1.

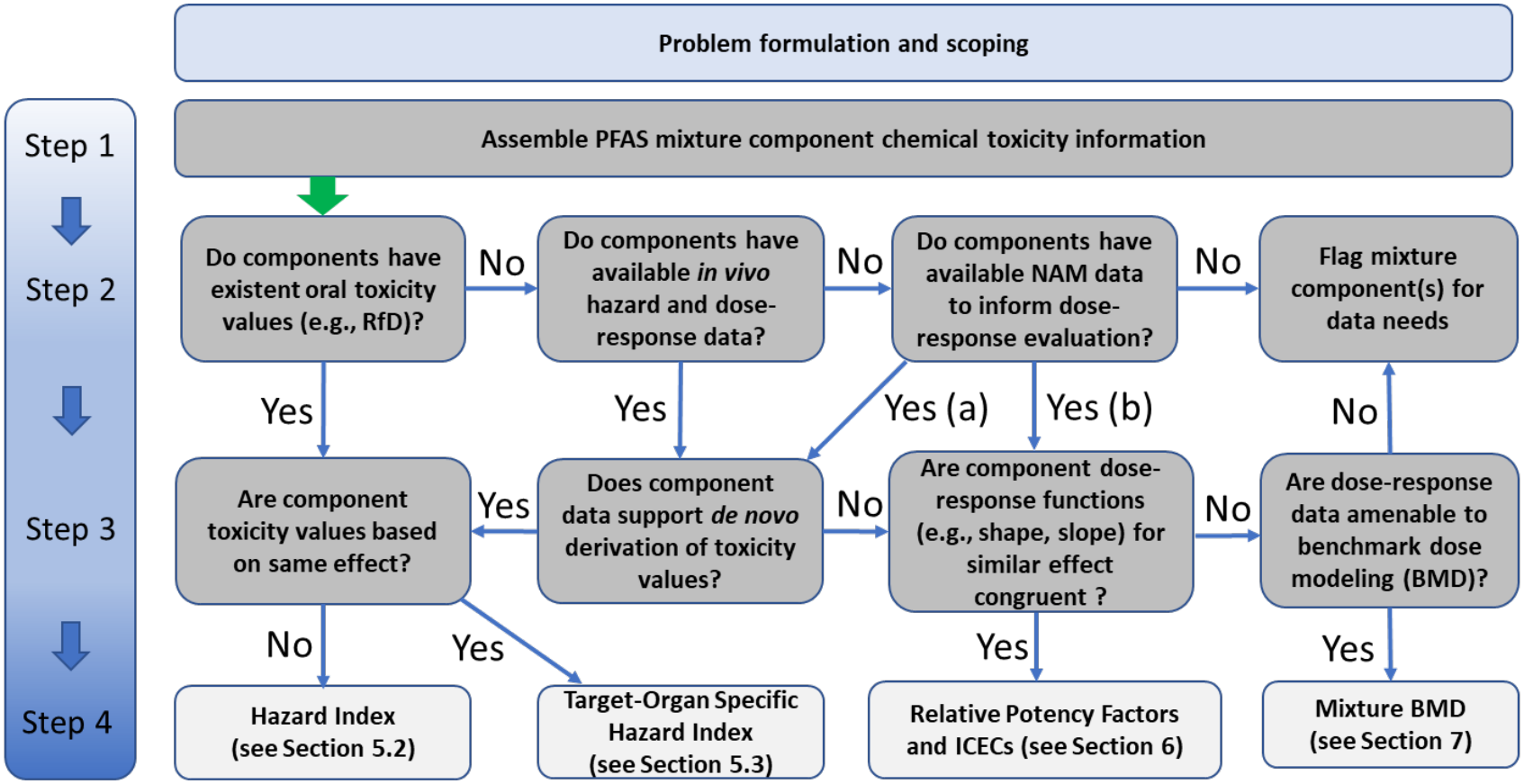


Figure 4-1. Framework for data-driven application of component-based assessment approaches for mixtures of PFAS based on an assumption of dose additivity.

The general steps of the component-based approach as shown in Figure 4-1 are as follows:

Problem formulation and scoping. Problem formulation is the part of the risk assessment framework that articulates the purpose of the assessment and defines the problem (e.g., PFAS source and occurrence, fate and transport, populations/subpopulations potentially at risk, health endpoints). Problem formulation also typically includes development of a conceptual model and analysis plan and includes engagement with potentially affected stakeholders (e.g., co-regulators like states and tribes, risk managers, affected community) to discuss foreseeable science and implementation issues.

Step 1: Assemble information.

Step 1 of the data-driven mixture assessment approach is to identify available hazard and dose-response information for: (1) a whole mixture of the PFAS of potential concern at component proportions consistent with the environmental sampling data; (2) a sufficiently similar mixture; and/or (3) data for the individual component PFAS. If whole toxicity data for the mixture itself or a sufficiently similar mixture are not available or are insufficient, then a structured search, collection, and assembly of all available toxicity data for mixture component PFAS of potential concern is conducted. Although the optimal approach would be to utilize formal systematic literature search and review principles as set forth by EPA (please see the systematic review protocol for PFBA, PFHxA, PFHxS, PFNA, and PFDA as an example[9]), the user of this framework may employ a structured literature search approach of their choosing so long as the underpinning decisions resulting in the literature inventory and data landscape used in steps 2–4 of the framework approach are transparent. It should be noted that while this step is primarily intended for identification and harvesting of traditional human epidemiological and/or experimental animal toxicity data, it is ideal to also assemble information such as toxicokinetic (TK)-relevant parameters (e.g., clearance, plasma/serum half-life, volume of distribution), mechanistic pathway data, empirical (or predicted) physicochemical properties, and if available, validated NAM data such as cell bioactivity, high(er)-throughput transcriptomics, and/or structure-activity/read-across.

Step 2: Evaluate data objectives.

Once harvested, curated, and arrayed, the user should be able to evaluate the following primary data objectives: (a) Existence of human health risk assessment values (e.g., EPA RfD; ATSDR MRL); duration-specific values (e.g., subchronic (note: ATSDR refers to this duration as "intermediate"), or chronic RfVs) may be available from various sources and should be assembled and incorporated into the data-driven mixture assessment approach(es) as deemed appropriate by the user; and (b) Development of health effect domain profiles (see example literature inventory in Figure 4-2 excerpted from a PFAS systematic review protocol[10]), and associated dose-response information sorted based on exposure duration (e.g., short[er]-term (i.e., acute, short-term, dev/repro), longer-term (i.e., subchronic, chronic)), across mixture PFAS supported by the assembled traditional (i.e., human epidemiological and/or experimental animal) toxicity data from Step 1.

[9] https://cfpub.epa.gov/ncea/iris_drafts/recordisplay.cfm?deid=345065
[10] https://cfpub.epa.gov/ncea/iris_drafts/recordisplay.cfm?deid=345065

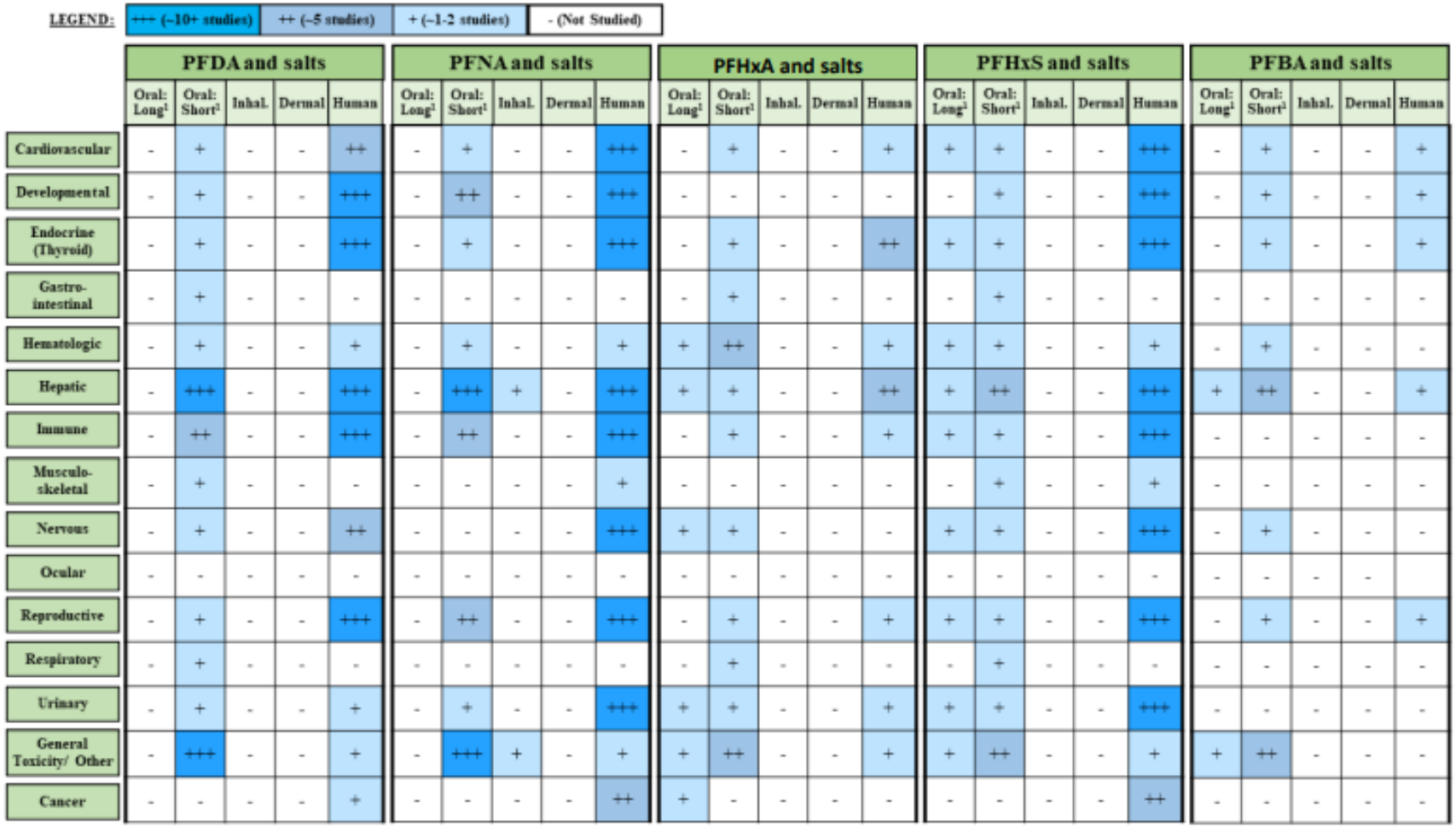


Figure 4-2. Example literature inventory heatmap for traditional epidemiological or experimental animal studies for five PFAS currently under development/review in EPA/ORD's IRIS program (heat map circa 2018). Health effects are based on groupings from the IRIS website (https://cfpub.epa.gov/ncea/iris/search/index.cfm).

Users of this framework may find that many PFAS of interest are data-poor (i.e., no 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 surrogate dose-response metrics (e.g., POD, EC_X, IC_X) for integration into the component-based mixtures assessment approaches presented in the subsections below. Analog-based read-across, in general, is a process in which chemicals (i.e., analogs) with relatively replete toxicity databases are compared to a data-poor target chemical across similarity domains including structural, physicochemical, TK, and/or toxicodynamic (TD) similarity (Wang et al., 2012; Wu et al., 2010). Based on weight-of-evidence for similarity between a data-poor target chemical and candidate analogs, hazard and dose-response data (e.g., POD) are then adopted from a selected (single-best) analog as surrogate for the target chemical. This read-across approach might facilitate incorporation of data-poor PFAS into the component-based methods presented in this framework, as surrogate PFAS data that inform similarity of toxic endpoint/health effect and dose-response could potentially: (a) be used in the derivation of a noncancer RfD (using uncertainty factors appropriate for the data-poor target chemical) and subsequent calculation of a hazard quotient (HQ); (b) be used in the calculation of RPF(s); or (c) the surrogate health-effect dose-response data could be BMD modeled and included in the calculation of an overall M-BMD. EPA's ORD has been employing expert-driven analog-based read-across in the evaluation of data-poor chemicals for over a decade; for an illustrative example human health assessment application please see Appendix A of the Provisional Peer-Reviewed Toxicity Value (PPRTV) document for 2,3-toluenediamine at https://cfpub.epa.gov/ncea/pprtv/recordisplay.cfm?deid=352932).

Another opportunity for integration of NAMs into the proposed mixture approaches involves cell bioactivity or transcriptomic data from experimental animals and/or *in vitro* cell cultures. For over a decade, EPA and NIEHS have invested significant resources into high(er)-throughput assay development and application to hundreds of chemicals (Richard et al., 2016; Kavlock et al., 2012; Dix et al., 2007). The assays are primarily targeted at nuclear receptor activity but also include several other assays that inform cell viability, enzyme activity, DNA reactivity, cell transport, and macromolecular/cellular dysfunction. The bioactivity information is quality assured, assembled, curated, and presented in a manner that is intended to facilitate incorporation into risk-based decision contexts such as human health assessment (see example bioactivity plot in Figure 4-3). Although there are inherent complexities and challenges associated with study designs and associated data interpretation using NAM assays/platforms, such as *in vitro* cell culture, recent investigation has demonstrated that quantitative data (e.g., PODs) from *in vitro* bioactivity is a reasonable estimate of *in vivo*-based PODs (Paul-Friedman et al., 2020). Likewise, over the past decade, systematic comparisons of pathway-based (e.g., Gene Ontology or 'GO'[11]) transcriptomic PODs to phenotypic health outcome PODs has illustrated that for most chemicals evaluated to date, the dose-response relationship between genotype and phenotype for toxic effects is typically within an order of magnitude (Johnson et al., 2020; Thomas et al., 2013; 2011). For many chemicals, bioactivity assays can also provide information on the potential to disrupt specific MIEs and KEs of known or postulated MOAs or AOPs and may inform the relevance of specific pathways to humans. While *in vitro* assays are critical they are not without limitations. For example, *in vitro* dose metrics in a component-based mixture context (e.g., RPFs

[11] http://geneontology.org/docs/ontology-documentation/

or BMDs) cannot be directly extrapolated to *in vivo* RPFs or BMDs and used for the prediction of PFAS mixture effects *in vivo* due to the lack of chemical-specific ADME processes. Rather, converting *in vitro* bioactivity concentrations to estimated human *in vivo* doses (i.e., Administered Equivalent Doses; AEDs) requires application of *in vitro*-to-*in vivo* extrapolation (IVIVE) and reverse toxicokinetics (rTK) which may not be possible for many data-poor PFAS. In addition, several *in vitro* cell-based assays to date employ truncated nuclear receptors with only the ligand binding domain and, as a consequence, the transcriptional events that follow binding may not be fully representative of quantitative chemical potencies compared to that seen with full length receptors in native *in vivo* systems. Further, for such approaches to gain widespread regulatory acceptance it will be important to demonstrate that the NAMs under consideration are reproducible, robust, and can be transferred to other laboratories and produce results that are relevant to *in vivo* adverse effects.

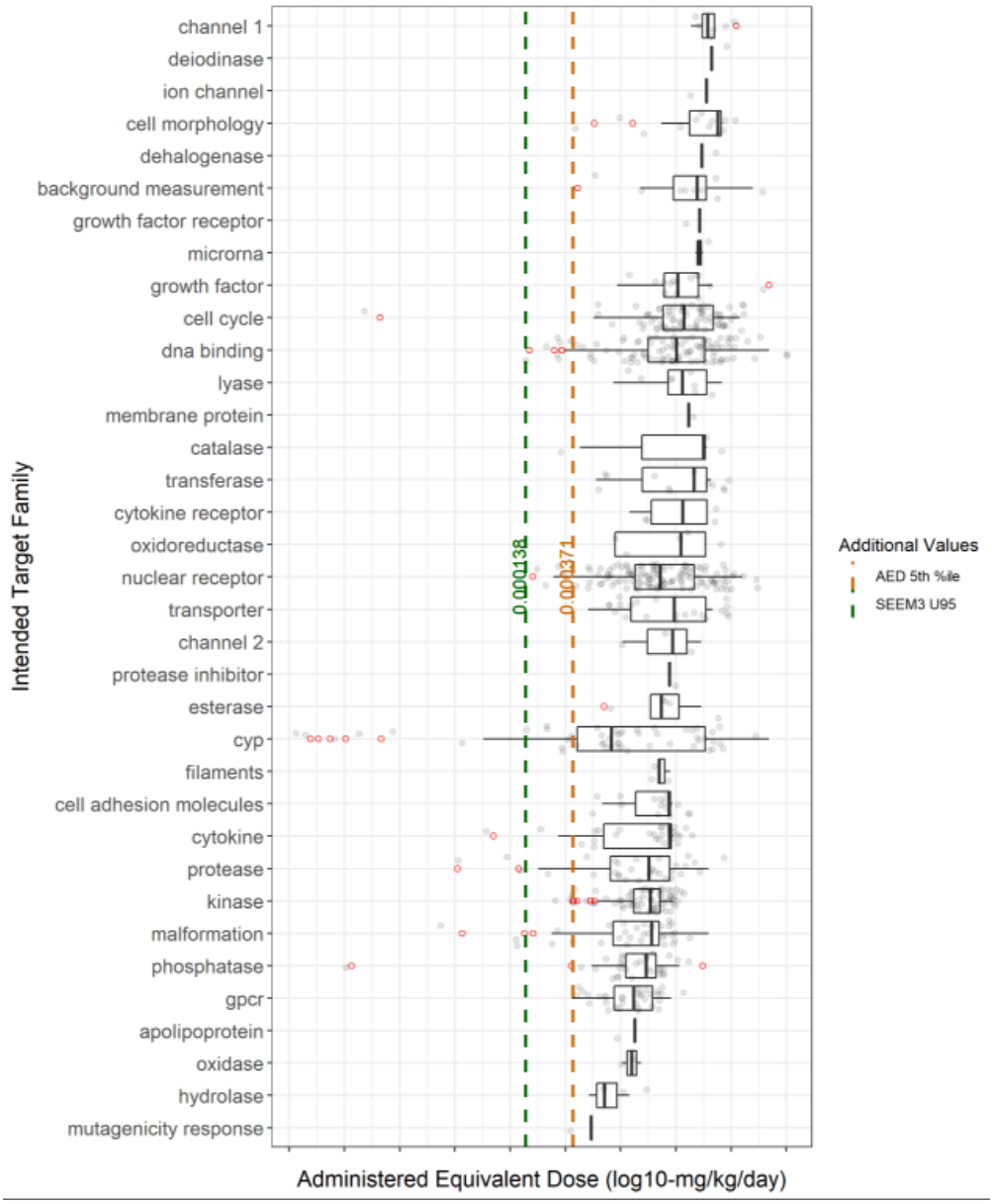


Figure 4-3. Example plot illustrating *in vitro* cell bioactivity expressed in AEDs which are an estimated oral exposure dose that results in an internal steady-state concentration consistent with the *in vitro* concentration associated with a biological perturbation or activity. The example shows the distribution of AEDs; the vertical orange dashed line indicates the 5th percentile on the bioactivity landscape. The green dashed line corresponds to an upper bound on the estimated general population-based median exposure (generated from EPA's Systematic Empirical Evaluation of Models (SEEM3); https://www.epa.gov/chemical-research/computational-toxicology-communities-practice-systematic-empirical-evaluation).

Step 3: Consider data landscape to select component-based approach(es).

Consideration of the data landscape across mixture component PFAS in Step 2 will help the user ascertain which option(s) in Step 4 is the most supportable. For example, dose-response data for PFAS mixture components may be applicable to more than one option in step 4, however characteristics of the data (e.g., shape or slope of dose-response between components) may not be congruent potentially leading the user to select a M-BMD approach over the RPF approach. Another example entails dose-response data for components that do not indicate "like" health outcome or MOA; in this case it may be more practicable to use the available data in a general HI approach (assuming human health assessment values already exist, or dose-response data support *de novo* derivation of assessment values). When using any of the approaches, it is optimal to calculate and use HEDs rather than oral administered doses in test animals where and when possible. The user is not precluded from applying available hazard and dose-response data across the entirety of the options in Step 4, however study data types, design (e.g., exposure duration, route), and confidence will likely dictate optimal selection of component-based approach for PFAS on a case-by-case basis. In most cases, it is likely that a user would select only one of the approaches, based on the data available for the PFAS components in a mixture of concern (e.g., if toxicity values and HBWCs are available or can be calculated, then the HI approach is appropriate). If instead toxicity values are not available or cannot be readily derived, then the RPF or M-BMD approach may be more suitable.

Step 4: Perform component-based approach(es).

a. HI and TOSHI. Although limited in number, for those mixture PFAS that have human health risk assessment value(s), the user in Step 3 should have determined if the critical effect(s) on which duration-specific assessment values were derived, for any two or more PFAS, fit into the same health effect domain (e.g., liver, thyroid, developmental). If not, then those PFAS are entered into a general HI approach (Section 5). For many PFAS, no formal health assessment exists, however human epidemiological and/or experimental animal hazard and dose-response data may be available in the public domain. Should such data be available, the user has the option of performing *de novo* derivation of duration-specific noncancer toxicity values using assessment guidance and practices accepted under their specific purview. Again, if the *de novo* derived values are not based on same/similar effect then the general HI approach is recommended. In brief, the HI approach entails use of duration-relevant exposure (E) and toxicity values (e.g., RfV), for each component PFAS, in a simple ratio (E/RfV) to calculate an HQ. The general HI involves the use of RfVs for each PFAS mixture component irrespective of health outcome domain. Because each mixture component HQ is calculated using a corresponding RfV, the mixture HI may represent a conservative indicator of potential mixture risk. The component PFAS HQs are then summed to generate a mixture HI (see Equation 5-1). A mixture HI approaching or exceeding 1.0 indicates potential concern for health risk(s) associated with a given environmental media or site. The HI provides an indication of: (1) risk associated with the overall PFAS mixture; and (2) potential driver PFAS (i.e., those PFAS with high(er) HQs). Conversely, those PFAS with low(er) HQs (e.g., $\leq$ 0.0X) might be deprioritized as they may not have significant impact(s) on overall mixture risk at the specific media concentrations identified. It should be noted that a user of this approach should consider the potential exposure (e.g., water concentration), the potency for toxic effect (e.g., low(er) or high(er) RfVs, PODs), the duration associated

with exposure and toxicity, and qualitative and quantitative uncertainty (i.e., totality of UF application) for each PFAS mixture component.

If hazard data across PFAS mixture components indicate same/similar effect, then a target-organ specific HI (TOSHI) is recommended (see Section 5.3). 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 RfD for a given PFAS). In practice, it is recommended to calculate TTDs for all health outcomes associated with a given mixture PFAS, where/when evidence support. This may facilitate calculation of TOSHIs for more mixture component PFAS across more health outcomes thus enriching the evaluation of PFAS co-occurring in a given environmental medium (e.g., drinking water).

If data are available that indicate and support deviations from dose additivity (e.g., synergy or antagonism), an interactions-based HI may be employed (see EPA, 2000b). However, in this framework, based on an assumption of dose additivity only the HI and TOSHI are included. Specifically, 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.

b. RPF. In contrast to the HI, the RPF approach provides a PFAS mixture risk estimate (see Section 6). In the RPF approach, potency for an effect across each mixture PFAS is scaled to a selected IC for critical health effect domains of concern. In the illustrative example in Section 6.2, application of the RPF method is demonstrated for liver, thyroid, and developmental effects associated with the hypothetical five component PFAS mixture. These three health effect domains were selected primarily because: (1) the effects are common across several PFAS assessed by EPA (and ATSDR) thus far; and (2) each hypothetical example PFAS has differing levels of hazard and dose-response data available across the three health effect domains, to best illustrate demonstration of the RPF methodology. 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 6-2), 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 an HBWC (e.g., Health Advisory, Maximum Contaminant Level Goal (MCLG)) based on the relevant health effect domain (e.g., liver, thyroid, developmental) for the IC. If the mixture ICEC for one or more of the selected 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 ICECs for all effect domains are below the corresponding IC HBWC, health 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.

c. M-BMD. An additional option entails calculation of a M-BMD (see Section 7) and is applicable even when PFAS in the mixture have dissimilar dose response curves. In contrast to the RPF approach, there is no need for identification of mixture ICs, calculation of RPFs or ICECs, or existence of HBWCs as the final determination of risk is based on comparison of the observed total mixture concentration with each effect-based M-BMD. Similar to the RPF approach, hazard dose response data across one or more health effect domains for each PFAS in the mixture are needed to determine the corresponding benchmark response (BMR) for each PFAS component (i.e., each component PFAS benchmark dose). Then the individual component chemical BMDs are scaled based on their proportion in the mixture and added using a simple dose-addition based equation to arrive at a total M-BMD. The M-BMD approach does not require that component chemicals meet an assumption of statistically similar dose response functions (i.e., slopes). The resulting M-BMD (i.e., POD) could be converted into a mixture RfD using appropriate UF application, and subsequently incorporated into the calculation of 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). The illustrative example in Section 7 utilizes data for a five-component hypothetical PFAS mixture with hazard and dose response data for the three selected effect domains. Similar to the RPF approach, if the total mixture concentration exceeds the M-BMD for one or more effect domains, then there may be cause for concern for the mixture at the reported/measured component water concentrations. If the total mixture concentration is below all M-BMDs calculated, then health risk is not anticipated.

4.2.2 Introduction to a Hypothetical Example with Five PFAS

In the following sections, the HI (Section 5), RPF (Section 6), and M-BMD (Section 7) approaches will be detailed and accompanied by demonstration of practical application to a hypothetical five component mixture of PFAS. As a reminder, PFAS 1–5 are as follows:

PFAS 1 = comprehensively studied, most potent for effect(s), and has formal noncancer human health assessment value(s) and an HBWC available;

PFAS 2 = well-studied, second most potent for effect(s) among PFAS 1–3, and has formal noncancer human health assessment value(s) and HBWC available;

PFAS 3 = studied, least potent for effect(s) among PFAS 1–3, and has formal noncancer human health assessment value(s) and HBWC available;

PFAS 4 = experimental animal toxicity data available but no formal human health assessment and no HBWC; and

PFAS 5 = data-poor.

To help introduce the illustrative case study, the hypothetical drinking water scenario is as follows: Periodic targeted and non-targeted analysis of drinking water samples obtained at the tap across a community revealed the presence of five PFAS, referred to as PFAS 1–5 (median

concentrations shown in Table 4-1), above hypothetical analytical quantitation limits[12] (Table 4-2).

Table 4-1. Drinking water concentrations for five hypothetical PFAS; the values represent the median of a distribution of sampling data collected across a community over time.

PFAS Exposure Estimates (Measured in Drinking Water) (ng/L)					
	PFAS 1	**PFAS 2**	**PFAS 3**	**PFAS 4**	**PFAS 5**
Median	4.8	52	172	58	69

Table 4-2. Analytical quantitation limits for drinking water for five hypothetical PFAS.

PFAS Analytical Quantitation Limits (ng/L)					
	PFAS 1	**PFAS 2**	**PFAS 3**	**PFAS 4**	**PFAS 5**
Analytical limit	3.0	5.0	5.0	4.0	5.0

Problem formulation and scoping. For simplicity, the problem formulation is scoped to 'What are the potential (noncancer) public health risks associated with exposure to the mixture of PFAS 1–5 in drinking water for the community?' A formal problem formulation and scoping exercise might include identification of population/community exposure details (e.g., distribution of sexes, ages, exposure frequency, exposure duration), seasonal variations in PFAS 1–5 levels in the drinking water, groundwater/surface water PFAS 1–5 concentrations, density of wells in the community, and other mitigating circumstances or factors.

Step 1: Assemble information.

The structured literature search for the mixture of PFAS 1–5 included comprehensive Boolean search strings and were applied across information databases such as PubMed, Web of Science, Toxline, and TSCATS (Figure 4-4). Please note that the specific PFAS names, synonyms, and CASRN in Figure 4-4 are for illustrative purposes only. In application, the search string(s) would need to be scoped and developed to optimize the literature search for specific mixture PFAS on a case-by-case basis.

[12] Analytical quantitation limits for chemicals are generally defined as the lowest detectable concentration of an analyte where the accuracy achieves the objectives of the intended purpose. For example, in EPA's UCMR Program, the agency establishes "minimum reporting levels" to ensure consistency in the quality of the information reported to the agency. Under UCMR 5, an analytical quantitation limit is the minimum quantitation level that, with 95% confidence, can be achieved by capable analysts at 75% or more of the laboratories using a specified analytical method.

- PubMed (National Library of Medicine)
- Web of Science (Thomson Reuters)
- Toxline (National Library of Medicine)
- TSCATS (Toxic Substances Control Act Test Submissions)

Search	Search strategy	Dates of search
PubMed		
Search terms	375-22-4[rn] OR "Heptafluoro-1-butanoic acid"[tw] OR "Heptafluorobutanoic acid"[tw] OR "Heptafluorobutyric acid"[tw] OR "Kyselina heptafluormaselna"[tw] OR "Perfluorobutanoic acid"[tw] OR "Perfluorobutyric acid"[tw] OR "Perfluoropropanecarboxylic acid"[tw] OR "2,2,3,3,4,4,4-heptafluoro-Butanoic acid"[tw] OR "Butanoic acid, 2,2,3,3,4,4,4-heptafluoro-"[tw] OR "Butanoic acid, heptafluoro-"[tw] OR "Perfluoro-n-butanoic acid"[tw] OR "Perfluorobutanoate"[tw] OR "2,2,3,3,4,4,4-Heptafluorobutanoic acid"[tw] OR "Butyric acid, heptafluoro-"[tw] OR "Fluorad FC 23"[tw] OR "H 0024"[tw] OR "NSC 820"[tw] OR ((PFBA[tw] OR "FC 23"[tw] OR HFBA[tw]) AND (fluorocarbon*[tw] OR fluorotelomer*[tw] OR polyfluoro*[tw] OR perfluoro-*[tw] OR perfluoroa*[tw] OR perfluorob*[tw] OR perfluoroc*[tw] OR perfluorod*[tw] OR perfluoroe*[tw] OR perfluoroh*[tw] OR perfluoron*[tw] OR perfluoroo*[tw] OR perfluorop*[tw] OR perfluoros*[tw] OR perfluorou*[tw] OR perfluorinated[tw] OR fluorinated[tw] OR PFAS[tw] OR PFOS[tw] OR PFOA[tw]))	No date limit

Figure 4-4. Example PFAS-specific literature search string applied to toxicity information databases such as the four listed (e.g., PubMed, WoS, Toxline, and TSCATS).

The assembled literature inventory was then screened at the level of title and abstract to determine preliminary relevance to informing human health risk assessment using defined Population, Exposure, Comparator, and Outcome (PECO) elements as illustrated in Figure 4-5. Again, specific details provided in Figure 4-5 are for illustrative purposes only; mention of PFAS other than the hypothetical PFAS 1–5 should not be construed to be the basis of the illustrative PFAS mixture example in subsequent sections.

PECO element	Evidence
Populations	Human: Any population and lifestage (occupational or general population, including children and other sensitive populations). The following study designs will be included: controlled exposure, cohort, case-control, and cross-sectional. (Note: Case reports and case series will be tracked as potential supplemental material.) Animal: Nonhuman mammalian animal species (whole organism) of any lifestage (including preconception, in utero, lactation, peripubertal, and adult stages). Other: In vitro, in silico, or nonmammalian models of genotoxicity. (Note: Other in vitro, in silico, or nonmammalian models will be tracked as potential supplemental material.)
Exposures	Human: Studies providing quantitative estimates of PFAS exposure based on administered dose or concentration, biomonitoring data (e.g., urine, blood, or other specimens), environmental or occupational-setting measures (e.g., water levels or air concentrations, residential location and/or duration, job title, or work title). (Note: Studies that provide qualitative, but not quantitative, estimates of exposure will be tracked as supplemental material.) Animal: Oral or Inhalation studies including quantified exposure to a PFAS of interest based on administered dose, dietary level, or concentration. (Note: Nonoral and noninhalation studies will be tracked as potential supplemental material.) PFAS mixture studies are included if they employ an experimental arm that involves exposure to a single PFAS of interest. (Note: Other PFAS mixture studies are tracked as potential supplemental material.) Studies must address exposure to one or more of the following: PFDA (CASRN 335-76-2), PFDA ammonia salt (CASRN 3108-42-7), PFDA sodium salt (CASRN 3830-45-3), PFNA (CASRN 375-95-1), PFNA ammonium salt (CASRN 4149-60-4), PFNA sodium salt (CASRN 21049-39-8), PFHxA (CASRN 307-24-4), PFHxA sodium salt (CASRN 2923-26-4), PFHxA ammonium salt (CASRN 21615-47-4), PFHxS (CASRN 355-46-4), PFHxS potassium salt (CASRN 3871-99-6), PFBA (CASRN 375-22-4), or PFBA ammonium salt (CASRN 10495-86-0). [Note: although while these PFAS are not metabolized or transformed in the body, there are precursor compounds known to be biotransformed to a PFAS of interest; for example, 6:2 fluorotelomer alcohol is metabolized to PFHxA and PFBA (Russell et al., 2015). Thus, studies of precursor PFAS that identify and quantify a PFAS of interest will be tracked as potential supplemental material (e.g., for ADME analyses or interpretations).]
Comparators	Human: A comparison or reference population exposed to lower levels (or no exposure/exposure below detection levels) or for shorter periods of time. Animal: Includes comparisons to historical controls or a concurrent control group that is unexposed, exposed to vehicle-only or air-only exposures. (Note: Experiments including exposure to PFAS across different durations or exposure levels without including one of these control groups will be tracked as potential supplemental material [e.g., for evaluating key science issues; Section 2.4].)
Outcomes	All cancer and noncancer health outcomes. (Note: Other than genotoxicity studies, studies including only molecular endpoints [e.g., gene or protein changes; receptor binding or activation] or other nonphenotypic endpoints addressing the potential biological or chemical

Figure 4-5. Example PECO criteria and considerations used to determine study relevance in the systematic review and evaluation of a literature inventory for chemicals such as PFAS.

Following removal of duplicate references and systematic screening of the initial inventory using the defined PECO, non-relevant studies/reports were excluded, and the remaining references were full text screened. The full-text screening resulted in three buckets of references: (1) studies or reports meeting PECO; (2) studies or reports tagged as supplemental (i.e., useful for risk assessment but not a toxicity study); and (3) studies or reports that upon further review were excluded as not PECO-relevant (bottom of Figure 4-6).

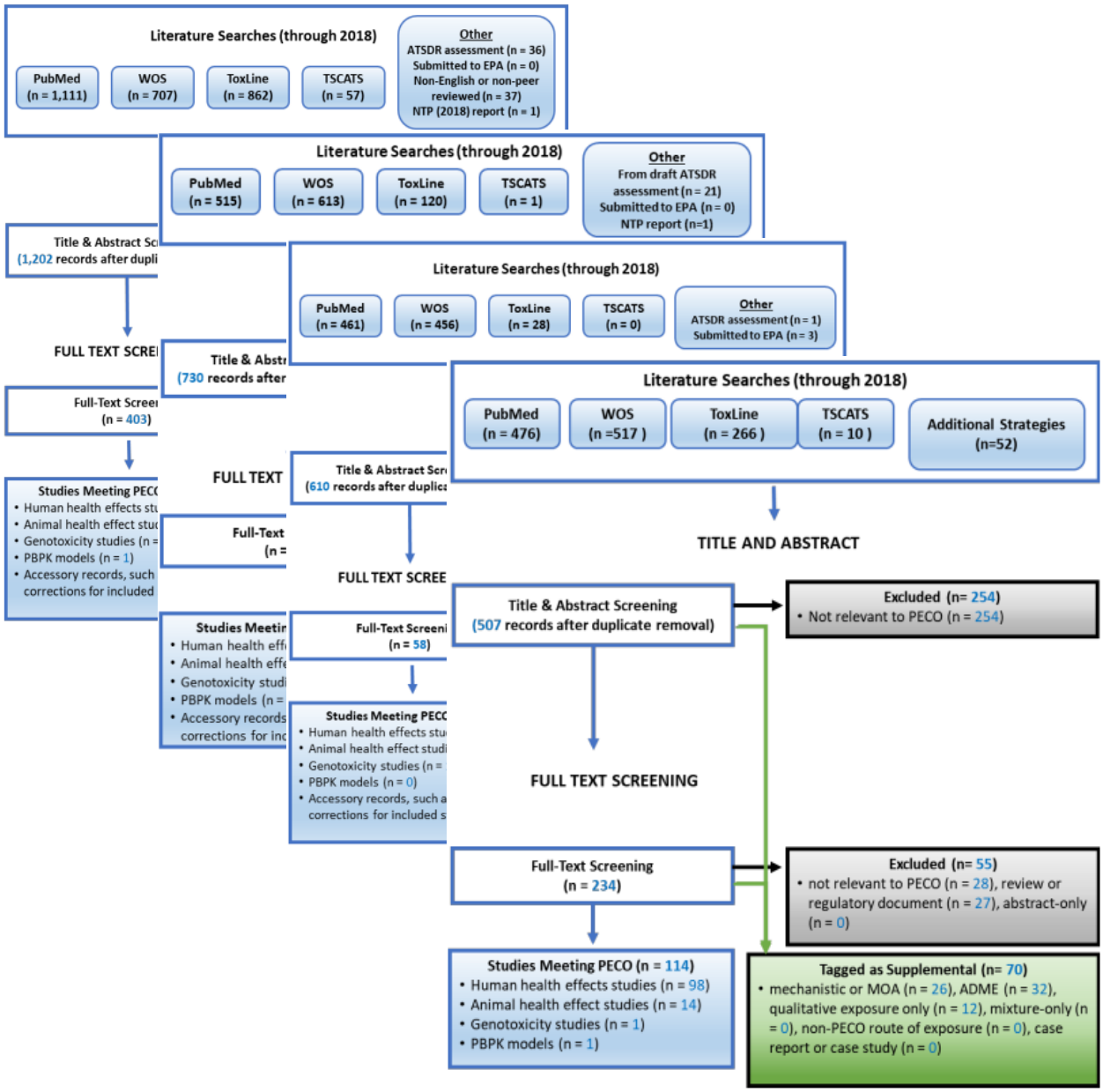


Figure 4-6. Example literature screening logic flow for hypothetical PFAS using an EPA systematic review approach. The figure depicts example PECO-dependent development of evidence bases to support human health assessment application(s). *Note*: only 4 of 5 hypothetical PFAS (i.e., PFAS 1–4) are represented as PFAS 5 is data-poor.

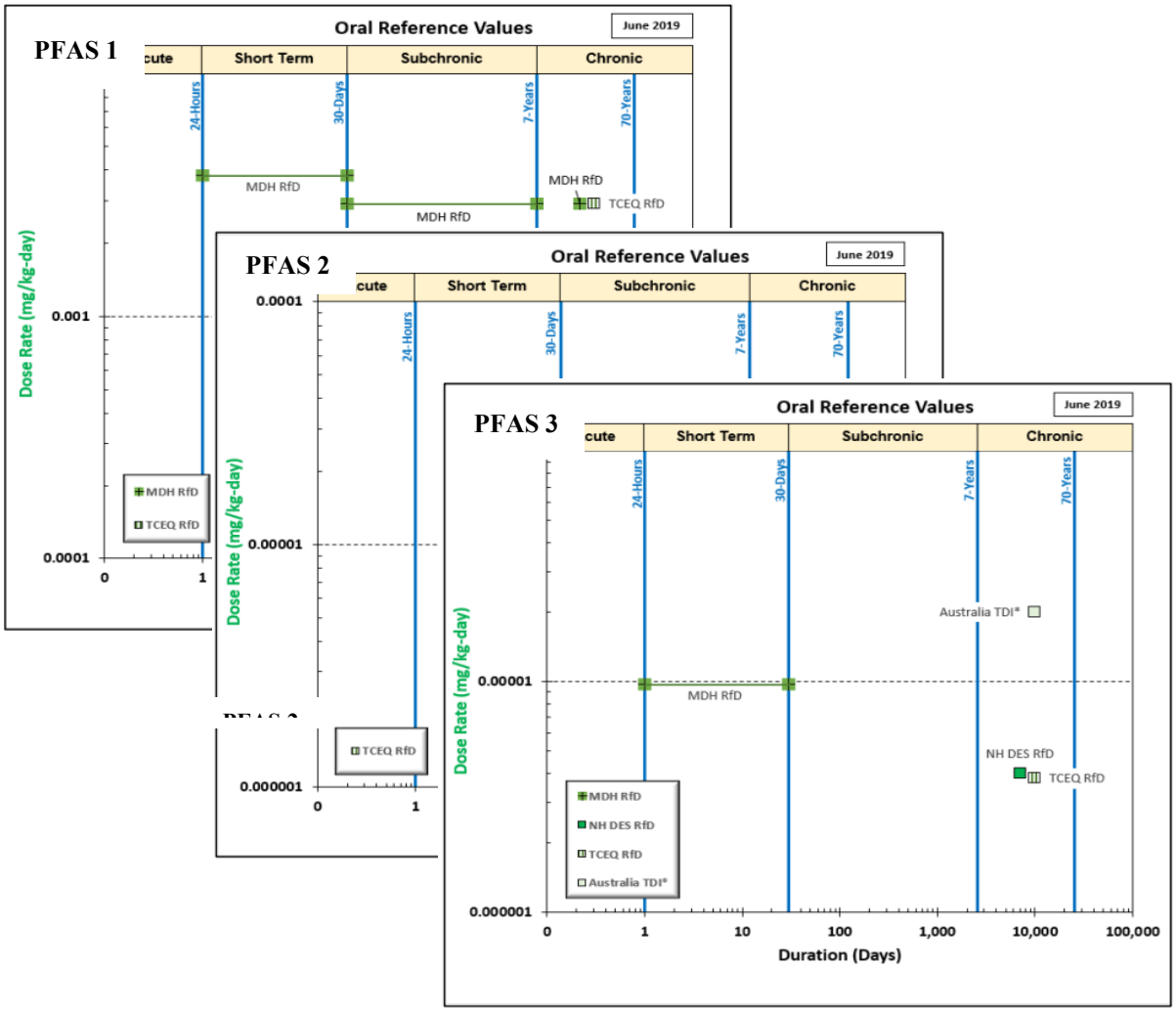
Step 2: Evaluate data objectives.

For the hypothetical example, the systematic literature search and screen resulted in PECO relevant studies for PFAS 1–4 only. PFAS 5 was identified as data-poor and further interrogated in EPA's Computational Toxicology Dashboard (https://comptox.epa.gov/dashboard/) for presence of alternative toxicity testing data (e.g., *in vitro* cell bioactivity assay data). At the conclusion of the data gathering exercise for the five PFAS in Step 1 of the framework approach, and once all of the hazard and dose-response studies and data were assembled and evaluated, it was determined under Step 2 that: (1) there are no whole mixture/sufficiently similar mixture studies available for the combination of PFAS of concern; (2) PFAS 1–3 have existing human health risk assessment values for the oral route of exposure (Figure 4-7A); (3) PFAS 4 has existing hazard and dose-response data but no known health assessment value(s); and (4) PFAS 5, although data-poor, has predicted physicochemical property and empirical *in vitro* cell bioactivity data. In addition, across the five hypothetical PFAS, different levels/types of toxicokinetic data were identified. Specifically, clearance[13] values for experimental rats and humans were located for example PFAS 1-3. No clearance or volume of distribution (Vd)[14] values were identified for PFAS 4; only serum half-life data were harvested where available. Lastly, only rat serum half-life data were located for PFAS 5 (Figure 4-7B).

[13] Clearance represents the combined intrinsic ability of organs and tissues to remove chemical(s) from the plasma and is commonly expressed in units of volume/time-body mass (e.g., L/day-kg body weight); it is typically calculated as the product of the elimination rate constant (k_e) × volume of distribution (Vd). An elimination rate constant represents the faction of chemical eliminated from the body per unit of time, commonly expressed in units of hour(s) or day(s).

[14] The volume of distribution represents the degree to which a chemical is distributed in body tissues. For example, chemicals that are highly bound to plasma proteins and not broadly distributed in tissues have a low Vd; conversely, chemicals that have low affinity for plasma proteins typically have a high Vd and distribute broadly across tissues/compartments. Vd is commonly expressed in units of volume/body mass (e.g., L/kg).

(A)

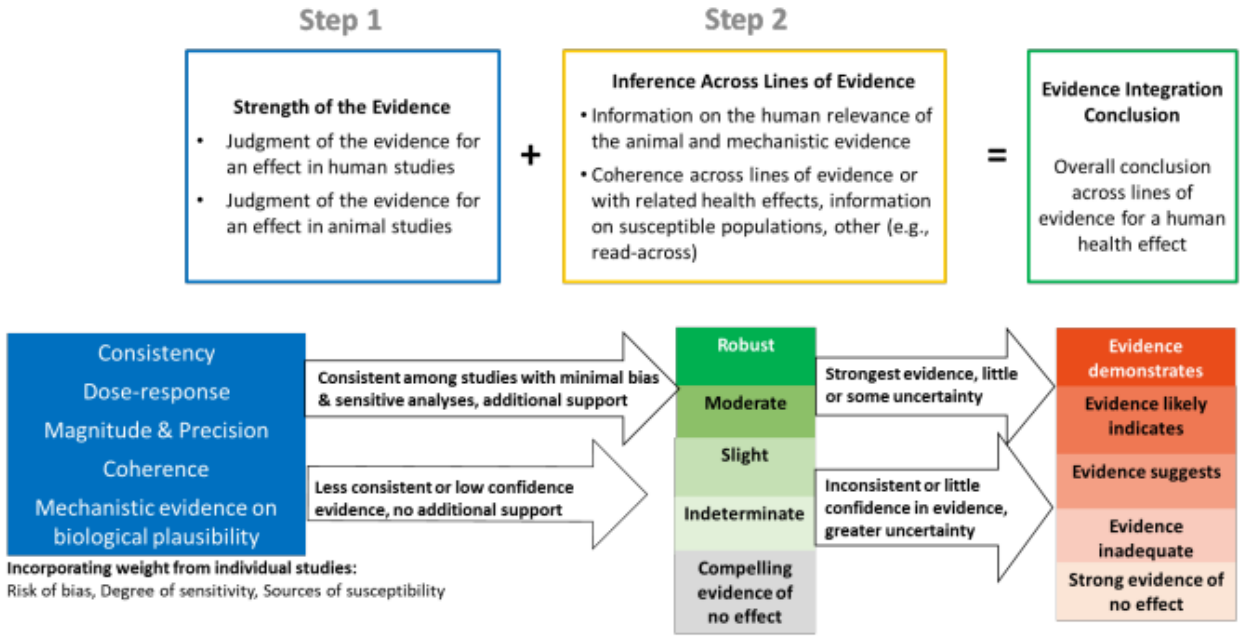


(B)

	PFAS 1		PFAS 2		PFAS 3		PFAS 4		PFAS 5	
	Clearance (L/day-kg)*						Plasma half-life (hours)**			
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
Rat	0.0031	0.0023	0.42	0.16	0.0024	0.00064	33.6	734.4	1406	958
Mouse	0.00057	0.00038	0.17	0.13	0.00017	0.00015	1128	1236	ND	
Monkey	0.000034		ND		0.00005	0.00003	ND		ND	
Human	1.8 E-5	0.9 E-5	2.7 E-4	1.2 E-4	0.00002†		24,528		ND	

Figure 4-7. (A). Example exposure-response arrays for the PFAS 1–3 identified as having existing human health risk assessment values for one or more exposure durations; (B). Empirical clearance or plasma half-life data for PFAS 1–5. ND = no data available. *Clearance = elimination rate constant (k_e) × volume of distribution (Vd); **Data needed to calculate clearance or Vd were not available for rodents or humans as such only plasma half-life is presented where available; †Male only

For brevity, in the evaluation of the hypothetical five component PFAS mixture, the health effect domains are truncated to three targets: Liver, Thyroid, and Developmental. Across these three target health effect domains, the PECO relevant studies and data were subjected to systematic review principles and practice (e.g., risk of bias analysis; evidence integration) across the available data sources/streams (Figure 4-8).



Effect domain	PFAS 1	PFAS 2	PFAS 3	PFAS 4	PFAS 5
Liver	++	+++	–	++	– *
Thyroid	++	++	+++	–	–
Developmental	+++	++	++	+++	–

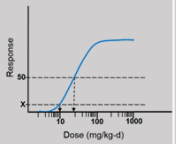
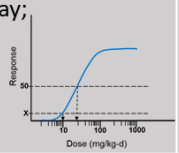
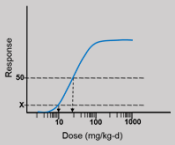
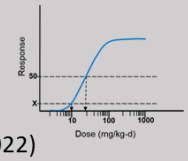
Figure 4-8. Evidence integration across three target health effect domains for a mixture of five hypothetical PFAS. The heat map indicates strength of evidence supporting an effect of the PFAS in a domain. (+++) indicates likely effect; (++) evidence suggests an effect in the domain; (–) evidence is inadequate to determine an effect in the domain; *Although PFAS 5 has no traditional human or experimental animal assay data available, *in vitro* cell bioactivity data are available from assays performed predominately in hepatocyte cell lines.

Step 3: Consider data landscape to select component-based approach(es).

As illustrated in the evidence heat map in Figure 4-8, PFAS 1, 2 and 4 have traditional experimental animal assay hazard and dose-response data available for component-based mixture assessment of liver effect(s); for thyroid effect(s), PFAS 1–3; and for developmental effect(s), PFAS 1–4. The landscape of duration relevant dose-response data (e.g., chronic) is then interrogated for identification of single best study/dataset for a critical effect to represent the PFAS in a given effect domain. "Single best" study/dataset will be subjective and user-

dependent, however considerations such as robustness of the study(ies) (e.g., power of study/high N per treatment group, comprehensiveness and transparency of toxicity evaluation; statistics), effect level identification (e.g., are both a LOAEL and NOAEL identifiable?), and are data amenable to benchmark dose modeling? are just a few factors for which a study might be selected. For those PFAS that have an existing health assessment (e.g., in the hypothetical example, PFAS 1–3), such decisions have already been made by the publishing authors. In practice, if the user of this framework deviates from use of existing health assessment dose-response metrics, clear rationale must be provided in the mixtures assessment. The dose-response data/metrics (e.g., PODs, dose-response curves) selected across mixture PFAS should be clearly presented, as in Table 4-3 for the hypothetical five component PFAS mixture.

Table 4-3. Data array to inform decisions in Steps 2 and 3 of the framework approach for component-based mixtures assessment of PFAS.

	Existent HHRA value (mg/kg-day)	Existent dose-response data for critical effect from principal study	Existent NAM data
PFAS 1	RfD: 3 E-8 POD_{HED}: 0.00001 (BMDL0.1ER) UF_C: 300 Critical effect: Delayed growth and development in offspring (ABC et al. 2022)	S-D rat; single generation repro/dev study; Daily gavage GD 1-20 (ABC et al. 2022) 	–
PFAS 2	RfD: 1 E-5 POD_{HED}: 0.0013 (BMDL10) UF_C: 100 Critical effect: Liver necrosis (DEF et al. 2022)	S-D rat; 2-year bioassay; DW ad libitum (DEF et al. 2022) 	Bioactivity profile in ToxCast; Biological perturbation at 5% AED (2.8 E-4 mg/kg-day) = ↓ cyt c oxidase in HepaRG cells; ↑ ox. Stress
PFAS 3	RfD: 7 E-4 POD_{HED}: 0.21 (BMDL1SD) UF_C: 300 Critical effect: Decreased thyroid hormones (T4/T3) (GHI et al. 2022)	C57BL6 mouse; 90-day gavage; (GHI et al. 2022) 	–
PFAS 4	–	F344 Rat; two generation repro/dev study; Multiple dev outcomes in offspring; feed ad libitum (JKL et al. 2022) 	–
PFAS 5	–	–	Bioactivity profile in ToxCast; Biological perturbation at 5% AED (8 E-5 mg/kg-day) = ↓ epoxide hydrolase in HepaRG cells; ↑ ox. stress

Notes: AED = administered equivalent dose[15]; GD = gestational day; HHRA = human health risk assessment; POD_{HED} = human equivalent point of departure; RfD = oral reference dose; UF_C = composite uncertainty factor.

Step 4: Perform component-based mixture assessment approach(es).

At this juncture in the data-driven framework approach, information has been assembled to facilitate application in Step 4. In practice, the user may choose to select one component-based mixture approach over others, based on data evaluation/interpretation, or apply data where appropriate to more than one of the approaches. For the purposes of demonstrating practical application using the PFAS 1–5 mixture, all approaches (i.e., Step 4/bottom row of Figure 4-1) will be selected and demonstrated in the following sections.

[15] An AED is an estimated oral exposure dose that results in an internal steady-state concentration in humans consistent with the *in vitro* concentration associated with a biological perturbation or activity.

Within each component-based approach (i.e., HI/TOSHI, RPF, and M-BMD), these five general steps apply:

1. Assemble/derive component health effects endpoints (e.g., POD_{HED}, RfD);
2. Assemble/derive health-based media concentrations (e.g., HBWC);
3. Select exposure estimates (e.g., measured water concentrations);
4. Calculate PFAS mixture potency; and
5. Compare PFAS mixture potency to existing health-based value (e.g., HI = 1.0, HBWC).

5.0 Hazard Index (HI) Approach

5.1 Background on the HI Approach

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 risk(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, 2000b; Svendsgaard and Hertzberg, 1994). In the HI approach, an HQ is calculated as the ratio of human exposure to a health-based RfV for each mixture component chemical (i) (EPA, 1986). The HI is unitless, so in the HI formula, E and the RfV must be in the same units (Equation 5-1). For example, if E is the oral intake rate (mg/kg-day), then the RfV could be the RfD, which has the same units. Alternatively, the exposure metric can be a media-specific metric such as water concentration and the toxicity value is best represented as a duration-specific HBWC, for example, an EPA lifetime drinking water Health Advisory (e.g., EPA 2022a,b) or MCLG, or a similar value (e.g., developed by a state). In this case, the HQ is calculated as the ratio of water concentration (in mass/volume) to a HBWC (also in mass/volume). The component chemical HQs are then summed across the mixture to yield the HI, as illustrated in Equation 5-1.

$$HI = \sum_{i=1}^{n} HQ_i = \sum_{i=1}^{n} \frac{E_i}{RfV_i} \qquad \text{(Eqn. 5-1)}$$

Where:

HI = Hazard Index

HQ_i = Hazard Quotient for chemical i

E_i = Exposure, i.e., dose (mg/kg-day) or media concentration, such as in drinking water (ng/L), for chemical i

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

Because the numerator of each component chemical HQ is the estimated population-level human exposure, the noncancer health RfVs used in the denominator must be based on human toxicity.

These RfVs are derived either directly from human epidemiological/occupational study-based PODs (or measured or modeled 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 "general" HI and a "target-organ specific" HI (TOSHI). In either case, following the logic flow in Figure 4-1 to the general HI or the TOSHI, they are both applied under an assumption of dose additivity. In the 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 HI is generally a health-protective 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 whereas for other components, the liver might be among the least sensitive of effects. To use this approach, organ-specific reference values (osRfVs) or TTDs are needed (note: these are the same type of noncancer 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.

A HI greater than 1.0 is generally regarded as an indicator of potential adverse health risks associated with exposure to the mixture. A HI less than or equal to 1.0 is generally regarded as having no appreciable risk (recall that an RfV, such as an oral RfD, represents an estimate at which no appreciable risk of deleterious effects exists), typically requiring no further analysis (EPA, 1986, 1991, 2000b). However, in some circumstances the user may want to consider a HI less than 1.0, for example, for screening when multiple contaminants of concern are present at a site or one or more are present in multiple exposure media. In the case of PFAS, final peer-reviewed toxicity assessments are only available for a small proportion of the approximately 11,000 environmentally relevant PFAS (e.g., see summary of EPA and ATSDR PFAS assessments in Table 4-4). EPA's primary source of peer-reviewed toxicity assessments is its 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 human health assessments, such as EPA's IRIS[16], PPRTV[17], EPA Office of Water toxicity assessments[18], TSCA risk evaluations[19], and ATSDR's ToxProfiles[20], undergo rigorous peer and public review processes; as a result, they are considered to be of *high* scientific quality. The chronic RfDs for

[16] https://www.epa.gov/iris
[17] https://www.epa.gov/pprtv
[18] e.g., https://www.epa.gov/system/files/documents/2021-10/genx-chemicals-toxicity-assessment_tech-edited_oct-21-508.pdf
[19] https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/risk-evaluations-existing-chemicals-under-tsca
[20] https://www.atsdr.cdc.gov/toxprofiledocs/index.html

PFBS (EPA, 2021a), HFPO-DA (EPA, 2021b), and PFBA (EPA, 2022e) represent the only currently available/final EPA toxicity values, although the agency is updating its PFOA and PFOS assessments (EPA, 2023b,c,d,e) and several more PFAS assessments are under development in EPA/ORD (e.g., PFHxA, PFHxS, PFNA, and PFDA; see Table 5-1 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.

Table 5-1. EPA and ATSDR Peer-Reviewed Human Health Assessments Containing Noncancer Toxicity Values (RfDs or MRLs) for PFAS that Are Either Final or Under Development

Chemical	EPA Chronic Oral RfD	ATSDR Intermediate MRL[a]
PFOA	Draft 2023 RfD = 3 x 10^{-8} mg/kg/day	2021 MRL = 3 x 10^{-6} mg/kg/day
PFOS	Draft 2023 RfD = 1 x 10^{-7} mg/kg/day	2021 MRL = 2 x 10^{-6} mg/kg/day
PFNA	Under development in the EPA IRIS program	2021 MRL = 3 x10^{-6} mg/kg/day
PFDA	Under development in the EPA IRIS program	N/A
PFBA	2022 RfD = 1 x 10^{-3} mg/kg/day	N/A
PFBS	2021 RfD = 3 x 10^{-4} mg/kg/day	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/day
HFPO-DA	2021 RfD = 3 x 10^{-6} mg/kg/day	N/A

Notes: N/A = Not available.

[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.

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 for scientific input and review, and the associated uncertainties (e.g., data gaps) be transparently characterized. 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 *in vitro* cell bioactivity and *in silico* platforms (e.g., read-across) might inform identification or prediction of data 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 IVIVE and rTK (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), and with expert-driven application of appropriate UFs, be leveraged into the derivation of corresponding noncancer 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. 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) 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, M-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, incorporating 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.

5.2 Illustrative Example Application of the General HI to a Hypothetical Mixture of Five PFAS

As mentioned previously, final human health assessments with chronic oral RfDs exist for hypothetical PFAS 1–3. Based on the RfDs for PFAS 1–3 (Table 4-3), PFAS 1 is a comprehensively studied chemical that is most potent for effect(s); PFAS 2 is also well-studied, but is less potent than PFAS 1 for effect(s); and PFAS 3 has been studied and is even less potent than PFAS 1 or 2. PFAS 4 has experimental animal toxicity data available but no formal human health assessment. Finally, PFAS 5 is data-poor, and was identified as having only bioactivity data available under step 2 of the framework approach to inform hazard and dose-response (Table 4-3). As PFAS 1–3 have existing human health assessment values, integration into the HI approach is simplified. However, for both PFAS 4 and 5, integration would necessitate *de novo* calculation of values in order to develop component HQs and an overall PFAS mixture HI (Equation 5-1). For the purposes of the defined illustrative example for the hypothetical five component PFAS mixture, this process is as follows:

5.2.1 General HI Step 1: Assemble/derive component health effects endpoints (Chronic oral RfDs)

PFAS 1–3: Upon review of the available information harvested in the literature search in Step 1 of the framework approach, formal human health assessments containing oral RfDs were identified (Table 4-3). However, the critical effect on which each corresponding RfD was

derived are in different effect domains; PFAS 1 critical effect = developmental effect in offspring; PFAS 2 critical effect = liver effect in adults; and PFAS 3 critical effect = thyroid hormone effect in adult females (in a repro/developmental life stage). As such, application of the general HI is optimal in this scenario and will entail use of the overall RfD (or MRL), regardless of underlying critical health effect. If a subchronic RfD or an MRL is only available for an intermediate duration (akin to subchronic for EPA purposes), additional uncertainty (e.g., subchronic-to-chronic duration) may be considered for extrapolation to a corresponding chronic duration value, unless subchronic/intermediate duration is the target.

PFAS 4: No federal, state, or other assessments with a RfV are available, but traditional hazard and dose-response (e.g., experimental animal study) data were judged adequate to support derivation. Systematic review and evaluation of the animal study data led to identification of a single best study (e.g., hypothetical 2-Gen repro/dev rat study; Table 4-3) and multiple developmental health outcomes as candidate critical effects such as delayed growth and development at post-natal day 1 (PND 1) and decreased neonatal viability and thyroid hormone levels at PND 4. Thus, the user may choose to calculate a RfV using appropriate dose-response metrics (i.e., POD) and application of UFs. Appropriate characterization of hazard conclusions and qualitative and quantitative confidence and uncertainty(ies) in *de novo* derivation of RfVs for PFAS in this category is imperative. For the specific hypothetical PFAS example, the dose-response data associated with delayed growth and development in PND 1 offspring provided the most robust endpoint and confidence in dose-response for PFAS 4; following benchmark dose modeling (as per EPA BMD guidance (EPA, 2012)), a rat lower statistical bound on a BMD ($BMDL_{1SD}$) of 1.06 mg/kg-day was calculated.

As shown in Figure 4-7B, TK data exists for PFAS 4 in relevant animal species (i.e., rats) and humans, such that a data-informed adjustment approach for estimating the dosimetric adjustment factor (DAF) can be used. In *Recommended Use of Body Weight$^{3/4}$ as the Default Method in Derivation of the Oral Reference Dose* (EPA, 2011b), EPA endorses a hierarchy of approaches to derive human equivalent oral exposures using data from laboratory animal species, with the preferred approach being physiologically based TK modeling. Other approaches might include using chemical-specific information, without a complete physiologically based TK model. In the absence of chemical-specific models or data to inform the derivation of human equivalent oral exposures, EPA endorses $BW^{3/4}$ as a default to extrapolate toxicologically equivalent doses of orally administered agents from laboratory animals to humans for the purpose of deriving an RfD under certain exposure conditions. In this illustrative hypothetical mixture example, it was determined that: (1) Clearance values were included in the dosimetric adjustment of PODs used in the derivation of non-cancer human health assessment values for PFAS 1-3; and (2) kinetic data for PFAS 4 are sufficient to support a data-informed dosimetric adjustment of the rat POD. Briefly, while specific TK data needed to estimate clearance or volume of distribution in rodents or humans for PFAS 4 were not available, clearance values for humans and rats could be estimated, under the assumption that the volume of distribution in human females is equal to female adult rats (i.e., the PFAS-exposed unit leading to effects in PND1 offspring), as follows:

> Clearance = elimination rate constant (k_e) × volume of distribution (Vd)
>
> Where k_e = (ln2/plasma half-life) = (0.693/plasma half-life), and Vd is assumed equivalent between female rats and humans

Table 5-2. Calculation of estimated clearance values for PFAS 4 in female rats and humans.

PFAS 4	Plasma half-life (hr)	Elimination rate constant (hr^{-1})	Volume of Distribution (L/day)*	Estimated Clearance (L/day-kg)
Female rats	33.6	0.021	1.0	0.021
Humans	24,528	0.000028	1.0	0.000028

*The value of 1.0 was used for volume of distribution (Vd) strictly for the purpose of calculation of an estimated clearance value; the Vd of 1.0 is not based on empirical evidence for PFAS 4. Having made this estimate, the ratio of clearance values (Table 5-2) in human females to that in female rats, CL_H:CL_A, can be used to calculate the DAF, and the resulting human equivalent dose (HED) can be calculated using equation 5-2 as follows:

$$HED = POD \times \frac{CL_H}{CL_A} \quad \text{(Eqn. 5-2)}$$

Where:

POD = the rat $BMDL_{1SD}$ of 1.06 mg/kg-day

DAF = CL_H/CL_A

CL_A = 0.021 L/day-kg (female adult rat; the effect in offspring is a function of maternal intake)

CL_H = 0.000028 L/day-kg

The application of the DAF of 0.001 to the rat POD results in a POD_{HED} of 0.0011 mg/kg-day. This POD_{HED} was then divided by a composite UF of 100 which included a human interindividual variability UF (UF_H) of 10 for human interindividual variability as there were no data to inform this parameter, an interspecies UF (UF_A) of 3 for extrapolation from rats to humans, as the kinetic differences between species were accounted for in part by the dosimetric adjustment above, a LOAEL-to-NOAEL UF (UF_L) of 1 because the POD is a BMDL, an extrapolation from subchronic to a chronic exposure duration UF (UF_S) of 1 for subchronic-to-chronic extrapolation because the effect was observed in a developmental population following gestational exposure (developmental exposures are considered duration independent in EPA), and a database UF (UF_D) of 3 as the totality of the hazard and dose-response database included repeat-dose studies in rats and mice of longer-term duration (i.e., subchronic), as well as single- and two-generation reproductive and developmental studies in rats. The resulting RfD for PFAS 4 = POD_{HED}/UF = 0.0011 mg/kg-day/100 = 1 E-5 mg/kg-day.

PFAS 5: 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 UFs 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 RfVs using accepted approaches and practice. Unfortunately, no formal EPA technical guidance or guidelines currently exist to guide the approach for use of NAM-based PODs in quantitative human health risk assessment applications. However, for the purposes of demonstrating potential application of NAM data (e.g., *in vitro* cell bioactivity) in the

hypothetical PFAS mixture evaluation, the general process within the context of this framework approach is as shown in Figure 5-1.

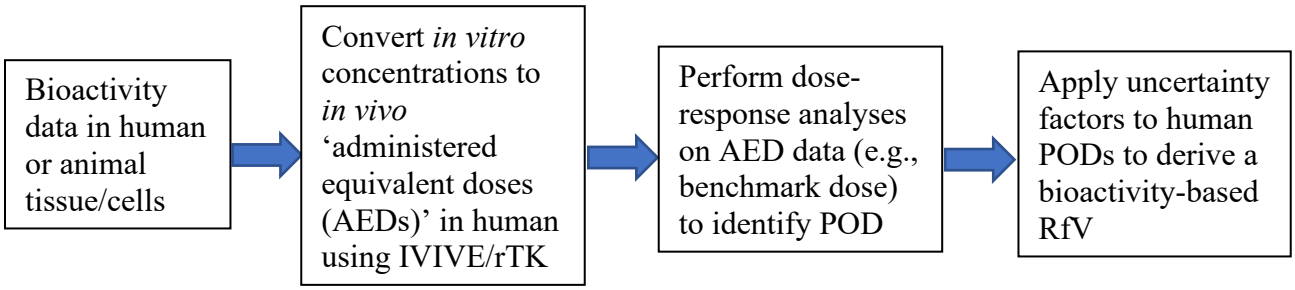


Figure 5-1. General steps to derive bioactivity-based reference value (RfV) using bioactivity data in human or animal tissue/cells.

The detailed steps and mechanics of the bioactivity > IVIVE/rTK > AED process above are beyond the scope of this framework document; the reader is referred instead to (Paul-Friedman et al., 2020; Wambaugh et al., 2018; Wetmore et al., 2012, 2014, 2015) for better context for the conversion of *in vitro* cell-based exposure concentrations to approximately equivalent human external exposure doses using IVIVE and rTK. The AED identified as a NAM-based human POD for PFAS 5 is a BMD, modeled off of the AED-based dose-response data for the decreased epoxide hydrolase endpoint in liver (HepaRG) cells; the BMD_{AED1SD} = 0.004 mg/kg-day. This human POD was then divided by a composite UF of 100 which included a UF_H of 1 for human interindividual variability as the rTK model used in the conversion of *in vitro* cell-based concentrations to corresponding human AEDs takes into account population level human variability, a UF_A of 1 for extrapolation from animals to humans since the resulting BMD_{AED1SD} is an estimated human POD, a UF_L of 1 since the POD is a BMD, a UF_S of 10 for subchronic-to-chronic extrapolation since the effect was observed in a short-term exposure duration and it is unclear how longer exposure durations might impact underlying biology in a liver health outcome, and a UF_D of 10 as the database is limited to NAM data; no traditional toxicity studies were identified. The resulting RfD for PFAS 5 = 0.004 mg/kg-day/100 = 4 E-5 mg/kg-day. Appropriate characterization and denoting of confidence and qualitative and quantitative uncertainty(ies) in RfVs derived for PFAS in this category is imperative. Consultation with experts in the field of NAM data interpretation and risk assessment application is recommended.

Summary of RfDs

In summary, as shown in Table 5-3, RfDs for PFAS 1–5 range from 10^{-5} to 10^{-8} mg/kg-day, with PFAS 1 being the most potent overall. Note that PFAS 4 and 5 have similar RfDs despite their data limitations.

Table 5-3. Summary of POD_{HED}s and RfDs for hypothetical PFAS in a mixture.

	Liver POD_{HED} (mg/kg-day)	Thyroid POD_{HED} (mg/kg-day)	Developmental POD_{HED} (mg/kg-day)	RfD (mg/kg-day)	Confidence
PFAS 1	0.044	0.24	**0.00001 ($BMDL_{0.1ERHED}$)**	3 E-8	High (formal toxicity assessment)
PFAS 2	**0.0013 ($BMDL_{10HED}$)**	0.23	0.0051	1 E-5	High (formal toxicity assessment)
PFAS 3	N/A	**0.21 ($BMDL_{1SDHED}$)**	2.1	7 E-4	High (formal toxicity assessment)
PFAS 4	50	N/A	**0.0011 ($BMDL_{1SDHED}$)**	1 E-5	Medium (high quality *in vivo* data)
PFAS 5	**0.004 (BMD_{1SDAED})**	N/A	N/A	4 E-5	Low (bioactivity-based)

Note:
Bold indicates lowest (most sensitive) health outcome selected for RfD derivation.

5.2.2 General HI Step 2: Assemble/derive health-based media concentrations (HBWC)

Dependent upon the problem formulation, the user has the option to either use the oral RfV calculated for mixture components, or to leverage such values in the calculation of media-specific values, such as HBWCs for drinking water. Care should be taken to ensure that all HBWCs are applicable to the same exposure duration. In the following examples, the HBWCs are derived using chronic oral RfDs and thus are considered health protective values *over a lifetime* of exposure.

How to Calculate a HBWC for Drinking Water

The following equation is used to derive a noncancer HBWC. A noncancer HBWC, such as a lifetime HA or MCLG, is designed to be protective of noncancer effects over a lifetime of exposure, including sensitive populations and life stages, and is typically based on data from chronic experimental animal toxicity and/or human epidemiological studies. The calculation of a HBWC includes an oral RfV such as a RfD (or chronic MRL or duration relevant user-provided value), body weight-based drinking water intake (DWI-BW), and a relative source contribution (RSC) factor as presented in equation 5-3.

$$\text{Noncancer HBWC} = (\text{RfD}/(\text{DWI-BW})) * \text{RSC} \qquad \text{(Eqn. 5-3)}$$

Where:

RfD = chronic reference dose—an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily oral exposure of the human population to a substance that is likely to be without an appreciable risk of deleterious effects during a lifetime. (see HI step 1 above).

DWI-BW = the 90th percentile drinking water intake (DWI) for the selected population or life stage, adjusted for body weight (BW), in units of liters of water consumed per kilogram body weight per day (L/kg bw-day). The DWI-BW considers both direct and

indirect consumption of drinking water (indirect water consumption encompasses water added in the preparation of foods or beverages, such as tea or coffee).

RSC = relative source contribution—the percentage of the total oral exposure attributed to drinking water sources (EPA, 2000b), with the remainder of the exposure allocated to all other routes or sources.

When developing HBWCs, the goal is to protect all ages of the general population including potentially sensitive populations or life stages such as children. The approach to select the DWI-BW and RSC for the HBWC includes a step to identify sensitive population(s) or life stage(s) (i.e., populations or life stages that may be more susceptible or sensitive to a chemical exposure) by considering the available data for the contaminant. Although data gaps can make it difficult to identify the most sensitive population (e.g., not all windows or life stages of exposure or health outcomes may have been assessed in available studies), the critical effect and POD that form the basis for the RfD can provide some information about sensitive populations because the critical effect is typically observed at the lowest tested dose among the available data. Evaluation of the critical study, including the exposure interval, may identify a particularly sensitive population or life stage (e.g., pregnant women, formula-fed infants, lactating women). In such cases, the user can select the corresponding DWI-BW for that sensitive population or life stage from the Exposure Factors Handbook (EPA, 2019a) to derive the HBWC. In practice, when multiple populations or life stages are identified based on the principal study design and critical effect or other health effects data (from animal or human studies), EPA selects the population or life stage with the greatest DWI-BW because it is the most health protective. This approach ensures that all populations and life stages are protected at the HBWC, and in the case of the HI approach, that each component HQ and the overall HI is protective of all populations and life stages. In the absence of information indicating a sensitive population or life stage (e.g., non-developmental critical effect as in PFAS 2 and 3, or NAM-based reference dose as in PFAS 5), the DWI-BW corresponding to all ages of the general population may be selected (Table 5-4).

Table 5-4 shows EPA exposure factors for DWI for some sensitive populations and life stages. Other populations or life stages may also be considered depending on the available information regarding study design and sensitivity to health effects after exposure to a contaminant.

Table 5-4. EPA Exposure Factors for Drinking Water Intake.

Population or Life Stage	DWI-BW (L/kg bw-day)	Description of Exposure Metric	Source
General population (all ages)	0.0338	90th percentile direct and indirect consumption of community water, consumer-only two-day average, all ages.	2019 Exposure Factors Handbook Chapter 3, Table 3-21, NHANES 2005–2010 (EPA, 2019a)
Children	0.143	90th percentile direct and indirect consumption of community water, consumer-only two-day average, birth to < 1 year.	2019 Exposure Factors Handbook Chapter 3, Table 3-21, NHANES 2005–2010 (EPA, 2019a)

Population or Life Stage	DWI-BW (L/kg bw-day)	Description of Exposure Metric	Source
Formula-fed infants	0.249	90th percentile direct and indirect consumption of community water, formula-consumers only, 1 to < 3 months. Includes water used to reconstitute formula plus all other community water ingested.	Kahn et al. (2013), Estimates of Water Ingestion in Formula by Infants and Children Based on CSFII 1994–1996 and 1998[a,b]
Pregnant women	0.0333	90th percentile direct and indirect consumption of community water, consumer-only two-day average.	2019 Exposure Factors Handbook Chapter 3, Table 3-63, NHANES 2005–2010 (EPA, 2019a)
Women of childbearing age	0.0354	90th percentile direct and indirect consumption of community water, consumer-only two-day average, 13 to < 50 years.	2019 Exposure Factors Handbook Chapter 3, Table 3-63, NHANES 2005–2010 (EPA, 2019a)
Lactating women	0.0469	90th percentile direct and indirect consumption of community water, consumer-only two-day average.	2019 Exposure Factors Handbook Chapter 3, Table 3-63, NHANES 2005–2010[c] (EPA, 2019a)

Notes: CSFII = continuing survey of food intake by individuals; L/kg bw-day = liter per kilogram body weight per day.
[a] The sample size does not meet the minimum reporting requirements as described in the Third Report on Nutrition Monitoring in the United States (LSRO, 1995).
[b] Chapter 3.2.3 in EPA (2019a) cites Kahn et al. (2013) as the source of drinking water ingestion rates for formula-fed infants. While EPA (2019a) provides the 95th percentile total direct and indirect water intake values, Office of Water/Office of Science and Technology (OW/OST) policy is to utilize the 90th percentile DWI-BW.
[c] Estimates are less statistically reliable based on guidance published in the Joint Policy on Variance Estimation and Statistical Reporting Standards on NHANES III and CSFII Reports: Human Nutrition Information Service (HNIS)/National Center for Health Statistics (NCHS) Analytical Working Group Recommendations (NCHS, 1993).

To account for potential aggregate risk from exposures and exposure pathways other than oral ingestion of drinking water, EPA applies an RSC when calculating HBWCs to ensure that total human exposure to a contaminant does not exceed the daily exposure associated with the RfD. When data are available for multiple sensitive populations or life stages, the most health-protective RSC is selected. The RSC represents the proportion of an individual's total exposure to a contaminant that is attributed to drinking water ingestion (directly or indirectly in beverages like coffee, tea, or soup, as well as from transfer to dietary items prepared with drinking water) relative to other exposure pathways. The remainder of the exposure equal to the RfD is allocated to other potential exposure sources (EPA, 2000a). The purpose of the RSC is to ensure that the level of a contaminant (e.g., HBWC value), when combined with other identified potential sources of exposure for the population of concern, will not result in exposures that exceed the RfD (EPA, 2000a).

To determine the RSC, EPA follows the Exposure Decision Tree for Defining Proposed RfD (or POD/UF) Apportionment in EPA's guidance, *Methodology for Deriving Ambient Water Quality Criteria for the Protection of Human Health* (EPA, 2000a). EPA considers whether there are

significant known or potential uses/sources other than drinking water, the adequacy of data and strength of evidence available for each relevant exposure medium and pathway, and whether adequate information on each source is available to quantitatively characterize the exposure profile. The RSC is developed to reflect the exposure to the general population or a sensitive population within the general population exposure.

Per EPA's guidance, in the absence of adequate data to quantitatively characterize exposure to a contaminant, EPA typically recommends an RSC of 20%. When scientific data demonstrating that sources and routes of exposure other than drinking water are not anticipated for a specific pollutant, the RSC can be raised as high as 80% based on the available data, thereby allocating the remaining 20% to other potential exposure sources (EPA, 2000a). For the illustrative hypothetical PFAS mixture, an RSC of 0.2 (i.e., 20%) is selected as no information was identified to suggest a higher value. The calculation of HBWCs for PFAS 1–5 are presented in Table 5-5.

Table 5-5. Calculation of HBWCs for hypothetical PFAS in a mixture.

Chemical	Oral Reference Dose (mg/kg-day)	DWI-BW (L/kg-day)	RSC	HBWC (ng/L)
PFAS 1	3 E-8	0.0354	0.2	0.2
PFAS 2	1 E-5	0.0338	0.2	60
PFAS 3	7 E-4	0.0338	0.2	4,000
PFAS 4	1 E-5	0.0469	0.2	43
PFAS 5	4 E-5	0.0338	0.2	200

5.2.3 General HI Step 3: Select exposure estimates (measured water concentrations)

Select appropriate exposure estimates consistent with the problem formulation. Specifically, the user may choose to calculate or use exposure estimates that are for the oral route in general (i.e., total intake in mg/kg-day) or media-specific concentrations. In the hypothetical PFAS mixture example, 'exposure' is represented by the drinking water monitoring data in Table 4-1.

5.2.4 General HI Step 4: Calculate PFAS mixture potency (component HQs and overall HI)

Using the median of the drinking water monitoring data (Table 4-1) and the calculated HBWCs for PFAS 1–4 (Table 5-5), individual component HQs are derived as shown in Table 5-6.

Table 5-6. Calculation of individual component hazard quotients (HQs) for the hypothetical PFAS mixture.

	Hypothetical Drinking Water Exposure Estimate (ng/L)	HBWC (ng/L)	General HQ
PFAS 1	4.8	0.2	24.0
PFAS 2	52	60	0.9

	Hypothetical Drinking Water Exposure Estimate (ng/L)	HBWC (ng/L)	General HQ
PFAS 3	172	4,000	0.04
PFAS 4	58	43	1.3
PFAS 5	69	200	0.3
MIXTURE GENERAL HAZARD INDEX (HI)			**27**

Notes: HQ = DW Exposure Estimate/HBWC; HI = the sum of individual HQs.

5.2.5 General HI Step 5: Compare PFAS mixture potency (HI) to existing health-based value (1.0)

The HI (27) is significantly greater than 1.0, indicating health risks to the mixture of PFAS at the measured drinking water concentrations. Further, as illustrated by the individual component HQs, PFAS 1 and 4 are risk drivers of the mixture HI with individual HQs greater than 1.0; PFAS 2 and 5 also appear to be contributors with HQs of 0.9 and 0.3, respectively. Assessment of PFAS 2 and 5 in isolation (individually) would indicate no/low risk (i.e., HQs < 1), but assessment of the binary mixture of PFAS 2 and 5 would indicate appreciable risk (HI = 1.2). Conversely, with a HQ of 0.04, PFAS 3 is less influential compared to the other mixture components. In this hypothetical scenario, clearly PFAS 1 and 4, and potentially PFAS 2 and 5, might be prioritized for remediation activity(ies).

It should be noted that in the hypothetical PFAS mixture, the HBWC for PFAS 1 (0.2 ng/L) is lower than its corresponding hypothetical drinking water analytical quantitation limit of 4 ng/L by over an order of magnitude. In such cases, any detectable level (i.e., of PFAS 1) will result in an HI greater than 1.0 for the whole mixture.

5.3 Illustrative Example Application of the Target-Organ Specific HI (TOSHI) to a Hypothetical Mixture of Five PFAS

5.3.1 TOSHI Step 1: Assemble/derive component health effects endpoints (RfDs or target-organ toxicity doses)

Application of the TOSHI is essentially identical to the steps for the general HI. The critical nuance is that use of human health/toxicity values across mixture components are effect/endpoint specific. For some PFAS, this might be 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, although in some federal and state purviews this practice is changing. In those instances where only an overall RfD (or MRL) has been derived, 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. TTDs are derived identically to RfDs; however, there may be differing circumstances to consider such as type of POD (e.g., BMD vs NOAEL or LOAEL), cross-species TK dosimetric adjustment (e.g., RfD may have been derived from a POD based on an adjustment of rat kinetics to human kinetics, whereas a TTD for the same chemical might be mouse to human resulting in different POD_{HED}),

and/or different qualitative and quantitative uncertainties. In practice, human health assessment applications including mixtures assessment may be more robust if TTDs are derived across all health outcome domains that are supported by evidence. For the purposes of the illustrative hypothetical PFAS mixture example, the calculation of TTDs is limited to the three selected health effect domains listed in Table 5-7. Several more TTDs could potentially be derived based on availability of data and confidence in the evidence conclusions.

Table 5-7. Target Organ Toxicity Doses (TTDs) for the hypothetical mixture PFAS; the bolded numbers represent the overall RfD for each respective PFAS.

Target Organ Toxicity Doses (mg/kg-day)					
Effect domain	PFAS 1	PFAS 2	PFAS 3	PFAS 4	PFAS 5
Liver	7 E-6	**1 E-5**	–	5 E-5	4 E-5*
Thyroid	2 E-6	4 E-4	**7 E-4**	–	–
Developmental	**3 E-8**	9 E-3	2 E-3	**1 E-5**	–

Note:
* TTD_{NAM} based on *in vitro* perturbation indicative of oxidative stress in liver cells.

5.3.2 TOSHI Step 2: Assemble/derive health-based media concentrations (HBWC)

To calculate HBWCs for the TOSHI, the TTDs for a specific effect domain across mixture components are used in the calculation of HQs and a TOSHI. For example, a TOSHI for developmental effects ($TOSHI_{DEV}$) for the hypothetical PFAS mixture can be calculated using the developmental TTDs, appropriate DWI-BWs and RSCs (Table 5-8). For this developmental example, the DWI-BW is the 90th percentile direct and indirect consumption of community water, consumer-only two-day average, for women of childbearing age (13 to < 50 years).

Table 5-8. Calculation of Developmental Effect-Specific HBWCs for hypothetical PFAS in a mixture using TTDs.

Chemical	Target Organ Toxicity Dose (mg/kg-day)	DWI-BW (L/kg-day)	RSC	$TOSHI_{DEV}$ HBWC (ng/L)
PFAS 1	3 E-8	0.0354	0.2	0.2
PFAS 2	9 E-3	0.0354	0.2	50,000
PFAS 3	2 E-3	0.0354	0.2	10,000
PFAS 4	1 E-5	0.0469	0.2	43
PFAS 5	--	N/A	N/A	ND

Notes: N/A = not applicable; ND = not determined.
Bolded numbers indicate that the TTD for developmental effects is the overall RfD for that PFAS.

5.3.3 TOSHI Step 3: Select exposure estimates (measured water concentrations)

Select appropriate exposure estimates consistent with the problem formulation. Specifically, the user may choose to calculate or use exposure estimates that are for the oral route in general (i.e., total intake in mg/kg-day) or media-specific concentrations. In the hypothetical PFAS mixture example, 'exposure' is represented by the drinking water monitoring data in Table 4-1.

5.3.4 TOSHI Step 4: Calculate PFAS mixture potency (component HQs and overall TOSHI)

Using the median of the drinking water monitoring data (Table 4-1) and the calculated HBWCs for PFAS 1–4 derived from TTDs for the developmental effect domain (Table 5-8), individual component HQs are derived as shown in Table 5-9.

Table 5-9. Calculation of individual component hazard quotients (HQs) specifically for developmental effects associated with the hypothetical PFAS mixture. The HBWCs in this TOSHI application are derived from TTDs for the developmental effect domain.

Chemical	Hypothetical Drinking Water Exposure Estimate (ng/L)	$TOSHI_{DEV}$ HBWC (ng/L)	$TOSHI_{DEV}$ HQ
PFAS 1	4.8	0.2	24
PFAS 2	52	50,000	0.001
PFAS 3	172	10,000	0.01
PFAS 4	58	43	1.3
PFAS 5	69	ND	ND
MIXTURE $TOSHI_{DEV}$			**25**

Notes: HQ = DW Exposure Estimate/HBWC; HI = the sum of individual HQs. ND = not determined.

5.3.5 TOSHI Step 5: Compare PFAS mixture potency (HI) to existing health benchmark (1.0)

The $TOSHI_{DEV}$ of 25 indicates concern for developmental effects associated with exposure to the hypothetical PFAS mixture at the measured drinking water concentrations (Table 4-1). While this example application shows that use of TTDs did not meaningfully diminish indication of health risk associated with the mixture (compared to a general HI approach), the individual HQs clearly demonstrate drivers (PFAS 1 and 4) and relative inerts (PFAS 2 and 3) for developmental health outcomes. The converse is possible dependent on the TTDs for different health outcomes, and differing PFAS concentrations in environmental media. Please note that the magnitude of an HI, TOSHI, 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 20 is not necessarily of 10-fold greater concern than a mixture HI of 2.0. The practical interpretation is that both mixture approaches would indicate appreciable risk of health effects in exposed populations.

5.4 Advantages and Challenges of the General HI Approach

The HI approach provides an indication of the joint 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 $\geq$ 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 general HI (e.g., all liver or all kidney effects). Specifically, the 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 noncancer RfV (e.g., oral RfD). As such, a HI will typically represent the most health-protective indicator of mixture risk, as each component HQ is based on its overall RfV.

The HI is an indication of appreciable risk, 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 composite UF, then the associated HI is also highly uncertain.

Another challenge 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 typically 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, 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 comprehensive and transparent systematic approaches and standardized protocols. 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 bioactivity-based assays/platforms (or read-across) may 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, NAMs may represent the only opportunity to integrate a data-poor PFAS into mixtures assessment. Further, while the integration of NAMs into applications such as mixtures risk assessment was demonstrated in the hypothetical example using a POD from a specific assay-type (*in vitro* cell bioactivity), available NAM data could be leveraged from a diverse assay portfolio. For example, transcriptomic data from whole animals or cells *in vitro* using platforms such as BioSpyder (i.e., TempO-Seq; see https://www.biospyder.com/), microarrays, and/or RT-PCR may represent additional opportunities to integrate validated methods and data into assessment application. A potential future improvement using NAMs such as cell bioactivity (including transcriptomics) may be the categorical integration of qualitative and quantitative information from across platforms to develop more comprehensive NAM-based hazard determinations and identification of candidate PODs (i.e., consensus lower bound BMD; cross-NAM platform mean; etc.). 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 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.

5.5 Advantages and Challenges of the 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 HI, a TOSHI approach might benefit from consideration of NAM data and approaches that can inform organ/tissue-specific dose-response.

6.0 Relative Potency Factor (RPF) Approach

6.1 Background on RPF Approach

RPF approaches comprise another 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 (Simmons et al., 2004), 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 and most robust toxicological database and is considered to be most representative of the type of toxicity caused by the mixture components (EPA, 1986, 2000b). The role of the IC in the RPF approach is to serve as the point of reference for standardizing the common toxicity (i.e., scaling the potencies) of all component chemicals in the analysis. The most important consideration in selecting one mixture component over another as an IC is that *high*-quality dose-response data are available (e.g., for the common toxic effect/species/sex) for the exposure route, duration, and pathways of interest. Further, the IC must have dose-response data for the dose range of interest; chemicals with steep slopes that cause an effect and/or induce significant toxicity at all doses tested are not ideal for IC selection. In most cases, identification of a single best mixture component IC will be evident. However, in the event that two or more mixture components are identified as candidate ICs, the user must judge which candidate is most representative of the mixture, or subgroupings within a mixture, and has the most robust toxicity database. It should be noted that selection of an IC can be duration-, exposure route-, and/or health outcome-specific. That is, in practical application, it is possible that different mixture components may be optimal ICs under different scenarios; for example, mixture components A and B may both be identified as candidate ICs in general, however, candidate A may be selected as the IC if it has a more robust evidence base for a specific application of interest (e.g., oral/subchronic duration). 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, 2000b). 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 LOAELs, 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 (e.g., 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 BMR level (e.g., 0.5 SD or 1 SD 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 are sometimes not amenable to BMD modeling. Human equivalent LOAELs or ED_X values are 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 6-1:

$$RPF_j = \frac{ED10_{IC}}{ED10_j} \quad \text{(Eqn. 6-1)}$$

where IC refers to the index chemical.

For example, if mixture component chemical 2 is twice as potent as the IC, its LOAEL, 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 LOAEL, 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 vs. chronic exposure, oral vs. 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 in Equation 6-1, ICECs are then calculated by multiplying each respective RPF_j by the corresponding component chemical's concentration (d_j), as shown in Equation 6-2:

$$ICEC_{MIX} = \sum_{j=1}^{n} d_j * RPF_j \quad \text{(Eqn 6-2)}$$

The total mixture ICEC ($ICEC_{MIX}$) is then obtained by taking the sum of the component chemical ICECs (including that of the IC) (Equation 6-3). A numerical estimate of risk for noncancer 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:

$$y_{MIX} = f(ICEC_{MIX}) \quad \text{(Eqn 6-3)}$$

where the ICEC is derived from Equation 6-3. In the context of this PFAS mixture framework, there are important modifications or adaptations of this approach to note that include: (1) use of ICECs, which are water-specific, correlates to index chemical equivalent doses (ICEDs) (EPA, 2000b) 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, 2000b) 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 (organophosphorus pesticides, triazines, N-methyl carbamates, chloroacetanilides, and pyrethrins/pyrethroids), which in each case were based on MOA-level information (EPA, 2018). However, 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. Further, as empirically demonstrated by Conley et al. (2022b), due to potential variability of potency for health effects across PFAS, RPFs can vary by more than an order of magnitude. Thus, where possible, it is preferable for a given PFAS mixture to evaluate multiple common effect domains or endpoints, where and when dose-response data are available, to identify the most sensitive endpoint for evaluation of risk. Using the most sensitive endpoint(s) for the RPF analysis helps to ensure that risks are not underestimated, and, providing a landscape of candidate RPFs across PFAS and health effects ensures transparent communication of mixtures risk assessment for decision-making. 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 (NRC, 2008).

6.2 Illustrative Example Application of RPF to a Hypothetical Mixture of Five PFAS

The example application of the RPF approach incorporates hazard and dose-response information for the hypothetical five PFAS mixture presented in the HI sections above. However, in this context only dose-response data for like/similar health effect(s) are needed. Recall that PFAS 1–3 have existing hazard and dose-response data that have been formally evaluated for human health risk assessment purposes; these three PFAS also have existing HBWCs. PFAS 4 has not undergone risk assessment but has existing experimental animal assay data. Lastly, PFAS 5 is data-poor with only physicochemical, TK, and *in vitro* cell-based bioactivity data. This example focuses on development of RPFs for liver, thyroid, and developmental effects only (Figure 4-8), which have been reported as toxicity targets of several compounds within the broader class of PFAS (EPA, 2021a,b; ATSDR, 2021; EFSA et al., 2020; Section 7.1 in ITRC, 2022). The approach here is to use a construct that allows for combination of PFAS with shared, common health outcome (e.g., such as delayed growth and development in offspring), 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 PFAS 1 relative to PFAS 2 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 optimize identification of those that are shared among the PFAS in the mixture being assessed. However, for purposes of evaluating mixture risk using the RPF approach in a specific environmental medium (e.g., drinking water), it is critical to have an IC effect-specific value or metric (e.g., an HBWC) so that the mixture ICEC can be compared to a benchmark point. 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. However, leveraging available NAM data, such as *in vitro* cell bioactivity, may provide opportunities to integrate those PFAS with poor(er) hazard and dose-response databases.

6.2.1 RPF Step 1: Assemble/Derive component health effects endpoints (select ICs, POD_{HEDs})

As PFAS 1–3 are toxicologically well-characterized and have existing HBWCs, all three are identified as candidate ICs for the mixture. PFAS 4 is also reasonably well characterized toxicologically and might be considered as a candidate IC in some RPF contexts, however in a drinking water specific application another key consideration for IC selection is the existence of a quantitative benchmark such as a HBWC. This is necessary such that the index chemical equivalent concentration for the mixture ($ICEC_{MIX}$) can be compared to the IC's corresponding HBWC to determine potential for health risk(s). As such, PFAS 1–3 are the only candidate ICs identified for the hypothetical five component mixture. Based on the strength of toxicological evidence (see figure below; note, this is a repeat of Table 4-8), not necessarily the quantitative potency for effect, ICs were selected as follows: Liver IC = PFAS 2; Thyroid IC = PFAS 3; and Developmental IC = PFAS 1.

Effect domain	PFAS 1	PFAS 2	PFAS 3	PFAS 4	PFAS 5
Liver	++	+++	–	++	– *
Thyroid	++	++	+++	–	–
Developmental	+++	++	++	+++	–

The dose-response metrics for this RPF example are the same as those used above in the HI example (Table 5-3). The POD_{HEDs} for three effect domains used in the calculation of the effect-specific RPFs and corresponding ICECs are presented below (Table 6-1).

Table 6-1. Summary of POD_{HEDs} for three selected health effect domains for a mixture of five hypothetical PFAS.

	Liver POD_{HED} (mg/kg-day)	Thyroid POD_{HED} (mg/kg-day)	Developmental POD_{HED} (mg/kg-day)
PFAS 1	0.044	0.24	**0.00001 ($BMDL_{0.1ERHED}$)**
PFAS 2	**0.0013 ($BMDL_{10HED}$)**	0.23	0.0051
PFAS 3	N/A	**0.21 ($BMDL_{1SDHED}$)**	2.1
PFAS 4	50	N/A	**0.0011 ($BMDL_{1SDHED}$)**
PFAS 5	**0.004 (BMD_{1SDAED})**	N/A	N/A

Note:
Bolded numbers represent those PODs used in the derivation of corresponding oral RfVs.

6.2.2 RPF Step 2: Assemble/derive health-based media concentrations (HBWCs for the Index Chemicals)

For this illustrative RPF example, the HBWCs are the same as those used above in the General HI example (Table 5-5). Specifically, the PFAS 1 HBWC is 0.2 ng/L (IC for developmental

effects), PFAS 2 HBWC is 60 ng/L (IC for liver effects), and PFAS 3 HBWC is 4,000 ng/L (IC for thyroid effects).

6.2.3 RPF Step 3: Select exposure estimates (measured water concentrations)

Select appropriate exposure estimates consistent with the problem formulation. Specifically, the user may choose to calculate or use exposure estimates that are for the oral route in general (i.e., total intake in mg/kg-day) or media-specific concentrations. In the hypothetical PFAS mixture example, 'exposure' is represented by the drinking water monitoring data in Table 4-1.

6.2.4 RPF Step 4: Calculate PFAS mixture potency (RPFs and ICECs for each effect domain)

Liver: Available traditional animal assay data indicate liver effects for PFAS 1, 2, and 4. PFAS 5 has only bioactivity data however the molecular and cellular perturbations were observed primarily in hepatocyte cell cultures (e.g., HepG2; HepaRG). As such, there is an opportunity to integrate NAM-based information into the RPF approach specifically for the liver effect domain. Across the landscape of experimental rodent studies that inform liver toxicity for hypothetical PFAS 1, 2 and 4, several effects were noted after oral exposures such as increased absolute and relative organ weights, increased incidence of macro- and microvesicular steatosis (i.e., lipid accumulation in hepatocytes), histopathological evidence of focal hepatocellular necrosis, and increased serum ALT, AST, and ALP, indicative of hepatocyte or biliary epithelium injury, respectively. In addition, *in vitro* cell bioactivity data for PFAS 2 and 5 indicate increased pro-oxidation/oxidative stress, mitochondrial stress, and altered lipid homeostasis in the lower tested concentration range. Many of these observed cellular effects are considered KEs in signal transduction pathways leading to liver tissue alteration and injury (Figure 6-1). Of the effects observed in experimental rodents across PFAS 1, 2, and 4, histopathological evidence of significantly *increased incidence of hepatocellular death* was common across studies. Further, this effect was the basis for the derivation of an oral RfD and corresponding HBWC for PFAS 2. As such, *increased incidence of hepatocellular death* is identified as the common effect for the liver domain for PFAS 1, 2 and 4. The liver effect-specific RPFs are calculated by dividing the selected liver effect POD_{HED} for the IC PFAS 2 by the POD_{HED} for PFAS 1 and 4 for the same effect (Table 6-2). Each RPF is multiplied by the corresponding chemical-specific measured water concentration to derive a PFAS 2 ICEC (Table 6-2). The example Mixture Total PFAS 2 $ICEC_{MIX}$ is then compared to the HBWC for PFAS 2, which is based on the effect of *increased incidence of hepatocellular death*.

For PFAS 5, the dose-response used in this specific example RPF application, obtained from IVIVE/rTK of the *in vitro* cell bioactivity data, is based on the lowest bioactivity event[21] associated with the IC; that is, noncancer bioactivity at the lower end of the distribution for the IC is the driver for identification of "like" effect for the data-poor mixture component PFAS. In this hypothetical example, the bioactivity for the IC (PFAS 2) in HepaRG cells was decreased mitochondrial cytochrome c oxidase activity (Table 4-3). Importantly, cytochrome c oxidase activity is a key component in proper mitochondrial respiration and function; disruption of this mitochondrial enzyme has been associated with increased oxidative stress, decreased ATP production, and cell death. Surveying the landscape of available cell bioactivity data for PFAS 5

[21] The "lowest" bioactivity for noncancer application purposes should not be a potential carcinogenic event (e.g., mutagenicity or clastogenicity).

revealed that the same effect occurred in hepatocytes, although it was not the most sensitive perturbation for PFAS 5. The objective of this NAM-based approach is to scale the potency of the selected bioactive event for the data-poor chemical(s) to the same/similar bioactive event for the IC, where or when available data allow. The NAM-based RPF (RPF_{NAM}) is calculated by taking the ratio of the BMD_{AED50} for the selected bioactivity event of the IC to the BMD_{AED50} for the same event associated with the data-poor mixture component chemical. The rationale for using the BMD_{AED50} is that the quantitative relationship between a KE and an adverse health outcome is typically unknown. As such, in NAM practice it is common to default to a 50th percentile for comparative biology purposes. The resulting RPF_{NAM} represents the relative potency between the data poor PFAS (PFAS 5) and the IC (PFAS 2) for the selected bioactive event. This RPF_{NAM} is then multiplied by the data-poor chemical (e.g., PFAS 5) exposure metric (e.g., measured water concentration) to obtain a NAM-based ICEC ($ICEC_{NAM}$); to convert the $ICEC_{NAM}$ to a mixture ICEC that comports with the other traditional assay-based component PFAS ICECs, the $ICEC_{NAM}$ is multiplied by the ratio of $BMD_{X\text{-}HED}$ for the critical effect of the IC (in this example, the BMD_{10HED} for *increased incidence of hepatocellular death*) to the BMD_{AED50} for the bioactive event of the IC. The resulting ICEC represents the estimated contribution of PFAS 5 to the overall risk of the liver-specific effect, but represented as a dose scaled for potency, relative to the IC, across different levels of biological organization (i.e., PFAS 5 *in vitro* to PFAS 2 *in vivo*). This process is illustrated in Figure 6-2.

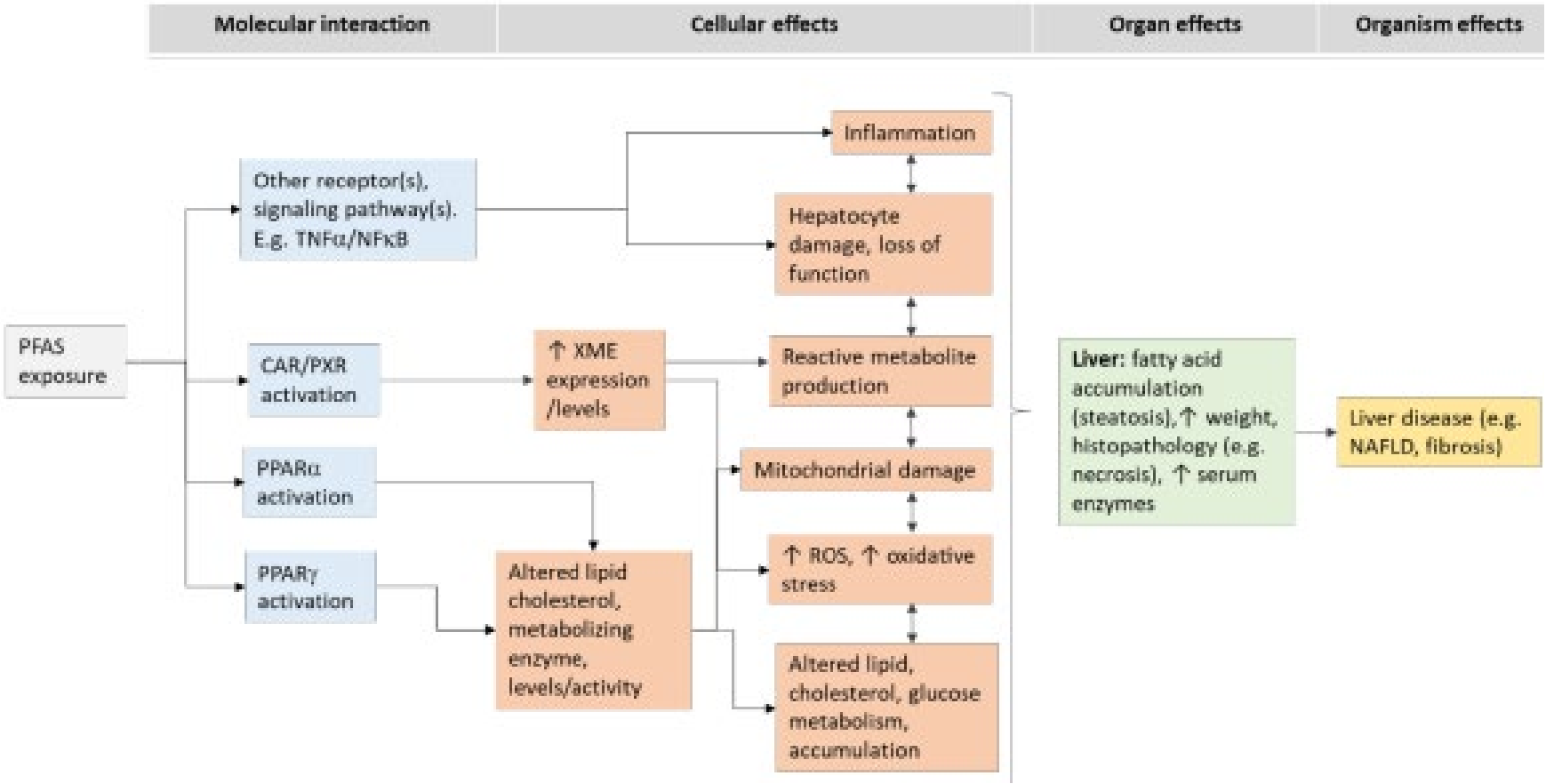
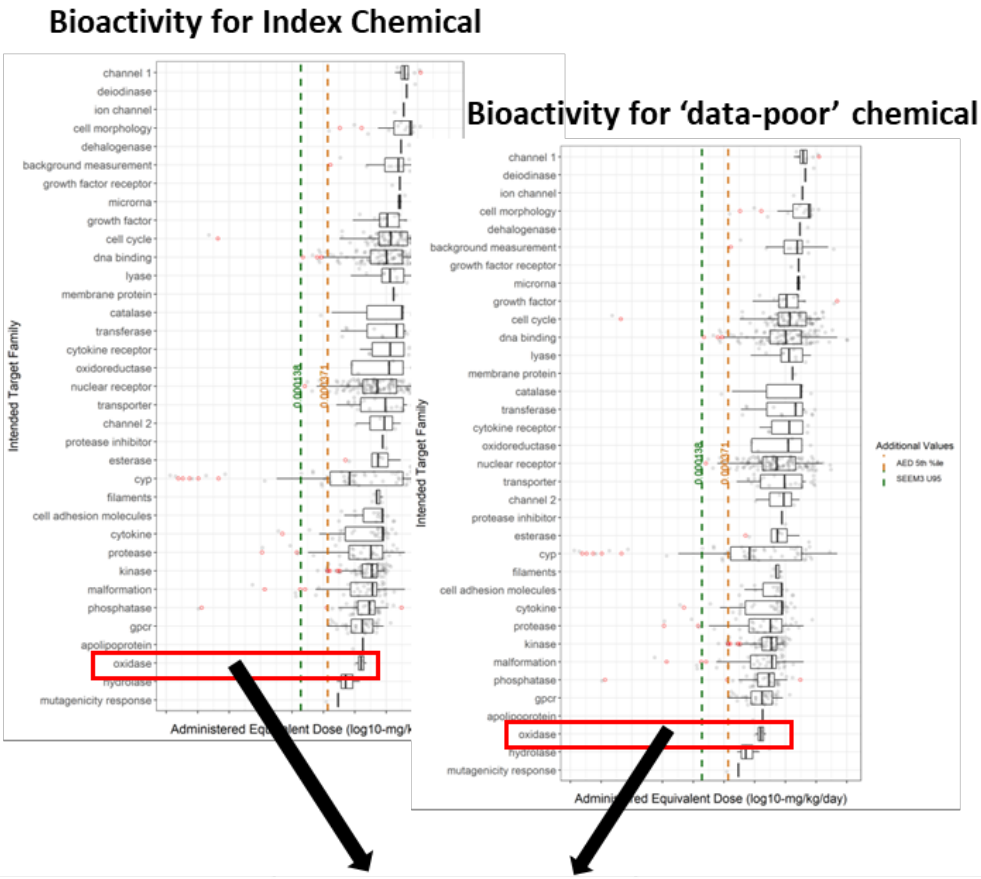


Figure 6-1. General cell signaling pathways associated with PFAS-induced liver injury.



Mixture Component	Selected Bioactivity AED	IC Critical Effect ($BMD_{x\text{-}HED}$)
Index Chem (IC)	Modeled BMD_{AED50}	Modeled $BMD_{x\text{-}HED}$
Data-poor member	Modeled BMD_{AED50}	–

$$RPF_{NAM} = \frac{\text{Selected Bioactivity } BMD_{AED50} \text{ for IC}}{\text{Selected Bioactivity } BMD_{AED50} \text{ for data-poor component}}$$

$$ICEC_{NAM} = RPF_{NAM} \times \text{Data-poor component exposure level (e.g., DW conc.)}$$

$$\textbf{ICEC} = ICEC_{NAM} \times BMD_{X\text{-}HED} \text{ for IC critical effect / Selected Bioactivity } BMD_{AED50} \text{ for IC}$$

Figure 6-2. Proposed process for integrating NAM-based RPFs and ICECs into mixtures assessment.

Table 6-2. Example Liver Effect RPFs and ICECs for a Hypothetical Mixture of Five PFAS

Mixture Component	POD_{HED} (mg/kg-day); *Increased incidence of hepatocellular death*	Example RPF	Hypothetical Exposure Estimate (ng/L)	PFAS 2 ICEC (ng/L)
PFAS 1	0.044 ($BMDL_{10HED}$)	0.03	4.8	0.1
PFAS 2 (IC)	0.0013 ($BMDL_{10HED}$)	1	52	52
PFAS 3	N/A	N/A	172	—
PFAS 4	50	0.00003	58	0.002
PFAS 5	0.037 (BMD_{AED50})[a]	1.4 (RPF_{NAM})[b]	69	24[c]
Mixture Total PFAS 2 ICEC (ppt)				**76**

Notes:
[a] The BMD was modeled from the AED-based dose-response for the selected bioactivity event (e.g., decreased mitochondrial cytochrome c oxidase activity) at a BMR of 50% over control.
[b] RPF_{NAM} for PFAS 5 was calculated as the ratio of the BMD_{AED50} for PFAS 2 (IC) / BMD_{AED50} for PFAS 5; in this example application, 0.0052 mg/kg-day / 0.0037 mg/kg-day = 1.4.
[c] The ICEC for PFAS 5 was calculated by first deriving the $ICEC_{NAM}$ as follows: RPF_{NAM} × Exposure estimate for PFAS 5 = 1.4 × 69 (ng/L) = 97 ng/L; the $ICEC_{NAM}$ was then multiplied by the ratio of the $BMDL_{10HED}$ for PFAS 2 / BMD_{AED50} for PFAS 2 = 97 ng/L × (0.0013/0.0052) = 24 ng/L.

Thyroid: Available traditional animal assay data indicate thyroid effects for PFAS 1, 2, and 3. PFAS 4 and 5 have no data available to support inclusion in the RPF analysis for this health effect domain. Across the landscape of experimental rodent studies that inform thyroid toxicity for hypothetical PFAS 1, 2 and 3, two effects were noted after oral exposures including decreased levels of total and free thyroxine (T4) and triiodothyronine (T3) and increased absolute and/or relative thyroid weight. Importantly, effects on thyroid hormone levels were observed across sexes and life stages (e.g., newborn and adult rats and mice) and was a common observation across PFAS 1–3. Further, decreased thyroid hormone levels, particularly total and free T4 (TT4 and FT4, respectively) in females, was the basis for derivation of the oral RfD and the corresponding HBWC for PFAS 3. As such, *decreased TT4 and FT4* is identified as the common effect for the thyroid domain for PFAS 1–3. The thyroid effect-specific RPFs are calculated by dividing the selected thyroid effect POD_{HED} for the IC PFAS 3 by the POD_{HED} for PFAS 1 and 2 for the same effect (Table 6-3). Each RPF is multiplied by the corresponding chemical-specific measured water concentration to derive a PFAS 3 ICEC (Table 6-3). The example Mixture Total PFAS 3 ICEC is then compared to the HBWC for PFAS 3, which is based on the effect of decreased TT4 and FT4. The calculation of the thyroid-specific RPFs and corresponding ICECs are presented in Table 6-3.

Table 6-3. Example Thyroid Effect RPFs and ICECs for a Hypothetical Mixture of Five PFAS

Mixture Component	POD_{HED} (mg/kg-day); *Decreased TT4 and FT4*	Example RPF	Hypothetical Exposure Estimate (ng/L)	PFAS 3 ICEC (ng/L)
PFAS 1	0.24 ($BMDL_{1SDHED}$)	0.9	4.8	4
PFAS 2	0.23 ($BMDL_{1SDHED}$)	0.9	52	47
PFAS 3 (IC)	0.21 ($BMDL_{1SDHED}$)	1	172	172
PFAS 4	N/A	N/A	58	—
PFAS 5	N/A	N/A	69	—
Mixture Total PFAS 3 ICEC (ppt)				**223**

Developmental: Developmental effects associated with oral exposures to PFAS 1, 2, 3, or 4 were observed in rats and mice; the studies available were predominately single-generation reproductive-developmental design however PFAS 4 also had a two-generation study in rats. PFAS 5 had no studies/data to suggest effects in the developmental domain. The landscape of developmental effects in newborns or neonates for PFAS 1–4 was broad and included decreased body weight at PND 0, delayed growth and development at PND 14 (e.g., reduced body weight, delayed eye opening and vaginal patency), decreased thyroid hormones at PND 0, and reduced ossification of phalanges. However, decreased body weight in offspring at birth was common across PFAS 1–4; further this effect was used as the basis for the derivation of the oral RfD and corresponding HBWC for the IC, PFAS 1. As such, *decreased body weight in offspring* was selected as the common developmental effect for the purposes of this RPF illustrative example. The developmental effect-specific RPFs are calculated by dividing the POD_{HED} for the selected effect associated with the IC PFAS 1 by the POD_{HED} for PFAS 2, 3, and 4 for the same effect (Table 6-4). Each RPF is multiplied by the corresponding chemical-specific measured water concentration to derive a PFAS 1 ICEC (Table 6-4).

Table 6-4. Example Developmental Effect RPFs and ICECs for a Hypothetical Mixture of Five PFAS

Mixture Component	POD_{HED} (mg/kg-day); *Decreased Body Weight in Offspring*	Example RPF	Hypothetical Exposure Estimate (ng/L)	PFAS 1 ICEC (ng/L)
PFAS 1 (IC)	0.00001 ($BMDL_{1SDHED}$)	1	4.8	5
PFAS 2	0.0051 ($BMDL_{1SDHED}$)	0.002	52	0.1
PFAS 3	2.1 ($BMDL_{1SDHED}$)	5 E-6	172	0.0009
PFAS 4	0.0011 ($BMDL_{1SDHED}$)	0.009	58	0.5
PFAS 5	N/A	N/A	69	—
Mixture Total PFAS 1 ICEC (ppt)				**6**

6.2.5 RPF Step 5: Compare PFAS mixture potency (Total $ICEC_{MIX}$) to existing health-based value (HBWC)

In the liver-specific RPF application (Table 6-2), the health risk(s) associated with the mixture is represented by comparing the PFAS 2 $ICEC_{MIX}$ to the IC HBWC which is based on the specified effect for that hazard domain (e.g., for this example, increased incidence of hepatocellular death). In this hypothetical example, the PFAS 2 $ICEC_{MIX}$ of 76 ppt exceeds the PFAS 2 HBWC of 60 ppt, indicating potential for risk of liver effects in individuals or populations exposed to a mixture of the five PFAS at the hypothetical water exposure estimates provided. Importantly, PFAS 2 and 5 appear to be drivers for the liver health risk associated with the hypothetical mixture.

In the thyroid-specific RPF application (Table 6-3), the health risk(s) associated with the mixture is represented by comparing the mixture total PFAS 3 $ICEC_{MIX}$ to the IC HBWC which is based on the specified effect for hazard domain (e.g., for this example, decreased TT4 and FT4). In this hypothetical example, the PFAS 3 $ICEC_{MIX}$ of 223 ppt is far below the PFAS 3 HBWC of 4000 ppt, indicating no apparent risk of thyroid effects in exposed individuals or populations to a mixture of the five PFAS at the hypothetical water exposure estimates provided.

In the developmental effect-specific RPF application (Table 6-4), the health risk(s) associated with the mixture is represented by comparing the mixture total PFAS 1 $ICEC_{MIX}$ to the IC HBWC which is based on the specified effect for hazard domain (e.g., for this example, decreased body weight in offspring). In this hypothetical example, the PFAS 1 $ICEC_{MIX}$ of 6 ppt exceeds the PFAS 1 HBWC of 0.2 ppt by over an order of magnitude, indicating significant potential for health risks in developmental populations exposed to a mixture of the five PFAS at the hypothetical water exposure estimates provided.

As illustrated in the RPF examples above, PFAS can have different potencies across health effect domains. Due to differences in both TK and TD, 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(ies). Thus, calculating RPFs for as many endpoints/effects as possible helps to ensure that subsequent PFAS risk management strategies are health protective. In the example above, risk would have been underestimated if the RPF analysis was limited to liver and thyroid effects: developmental effects are the risk driver in this scenario. 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; temporal and spatial variability). 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 7 offers an alternative to the RPF approach in such a scenario.

6.3 Advantages and Challenges of the Relative Potency Factor Approach

An 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 or necessitate use of effect data deriving from diverse study designs and exposure durations. As such, in some cases, there may be a need to normalize or adjust available quantitative metrics such that potency comparisons are at comparable points on a given dose-response. For example, there might be a need to selectively apply UFs in the RPF method, in particular, the LOAEL-to-NOAEL (UF_L) and/or subchronic-to-chronic duration (UF_S) factors to convert quantitative metrics to NOAELs from an estimated chronic-duration exposure. This flexibility is needed as in some cases, effect data for mixture component PFAS may come from a variety of study designs such as reproductive/developmental 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. 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) among component PFAS. 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, including PFAS, have limited-to-no MOA data available. 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, 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 framework, is that the same dose-response data that underpinned the derivation of corresponding RfVs (overall RfDs or organ-specific TTDs) for use as input(s) for HQs and HIs would also be leveraged in RPF and/or M-BMD approaches (see Sections 6 and 7)). 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).

Another challenge is that depending on data availability for the component PFAS, the effect domains used for the RPF analysis may not be the overall most sensitive out of the total constellation of common PFAS effects. In the RPF examples shown above, risk is indicated based on the liver and developmental RPFs, but not for the thyroid effect domain. To effectively use the RPF approach, the user needs effect data for at least one common endpoint among the effects for all component PFAS in the mixture. Ideally this would include the most sensitive effect across PFAS in the mixture of interest in order to provide a conservative (health protective) risk-based scenario.

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 in this hypothetical example 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 AED) is suitable for traditional RPF calculation simply based on the interpretation of "same" effect. Further investigation is needed to evaluate 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. This is particularly true of NAMs where the possible lumping or splitting of assay/data types to inform an integrated or more individualized interpretation of hazard and dose-response for data-poor mixture components is in its infancy; case studies using validated NAM assays and data are needed to help optimize application in mixtures risk assessment.

7.0 Mixture-BMD Approach

7.1 Background on the Mixture-BMD Approach

Given the broad range of PFAA congeners and structural diversity across the PFAS class, it is likely that for some effects used for mixture assessment the dose response functions (i.e., slopes) will be dissimilar across component chemicals. The use of an IC in the RPF approach assumes component chemicals have congruent dose response slopes for same health effect (and/or MOA). In addition, the HI approach requires human health assessment values, such as oral RfDs and individual HBWCs, because 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 congruent dose response curves or have available human health assessment values (e.g., RfDs). In these cases, a third approach, called the M-BMD, can be used to estimate health risk(s) associated with mixture exposure. This approach is described in EPA's supplementary guidance (2000b) (Section 4.2.6) and NRC (2008) (Appendix C) and employs a DA model-based calculation of a total M-BMD that corresponds to a defined BMR (e.g., BMD_{10}) for a PFAS mixture. Similar to the RPF approach, only effects/endpoints and associated dose-response metrics (e.g., BMD_X) are needed to perform the exercise. Further, the mixture evaluation is based on a similar toxicological effect for component chemicals and the equation provided can be used to define a single point estimate (e.g., POD) or derive a full dose response curve for the PFAS mixture of interest.

Because RPFs are special applications of the DA concept, such approaches can be a straightforward way of making quantitative assessments of the effects of chemicals, including PFAS. However, application of the RPF concept requires congruent dose-response curves for all component chemicals for the given effect. When this requirement is not met, RPFs will vary with the effect levels in the mixture and thus could not be considered a "dilution" of the IC across the full dose response range. For example, the RPF for a given PFAS may be different in the low dose range vs. the middle or high dose range depending on slope differences with the IC. In this regard, published data (Hass et al., 2007; Howdeshell et al., 2008; Metzdorff et al., 2007; and Rider et al., 2008) reveal that chemical dose-response curves for a common effect can display very different slopes and shapes even across related structures within a given class. In contrast to the RPF approach, other DA-based equations can be used for quantitative evaluations of the effects of chemical mixtures when the slopes for a common effect differ among chemicals in the mixture. As stated in the NRC (2008) report, "It is a widely held misconception (EPA 2000(b)) that dose addition is applicable only with congruent dose response curves (for a general discussion, see Gennings et al. 2005 and Kortenkamp et al. 2007)."

The following discussion compares the predictions of two DA models, one assumes that the individual chemicals in the mixture have congruent slopes, whereas the second DA model (described by NRC (2008)) does not require similar slopes to yield accurate predictions. As an example, these two models were recently used to predict the full dose-response curve of a mixture using the ED_{50}s and slopes of each component chemical in the mixture. The first model, similar to the RPF method (discussed above), assumes that the chemicals in the mixture have equal slopes, indicating that the relative potencies are constant across the entire dose response curve. Equation 7-1 is an example of such a model that uses an average slope value to calculate the joint toxicity of a mixture with the following equation (Olmstead and LeBlanc, 2005; Rider and LeBlanc, 2005; Rider et al. 2008):

$$R = \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^{n} \frac{D_i}{ED50_i}\right)^{\rho}}} \quad \text{(Eqn 7-1)}$$

where R is the response to the mixture, D_i is the dose of chemical i in the mixture, $ED50_i$ is the dose of chemical i that causes a 50% response, and ρ is the average power (Hillslope) associated with the chemicals.

Because the assumption of similar slopes is not always met, DA models, like the M-BMD method described below, that do not require parallel slopes for the chemicals in the mixture provide more accurate predictions of the mixture effects. Several of these DA-based models have been previously described (for example, Altenburger et al., 2000; Kortenkamp et al., 2007; Metzdorff et al., 2007) and by NRC (NRC 2008). The M-BMD equation below (Equation 7-2) is an example of such a DA model that calculates a given effect level for the total mixture ($EDx_{mixture}$) where p_i is the proportion of chemical i in the mixture and EDx_i denotes the dose producing the given level of response for the ith chemical in the mixture:

$$EDx_{mixture} = (p_1/EDx_1 + p_2/EDx_2)^{-1} \quad \text{(Eqn 7-2)}$$

When the slopes of the dose response curves differ among chemicals in the mixture, the two DA models (i.e., Equation 7-1 and Equation 7-2) can yield different dose response predictions (see Figure 7-1). Further, there is greater uncertainty in the accuracy of the DA mixture predictions when the assumption of parallelism among slopes is violated (NRC, 2008). The following example compares the accuracy of a DA model that assumes parallelism (Equation 7-1) with a DA model that does not (Equation 7-2), and these model predictions are compared with a RA model. The data used in this example are from a published binary mixture study, but the chemicals are not identified (Gray et al., 2022).

In this example two chemicals were mixed using a fixed-ratio design. The top dose of this mixture contained each chemical at a dose close to their respective ED_{50}s, but they have different dose response shapes using nonlinear 4 parameter logistic regression (Chemical A slope = 40, Chemical B slope = 5 using linear Y axis and log10 X axis). The mixture effect described in the figure below is percent reduction in a reproductive organ weight, ranging from 0% reduction in the control (0 dose of the mixture) to complete agenesis (100% reduced).

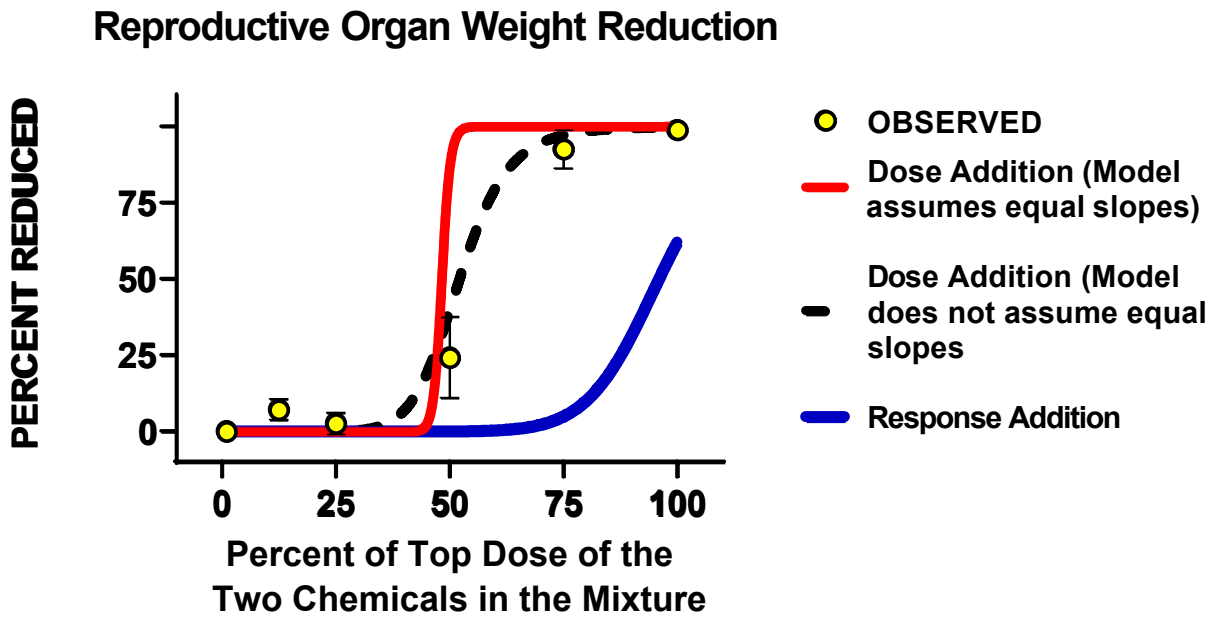


Figure 7-1. Example comparison of observed data with model predictions using two dose addition-based mixture models and a response addition model for a binary mixture study (adapted from Gray et al., 2022). The two chemicals displayed individual dose response curves with widely disparate slopes for the endpoint (reduced organ weight). The two dose addition models either assume component chemicals have similar dose response slopes (red solid line) for the effect or have non-congruent dose response curve slopes (black dashed line). For these chemicals with disparate slopes the dose addition model that does not assume equal slopes provided a better fit of the observed data (see table below).

The observed data were fit with the model parameters of the two DA and the RA model and Akaike Information Criteria (AIC) values were calculated to determine the best model of the observed data (Table 7-1). The lower AIC values indicate a better-fit model, and as a "rule of thumb" (Burnham and Anderson, 2004) there is little support for two of these models because the delta-AIC (the difference between the two AIC values being compared) is greater than 7.

Table 7-1. "Best Model" based upon AIC values

"Best Model" based upon AIC values	
Model	**AIC**
DA - does not assume equal slopes	164.1
DA - assumes equal slopes	203.3
RA	223.4

AIC: Akaike Information Criterion

In contrast to the above example of chemicals with disparate slopes, if the dose response curves of the component chemicals in a mixture have similar slope parameters from nonlinear regression, then there would be little or no difference between the predictions of the two DA

models shown here. Hence, if sufficient dose response information is available and the slopes are not parallel then it is preferable to model the data with the M-BMD equation (Equation 7-2) that does not assume parallelism, as stated by the NRC (2008).

Estimating PFAS mixture effects using the M-BMD method requires empirical data-driven or reasonable estimation (e.g., read-across between structures) of effect-equivalent endpoints for all PFAS in the mixture. Similar to the RPF approach above, ideally the dose-response functions used to calculate effect endpoints (e.g., BMD_X) 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 strongly recommended to convert experimental animal dose-response data to human equivalents where possible. Lastly, of the options for dose-response metrics to use across mixture PFAS, BMDs (e.g., 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 BMR 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 LOAELs or ED_X values are suitable alternatives.

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. The equation will produce an equivalent metric (i.e., BMD_{10}) for the total mixture with the given proportions of component PFAS being evaluated. In the illustrative example below, BMDs associated with a BMR of 10% are estimated for each chemical in a mixture and are used to determine a "M-BMD". The choice of a 10% BMR is for illustration only; other values may be selected. If available, it is preferable to calculate the M-BMDs using HED doses rather than oral mg/kg doses administered to test animals. Effect equivalent BMDs are more statistically robust, and the equation explanation and example below will reference BMD_X as the model components using Equation 7-3 (similar to Equation 7-2), where M-BMD is the total mixture dose in mg/kg/day, 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).

$$Mixture\ BMD = \left(\sum_{i=1}^{n} \frac{a_i}{BMD_i}\right)^{-1} \quad \text{(Eqn 7-3)}$$

The equation results in a single M-BMD dose for a given BMR, that could then be converted to an assessment value (e.g., oral RfD) and a corresponding HBWC for the mixture. Then the original observed PFAS mixture concentration (e.g., 38.6 ng PFAS Mix/L) is compared to the estimated M-BMD-HBWC from the M-BMD equation, and if the observed concentration is greater than the mixture-based HBWC there is potential for human health risk. If the observed concentration is below the mixture based HBWC then risk of health effects is not likely. Further, the calculation can be repeated at multiple BMR levels to allow for modeling of a full mixture dose response curve. Finally, similar to RPF, 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 for the mixture.

An example is described in the following sections for a mixture of five hypothetical PFAS from Table 4-1. The five hypothetical PFAS have existing dose response data for increased incidence of hepatocellular death (i.e., liver endpoint), reduced serum thyroid hormone concentrations (i.e., thyroid endpoint), and decreased body weight in offspring (i.e., developmental endpoint), and in rodent models for each compound. Dose responses for each chemical and each endpoint are modeled and BMD_X calculated for each compound. These values serve as the denominator values in Equation 7-3. The numerator values are the proportions of each component PFAS in the given mixture on a concentration basis. The total M-BMD is the inverse of the sum of the proportion divided by the BMD_X for each PFAS in the mixture. The total M-BMD_X represents an equivalent BMD_X as each of the individual chemical BMDs that were used in the calculations (i.e., if the individual chemical data were BMD_{10} values, the DA calculation derives a BMD_{10} for the mixture of PFAS with those specific proportions).

The M-BMD, which is in the same units as the component chemical BMDs (e.g., oral dose in a rodent study such as mg/kg-day), can then be adjusted based on user-defined extrapolation factors (e.g., application of dosimetric adjustment, RSC, 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 M-BMD-HBWC can then be compared to the actual (measured) mixture concentration and if the actual mixture concentration exceeds the M-BMD-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 effect domain for the mixture and this endpoint can then be used for the derivation of an equivalent M-BMD-HBWC and estimation of risk. This M-BMD is then used to derive a M-BMD HBWC for comparison to the actual total mixture concentration of the sample. If the total mixture concentration is greater than the M-BMD HBWC, then there is potential risk of developmental effects in the exposed population.

7.2 Illustrative Example Application of the Mixture Benchmark Dose Approach to a Hypothetical Mixture of Five PFAS

7.2.1 Mixture BMD Step 1: Assemble/derive component health effects endpoints (BMDx)

PFAS 1–5 have existing dose response data on increased incidence of hepatocellular death (i.e., liver endpoint), reduced serum thyroid hormone concentrations (i.e., thyroid endpoint), and decreased body weight in offspring (i.e., developmental endpoint), in rodent models for each compound (Note: Table 7-2 below is a repeat of Table 6-1; these data were also used in the RPF example). Dose responses for each chemical and each endpoint are modeled and BMD_X calculated for each compound.

Table 7-2. Summary of POD_{HED}s for three selected health effect domains for a mixture of five hypothetical PFAS.

	Liver POD_{HED} (mg/kg-day)	Thyroid POD_{HED} (mg/kg-day)	Developmental POD_{HED} (mg/kg-day)
PFAS 1	0.044	0.24	0.00001 ($BMDL_{0.1ERHED}$)
PFAS 2	0.0013 ($BMDL_{10HED}$)	0.23	0.0051
PFAS 3	N/A	0.21 ($BMDL_{1SDHED}$)	2.1
PFAS 4	50	N/A	0.0011 ($BMDL_{1SDHED}$)
PFAS 5	0.004 (BMD_{1SDAED})	N/A	N/A

7.2.2 Mixture BMD Step 2: Assemble/derive health-based media concentrations (HBWC)

In the case of the M-BMD approach (unlike the HI/TOSHI and RPF approaches) there is no need for pre-existing HBWC(s) because the goal of this approach is to develop a unique, mixture-specific HBWC for comparison to the Mixture Total PFAS concentration. Calculation of the M-BMD HBWC is shown in Section 7.2.4.

7.2.3 Mixture BMD Step 3: Select exposure estimates (measured water concentrations)

Select appropriate exposure estimates consistent with the problem formulation. Specifically, the user may choose to calculate or use exposure estimates that are for the oral route in general (i.e., total intake in mg/kg-day) or media-specific concentrations. In the hypothetical PFAS mixture example, 'exposure' is represented by the drinking water monitoring data in Table 4-1.

7.2.4 Mixture BMD Step 4: Calculate PFAS mixture potency (Mixture BMD HBWC)

In this example, M-BMDs are calculated for three effect domains: Liver, Thyroid, and Developmental (Table 7-3). Application of Equation 7-3 to the example water sample in Table 4-1 is used to derive the M-BMD. This example is for the developmental domain as it was the lowest M-BMD of the three effect domains.

$$Mixture\ BMD = \left(\sum_{i=1}^{4} \frac{a_i}{BMD_i}\right)^{-1} = \left(\frac{0.01}{0.00001} + \frac{0.15}{0.0051} + \frac{0.48}{2.1} + \frac{0.16}{0.0011} + \frac{0.19}{N/A}\right)^{-1} = 0.00085 \text{ mg/kg-day}$$

Table 7-3. M-BMD Approach: Hypothetical Water Sample

	Median Measured Water Concentration (ng/L)	Mixing Ratio (Proportion)	Liver BMD (mg/kg/d)	Thyroid BMD (mg/kg/d)	Developmental BMD (mg/kg/d)
PFAS 1	4.8	0.01	0.044	0.24	0.00001
PFAS 2	52	0.15	0.0013	0.23	0.0051
PFAS 3	172	0.48	N/A	0.21	2.1
PFAS 4	58	0.16	50	N/A	0.0011
PFAS 5	69	0.19	0.004	N/A	N/A
Mixture Total	**355.8**	1.0			
M-BMD Calculation			0.0061	0.34	**0.00085***

Notes: N/A = data not available.
*The lowest M-BMD is converted to a mixture-HBWC using Eqn. 7-3 for comparison to the measured concentration (i.e., 355.8 ng/L).

The developmental-effect produced the lowest M-BMD (i.e., 0.00085 mg/kg-day), representing the most sensitive effect domain; this value is selected for calculation of the M-BMD HBWC. The developmental-based Mixture BMD is first converted to an RfD by applying UFs consistent with the data being used. Selection of uncertainty factors will likely be different across mixture component chemicals based on the available hazard and dose-response data. As such there is no suggested standard application of quantitative uncertainty for a mixture of components although it is suggested that a user of this approach consider uncertainty across the five areas used in EPA human health risk assessment practice: (1) Human interindividual variability (UF_H); (2) extrapolation from animal-to-human (UF_A); (3) subchronic-to-chronic duration extrapolation (UF_S); (4) LOAEL-to-NOAEL extrapolation (UF_L); and (5) database uncertainty (UF_D). In the specific context of application of uncertainty to a M-BMD, a reasonable health protective approach is to apply factors consistent with the data status of the most data-poor member of the mixture. For example, in this hypothetical five-component PFAS mixture, a composite UF of 300 is applied based on the uncertainty associated with PFAS 5; this includes a UF_H of 10 (no availability of empirical data to inform human interindividual variability), UF_A of 3 (toxicokinetic extrapolation between experimental cells/animals and humans has been accounted for in the calculation of human equivalent doses), UF_S of 1 (developmental effects are considered duration-independent), UF_L of 1 (POD is based on a benchmark dose); and UF_D of 10 (significant lack of data across exposure durations and health outcome domains):

$$\text{RfD} = \left(\frac{\text{BMD}}{\text{UF}}\right) = \left(\frac{0.00085\frac{\text{mg}}{\text{kg}}/\text{d}}{300}\right) = 0.0000028 \text{ mg/kg-day}$$

An HBWC can then be derived using Eqn. 7-3. In this example, the DWI-BW is for women of childbearing age (i.e., 90th percentile direct and indirect consumption of community water, consumer-only two-day average, 13 to < 50 years), and the RSC is 20% (0.2). The M-BMD HBWC is calculated as follows:

$$\text{HBWC} = \left(\frac{\text{RfD}}{\text{DWI-BW}}\right) * \text{RSC} = \left(\frac{0.0000028\frac{\text{mg}}{\text{kg}}/\text{d}}{0.0354\frac{L}{kg}/d}\right) * 0.2 = 0.000016 \text{ mg/L} = 16 \text{ ng/L}$$

7.2.5 Mixture BMD Step 5: Compare PFAS mixture potency (total PFAS mixture concentration) to health-based value (Mixture BMD HBWC)

In the developmental effect-specific M-BMD application, the health risk(s) associated with the mixture is represented by comparing the mixture total PFAS concentration (355.8 ng/L) to the M-BMD HBWC which is based on the specified effect for hazard domain (e.g., for this example, decreased body weight in offspring). In this hypothetical example, the mixture total PFAS concentration 355.8 ng/L exceeds the M-BMD HBWC 16 ng/L by over an order of magnitude, indicating significant potential for health risks in developmental populations exposed to a mixture of the five PFAS at the hypothetical water exposure estimates provided.

Although not shown in the above example, if the M-BMD was instead based on the liver effect domain, M-BMD HBWC would be 115 ng/L, well below the measure PFAS concentration (355.8 ng/L), indicating the potential for liver effects in exposure populations. Alternatively, if the M-BMD was based on the thyroid effect domain, the resulting M-BMD HBWC would be 6,321 ng/L, well above the measured total PFAS concentration (355.8 ng/L), indicating unlikely risk for thyroid effects among the exposed population.

7.3 Advantages and Challenges of the Mixture BMD Approach

There are several advantages to the M-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) 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 POD for the whole mixture 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 (M-BMD) is different for each PFAS mixture depending on: (1) the specific PFAS in the mixture; (2) the mixing ratio; and (3) the effect or endpoint being modeled. For example, one could expect that a mixture of PFAS that has a greater concentration of a more potent compound, and a lower concentration of a less potent compound would have a lower (i.e., more potent) M-BMD than a similar assortment of compounds that has a lower concentration of the more potent PFAS and a greater concentration of the less potent PFAS. It is also advantageous that the M-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 NRC (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., BMD_{10}), and this is necessary if a goal is to model the entire dose response for the mixture. However, in the absence of such data, M-BMD 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 incidence of hepatocellular death) but use of this type of point data would impede the modeling of the full mixture dose response curve if desired.

There are also several challenges with the M-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. Ideally this would be for one of, if not the most sensitive, effect across PFAS in the mixture of interest in order to provide a conservative (protective) risk scenario. For some mixtures that contain less well-studied PFAS there may be limited or no available dose response data available to derive component chemical BMDs in order to calculate the M-BMD.

A limitation, that is not unique to this specific approach, is that PFAS mixtures may vary over time in environmental media. As proportions of component PFAS change in the mixture, the calculations would need to be recalculated as the composition of the mixture changed from site to site or over time within the same site. However, the calculation can be readily and easily repeated for different mixing ratios and mixture concentrations once the component chemical effect endpoint values have been determined. Finally, for both the RPF and M-BMD approaches, 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 developmental effects may be the most sensitive and would produce the lowest M-BMD, but data are only available for the component PFAS to calculate M-BMDs for liver and thyroid effects). In this example, the M-BMD HBWC is based on developmental effects because that is the most sensitive among the three assessed effect domains, and thus is protective of the other effects (i.e., liver, thyroid). As described in the previous section, if this M-BMD analysis was instead based on the thyroid effect, the user would conclude that potential risk is unlikely.

8.0 Comparison of Component-Based Approaches

This framework document describes the conceptual bases and practical application of data-driven options for estimating the noncancer health risks associated with human exposure to mixtures of PFAS. The component-based options described are included in prior EPA mixtures guidance (EPA, 1986, 2000b) and supported by NRC (2008). Although the approaches and illustrative examples are provided using drinking water as the exposure route, the technical basis of each approach could be readily applied or adapted to other sources of oral exposure (e.g., soil, fish/shellfish). Each of the approaches included require varying levels of data input and have relatively subtle but substantive differences in assumptions and ultimately produce risk estimations that may slightly differ based on those exposure assumptions. Importantly, interpretations of health risks associated with mixtures of PFAS will be highly dependent on the specific PFAS components within a given mixture and the individual concentrations or proportions of each of those components. Given the significant lack of toxicity data across the diverse structural landscape of compounds within the PFAS chemical class, it is likely that many users of this framework will need to incorporate information NAMs such as *in vitro* cell bioactivity, toxicogenomic platforms, and/or structure-activity/read-across in order to facilitate estimation of health risk for a PFAS mixture of interest; however, *in vivo* animal or human toxicity data are strongly recommended where available.

Given the range of data-driven options presented in this framework, an important consideration is under what circumstances the different options produce similar or dissimilar indications or estimates of health risk(s). The primary basis for differing risk estimates relates to differences in data input requirements, model assumptions, and final value derivation. Both the HI and TOSHI approaches necessitate availability (or *de novo* derivation) of health assessment values (e.g., oral RfDs) to calculate mixture component HQs. In contrast, the RPF and M-BMD approaches target dose-response data for same/similar effect, sans derivation of health assessment values, to inform mixture risk estimates (e.g., concentrations or doses) for comparison to measured media concentrations either for an anchor/index chemical (RPF) or across each mixture component (M-BMD). The general HI approach allows for component PFAS in the mixture to have different health effects or endpoints as the basis for the component chemical RfVs (see Figure 4-1) and thus this approach is likely a more health-protective indicator of risk (i.e., produce a component HQ or mixture HI of > 1) since the representation of toxicity will likely be the most sensitive, compared to the RPF and M-BMD approaches where similarity in toxicity does not have to *a priori* be the most sensitive effect domain. In contrast, the TOSHI approach is more targeted and assumes the component RfVs are based on the same organ or organ system. This more narrowed focus is likely to produce a less health-protective indicator of risk than the general HI (i.e., less likely than general HI to produce HQ > 1) because the range of potential effects has been scoped to a specific target organ or organ system; for example, for some mixture components the effect domain identified for TOSHI application may be one of the less potent across a profile of effects. This important nuance will be dependent on the availability of target organ specific RfVs, and case-by-case interpretations of "potency" for effect will be a function of both dose-response (e.g., POD) and uncertainty factor application. The user of the TOSHI approach would be advised to also perform the general HI for the same mixture and compare the HIs (and component HQs) across each approach. It should be noted that any component chemical HQ or mixture HI >1 indicates potential health risks; magnitude of HI is not an optimal comparator. A TOSHI risk estimate is likely less conservative than the general HI but may be more

conservative than the RPF and M-BMD approaches. However, the TOSHI and RPF approach will give essentially the same answer when the ratio of the POD values used to calculate the RPFs is equal to the ratio of the endpoint-specific PODs used in the derivation of RfVs used to calculate the TOSHI. The major difference between the RPF and M-BMD estimates is the RPF approach assumes congruent slopes whereas the M-BMD does not. If the mixture component chemicals have congruent dose response curves, for same effect, the RPF and M-BMD calculations produce nearly identical risk estimates for the same mixture. However, if the mixture component chemicals display a range of congruent and non-congruent dose response curves, then the assumptions for application of the RPF method are violated and the M-BMD approach should be used to produce a more accurate estimate of risk (NRC, 2008).

Another factor in the concordance (or not) of mixture risk estimates across the component-based approaches presented are the factors used in deriving RfVs or HBWCs from hazard data across components. The HI and TOSHI approaches are calculated based on an exposure metric divided by the component chemical RfVs or HBWCs. The input data for calculating RfV and HBWC, including the critical effect and POD (i.e., $BMDL_X$ or NOAEL or LOAEL), correction factors (i.e., UFs, DAF), and exposure route adjustments (i.e., DWI, RSC) used for each component PFAS will have impacts on the resulting risk estimates and should be carefully selected based on available data for each component PFAS. Similarly, when conducting RPF or M-BMD assessment, the PODs used across component PFAS in a mixture could potentially be derived from dissimilar response metrics (i.e., LOAEL vs $BMDL_X$) and the resulting M-BMD-HBWC or Total ICEC comparison will be somewhat dependent on the applicability of the correction and route adjustment factors used for the M-BMD HBWC or Total ICEC derivation across all component PFAS in the mixture. For example, the correction factors and route adjustment factors may not be appropriate for all component PFAS and thus the Total ICEC to IC RfV or HBWC comparison or M-BMD HBWC to measured concentration may be affected. Thus, it is strongly encouraged to use comparable PODs across component PFAS where possible and select adjustment factors given careful consideration of the components for the specific PFAS mixture being evaluated. It will be key to transparently present and communicate selection of uncertainty factors or exposure route adjustment factors, and associated rationale(s), such that interpretations and conclusions of mixture risk are supportable.

A critical consideration for use of the approaches in this framework document is that where data are available and support, it may be prudent to apply each approach to the same mixture. The purpose of the comparison is not necessarily to determine which approach provides the most conservative estimate of mixture risk but rather which approach entails the greatest level of confidence in the data underlying the components and support for the assumption of dose additivity in the evaluation of joint toxicity of PFAS.

References

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

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.

ATSDR (Agency for Toxic Substances and Disease Registry). 2022. *CDC/ATSDR PFAS Exposure Assessment Community Level Results: Hampden County (MA) near Barnes Air National Guard Base*. ATSDR, Atlanta, GA. https://www.atsdr.cdc.gov/pfas/communities/factsheet/Community-Level-Results-Factsheet.html.

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., B. Yamini., and S.J. Bursian. 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. 2021. 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.

Bil W, Zeilmaker MJ, Bokkers BGH. 2022. Internal Relative Potency Factors for the Risk Assessment of Mixtures of Per- and Polyfluoroalkyl Substances (PFAS) in Human Biomonitoring. *Environmental Health Perspectives* 130(7):77005. doi:10.1289/EHP10009.

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.

Bjork, J. A., J. L. Butenhoff and K. B. Wallace. 2011. Multiplicity of nuclear receptor activation by PFOA and PFOS in primary human and rodent hepatocytes. *Toxicology* 288(1-3):8–17. doi:10.1016/j.tox.2011.06.012

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.

Burnham, K. P., D. R. Anderson and K. P. Huyvaert. 2010. AIC model selection and multimodel inference in behavioral ecology: some background, observations, and comparisons. *Behavioral Ecology and Sociobiology* 65(1):23–35. doi:10.1007/s00265-010-1029-6.

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.

Cadwallader, A., A. Greene, H. Holsinger, A. Lan, M. Messner, M. Simic, and R. Albert. 2022. A Bayesian hierarchical model for estimating national PFAS drinking water occurrence. *AWWA Water Science* 4(3):e1284. doi:10.1002/aws2.1284.

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.

Carlson, L.M., M. Angrish, A.V. Shirke, E.G. Radke, B. Schulz, A. Kraft, R. Judson, G. Patlewicsz, R. Blain, C. Lin, N. Vetter, C. Lemeris, P. Hartman, H. Hubbard, X. Arzuaga, A. Davis, L.V. Dishaw, I.L. Druwe, H. Hollinger, R. Jones, J.P. Kaiser, L. Lizarraga, P.D. Noyes, M. Taylor, A.J. Shapiro, A.J. Williams, and K.A. Thayer. 2022.Systematic evidence map for one hundred and fifty per- and polyfluoroalkylsubstances (PFAS). *Environmental Health Perspectives* 130(5):056001. doi:10.1289/EHP10343

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):382–395.

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.S. Lambright, N. Evans, M.J. Strynar, J. McCord, B.S. McIntyre, G.S. Travlos, M.C. Cardon, E. Medlock-Kakaley, P.C. Hartig, V.S. Wilson and L.E. Gray, Jr. 2019. Adverse maternal, fetal, and postnatal effects of hexafluoropropylene oxide dimer acid (GenX) from oral gestational exposure in Sprague-Dawley rats. *Environmental Health Perspectives* 127(3):37008. doi:10.1289/EHP4372.

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.

Conley, J.M., C.S. Lambright, N. Evans, E. Medlock-Kakaley, D. Hill, J. McCord, M.J. Strynar, L.C. Wehmas, S Hester, D.K. MacMillan, and L.E. Gray, Jr. 2022a. Developmental toxicity of Nafion byproduct 2 (NBP2) in the Sprague-Dawley rat with comparisons to hexafluoropropylene oxide-dimer acid (HFPO-DA or GenX) and perfluorooctane sulfonate (PFOS). *Environment International* 160:107056. doi:10.1016/j.envint.2021.107056.

Conley, J.M., C.S. Lambright, N. Evans, E. Medlock-Kakaley, A. Dixon, D. Hill, J. McCord, M.J. Strynar, J. Ford, L.E. and Gray. 2022b. Cumulative maternal and neonatal effects of combined exposure to a mixture of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) during pregnancy in the Sprague-Dawley rat. *Environment International* 170:107631. doi.org/10.1016/j.envint.2022.107631.

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.

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 DHHS (Delaware Health and Social Services). 2021. *Delaware PFOA and PFOS MCL Implementation Plan.* Proposed implementation plan for the maximum contaminant levels (MCL) as required by Delaware House Bill 8 (HB-8), signed in October 2021. https://www.dhss.delaware.gov/dhss/dph/hsp/files/MCLimplementationPlanPFAS.pdf

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.

Ding, G., J. Zhang, Y. Chen, L. Wang, M. Wang, D. Xiong and Y. Sun (2013). Combined effects of PFOS and PFOA on zebrafish (Danio rerio) embryos. *Archives of Environmental Contamination and Toxicology* 64(4):668–675. doi:10.1007/s00244-012-9864-2.

Dix, D.J., K.A. Houck, M.T. Martin, A.M. Richard, R.W. Setzer, and R.J. Kavlock. 2007. The ToxCast program for prioritizing toxicity testing of environmental chemicals. *Toxicological Sciences* 95(1):5–12. doi:10.1093/toxsci/kfl103.

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

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 (U.S. 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 (U.S. 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 (U.S. 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 (U.S. 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 (U.S. Environmental Protection Agency). 1999. Policy on a Common Mechanism of Action: The Organophosphate Pesticides. Vol. 64. Federal Register, Washington, D.C., 1999, pp. 5795-5799.

EPA (U.S. Environmental Protection Agency). 2000a. *Methodology for Deriving Ambient Water Quality Criteria for the Protection of Human Health*. EPA/822/B-00/004. EPA, Office of Water, Washington, DC. https://www.epa.gov/sites/default/files/2018-10/documents/methodology-wqc-protection-hh-2000.pdf.

EPA (U.S. Environmental Protection Agency). 2000b. *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 (U.S. 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 (U.S. Environmental Protection Agency). 2006. *Organophosphorus Cumulative Risk Assessment (2006 Update).* EPA-HQ-OPP-2006-0618-0002. EPA, Office of Pesticide Programs, Washington, DC.

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

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

EPA (U.S. 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 (U.S. Environmental Protection Agency). 2011a. Pyrethrins/Pyrethroid Cumulative Risk Assessment. EPA-HQ-OPP-2011-0746-0003. https://www.regulations.gov/document/EPA-HQ-OPP-2011-0746-0003.

EPA (U.S. Environmental Protection Agency). 2011b. *Recommended Use of Body Weight$^{3/4}$ as the Default Method in Derivation of the Oral Reference Dose*. EPA/100/R-11/0001. EPA, Risk Assessment Forum, Washington, DC. https://www.epa.gov/risk/recommended-use-body-weight-34-default-method-derivation-oral-reference-dose.

EPA (U.S. Environmental Protection Agency). 2012. *Benchmark Dose Technical Guidance.* EPA/100/R-12/001. EPA, Risk Assessment Forum, Washington, DC. https://www.epa.gov/risk/benchmark-dose-technical-guidance.

EPA (U.S. Environmental Protection Agency). 2016a. *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 (U.S. Environmental Protection Agency). 2016b. *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 (U.S. 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 (U.S. 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 (U.S. 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 (U.S. 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 (U.S. 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 (U.S. 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 (U.S. Environmental Protection Agency). 2021c. *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). 2021d. *Fifth Unregulated Contaminant Monitoring Rule*. https://www.epa.gov/dwucmr/fifth-unregulated-contaminant-monitoring-rule.

EPA (U.S. Environmental Protection Agency). 2021e. *External Peer Review Draft Framework for Estimating Noncancer Health Risks Associated with Mixtures of Per- and Polyfluoroalkyl Substances (PFAS).* EPA 822D/21/003. EPA Office of Water and Office of Research and Development, Washington, DC.

EPA (U.S. Environmental Protection Agency). 2021f. National PFAS Testing Strategy: Identification of Candidate Per- and Polyfluoroalkyl Substances (PFAS) for Testing. EPA, Office of Chemical Safety and Pollution Prevention, Office of Pollution Prevention and Toxics. https://www.epa.gov/system/files/documents/2021-10/pfas-natl-test-strategy.pdf

EPA (U.S. Environmental Protection Agency). 2022a. *Interim Drinking Water Health Advisory: Perfluorooctanoic Acid (PFOA) CASRN 335-67-1.* EPA/822/R-22/003. EPA, Office of Water, Health and Ecological Criteria Division, Washington, DC. https://www.epa.gov/sdwa/drinking-water-health-advisories-has.

EPA (U.S. Environmental Protection Agency). 2022b. *Interim Drinking Water Health Advisory: Perfluorooctane Sulfonic Acid (PFOS) CASRN 1763-23-1.* EPA/822/R-22/004. EPA, Office of Water, Health and Ecological Criteria Division, Washington, DC. https://www.epa.gov/sdwa/drinking-water-health-advisories-has.

EPA (U.S. Environmental Protection Agency). 2022c. *Drinking Water Health Advisory: Hexafluoropropylene Oxide (HFPO) Dimer Acid (CASRN 13252-13-6) and HFPO Dimer Acid Ammonium Salt (CASRN 62037-80-3), Also Known as "GenX Chemicals."* EPA/822/R-22/005. EPA, Office of Water, Health and Ecological Criteria Division, Washington, DC. https://www.epa.gov/sdwa/drinking-water-health-advisories-has.

EPA (U.S. Environmental Protection Agency). 2022d. *Drinking Water Health Advisory: Perfluorobutane Sulfonic Acid (CASRN 375-73-5) and Related Compound Potassium Perfluorobutane Sulfonate (CASRN 29420-49-3).* EPA/822/R-22/006. EPA, Office of Water, Health and Ecological Criteria Division, Washington, DC. https://www.epa.gov/sdwa/drinking-water-health-advisories-has.

EPA (U.S. Environmental Protection Agency). 2022e. *IRIS Toxicological Review of Perfluorobutanoic Acid (PFBA, CASRN 375-22-4) and Related Salts.* EPA/635/R-22/277Fa. EPA, Office of Research and Development, Washington, DC. https://iris.epa.gov/static/pdfs/0701tr.pdf.

EPA (U.S. Environmental Protection Agency). 2023a. *EPA Response to Final Science Advisory Board Recommendations (August 2021) on Four Draft Support Documents for the EPA's Proposed PFAS National Primary Drinking Water Regulation.* EPA-815-D23-001. EPA Office of Water, Washington, DC.

EPA (U.S. Environmental Protection Agency). 2023b. PUBLIC COMMENT DRAFT Toxicity Assessment and Proposed Maximum Contaminant Level Goal for Perfluorooctanoic Acid (PFOA) in Drinking Water. EPA-822-P-23-005. EPA, Office of Water, Washington, DC.

EPA (U.S. Environmental Protection Agency). 2023c. PUBLIC COMMENT DRAFT Toxicity Assessment and Proposed Maximum Contaminant Level Goal for Perfluorooctane Sulfonic Acid (PFOS) in Drinking Water. EPA-822-P-23-007. EPA, Office of Water, Washington, DC.

EPA (U.S. Environmental Protection Agency). 2023d. PUBLIC COMMENT DRAFT Appendix - Toxicity Assessment and Proposed Maximum Contaminant Level Goal for Perfluorooctanoic Acid (PFOA) in Drinking Water. EPA-822-P-23-006. EPA, Office of Water, Washington, DC.

EPA (U.S. Environmental Protection Agency). 2023e. PUBLIC COMMENT DRAFT Appendix - Toxicity Assessment and Proposed Maximum Contaminant Level Goal for Perfluorooctane Sulfonic Acid (PFOS) in Drinking Water. EPA-822-P-23-008. EPA, Office of Water, Washington, DC.

EPA SAB (U.S. Environmental Protection Agency Science Advisory Board). 2022. *Review of EPA's Analysis to Support EPA's National Primary Drinking Water Rulemaking for PFAS*. EPA-SAB-22-008. https://sab.epa.gov/ords/sab/f?p=100:18:16490947993:::RP,18:P18_ID:2601#report

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.

Evans, N., J.M. Conley, M. Cardon, P. Hartig, E. Medlock-Kakaley, and L.E. Gray. 2022. *In vitro* activity of a panel of per- and polyfluoroalkyl substances (PFAS), fatty acids, and pharmaceuticals in peroxisome proliferator-activated receptor (PPAR) alpha, PPAR gamma, and estrogen receptor assays. *Toxicology and Applied Pharmacology* 449:116136. https://doi.org/10.1016/j.taap.2022.116136.

Evich, M.G., M.J.B. Davis, J.P. McCord, B. Acrey, J.A. Awkerman, D.R.U. Knappe, A.B. Lindstrom, T.F. Speth, C. Tebes-Stevens, M.J. Stryner, Z. Wang, E.J. Weber, W.M. Henderson, and J.W. Washington. 2022. Per- and polyfluoroalkyl substances in the environment. *Science* 375(512). doi:10.1126/science.abg9065.

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

Florida Health. 2020. *Per- and Polyfluoroalkyl Substances (PFAS).* https://www.floridahealth.gov/environmental-health/hazardous-waste-sites/contaminant-facts/_documents/final-faq-pfas-update-01132022.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, and L.E. 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):403–424.

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* 21:2477–2482.

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.

Gennings, C., W.H. Carter Jr., R.A. Carchman, L.K. Teuschler, J.E. Simmons, and E.W. Carney. 2005. A unifying concept for assessing toxicological interactions: changes in slope. *Toxicological Sciences* 88(2):287–297.

Goodrow, S.M., B. Ruppel, R.L. Lippincott, G.B. Post, and N.A. Procopio. 2020. Investigation of levels of perfluoroalkyl substances in surface water, sediment and fish tissue in New Jersey, USA. *Science of the Total Environment* 729:138839.

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.

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 vs. bisphenol C. *Toxicological Sciences* 168(2):632–643.

Gray, L. E., J. M. Conley, C. S. Lambright and J. R. Furr. 2022. In utero exposure to a mixture of the perfluoroalkyl-isopropyl pesticide pyrifluquinazon with dibutyl phthalate cumulatively disrupts male rat reproductive development via different mechanisms of action. *Toxicological Sciences* 188(2):234–247. doi:10.1093/toxsci/kfac059

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 (PFAS) 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* 223:315–321.

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):153–165.

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.

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). 2022. *PFAS Technical and Regulatory Guidance Document*. ITRC, Washington, DC. https://pfas-1.itrcweb.org.

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.

Kahn, H.D., K. Stralka, and P.D. White. 2013. Estimates of water ingestion in formula by infants and children based on USDA's 1994-1996 and 1998 continuing survey of food intakes by individuals. *Human and Ecological Risk Assessment: An International Journal* 19(4):888–889

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.

Kavlock, R., K. Chandler, K. Houck, S. Hunter, . Judson, N. Kleinstreuer, T. Knudsen, M. Martin, S. Padilla, D. Reif, A. Richard, D. Rotroff, N. Sipes, and D. Dix. 2012. Update on EPA's ToxCast program: providing high throughput decision support tools for chemical risk management. *Chemical Research in Toxicology* 25(7):1287–1302. doi:10.1021/tx3000939.

Kjeldsen, L.S., and E.C. Bonefeld-Jorgensen. 2013. Perfluorinated compounds affect the function of sex hormone receptors. *Environmental Science and Pollution Research International* 20(11):8031–8044. doi: 10.1007/s11356-013-1753-3.

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., M. Faust, M. Scholze, and T. Backhaus. 2007. Low level exposure to multiple chemicals: Reason for human health concerns? *Environmental Health Perspectives* 115(Suppl. 1):106–114.

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.

Lau, C., J. R. Thibodeaux, R. G. Hanson, M. G. Narotsky, J. M. Rogers, A. B. Lindstrom and M. J. Strynar. 2006. Effects of perfluorooctanoic acid exposure during pregnancy in the mouse. *Toxicological Sciences* 90(2):510–518. doi:10.1093/toxsci/kfj105.

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.

LSRO (Life Sciences Research Office, Federation of American Societies for Experimental Biology). 1995. *Third Report on Nutrition Monitoring in the United States: Volume 1.* Prepared for the Interagency Board for Nutrition Monitoring and Related Research. US Government Printing Office, Washington, DC.

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.

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.

Marques, E., M. Pfohl, W. Wei, G. Tarantola, L. Ford, O. Amaeze, J. Alesio, S. Ryu, X. Jia, H. Zhu, G. D. Bothun and A. Slitt. 2022. Replacement per- and polyfluoroalkyl substances (PFAS) are potent modulators of lipogenic and drug metabolizing gene expression signatures in primary human hepatocytes. *Toxicology and Applied Pharmacology* 442:115991. doi:10.1016/j.taap.2022.115991.

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.

Medvedev, A,L.A. Medvedeva, E. Martsen, M. Moeser, K.L. Gorman, B. Lin, B. Blackwell, D.L. Villeneuve, K.A. Houck, K.M. Crofton, and S.S. Makarov. 2020. Harmonized cross species assessment of endocrine disruptors by Ecotox FACTORIAL assay. *Environmental Science and Technology* 54(19):12142–12153.

Menger, F., J. Pohl, L. Ahrens, G. Carlsson and S. Orn. 2020. Behavioural effects and bioconcentration of per- and polyfluoroalkyl substances (PFASs) in zebrafish (Danio rerio) embryos. *Chemosphere* 245:125573. doi:10.1016/j.chemosphere.2019.125573.

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.

NASEM (National Academies of Sciences, Engineering, and Medicine. 2022. Guidance on PFAS Exposure, Testing, and Clinical Follow-up. Washington, DC: The National Academies Press. https://doi.org/10.17226/26156.

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

NCHS (National Center for Health Statistics). 1993. *Joint policy on variance estimation and statistical reporting standards on NHANES III and CSFII reports: HNIS/NCHS Analytic Working Group Recommendations. Human Nutrition Information Service (HNIS)/Analytic Working Group*. Agricultural Research Service, Survey Systems/Food Consumption Laboratory, Riverdale, MD.

Nielsen, G., W.J. Heiger-Bernays, J.J. Schlezinger, and T.F. Webster. 2022. Predicting the effects of per- and polyfluoroalkyl substance mixtures on peroxisome proliferator-activated receptor alpha activity *in vitro. Toxicology* 465:153024. doi:10.1016/j.tox.2021.153024.

NRC (National Research Council). 2008. *Phthalates and Cumulative Risk Assessment: The Tasks Ahead*. The National Academies Press, Washington, DC. doi:10.17226/12528.

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). 2019. *Per- and Polyfluoroalkyl Substances (PFAS)*. https://epa.ohio.gov/pfas#184345314-public-drinking-water.

Ojo, A.F., C. Peng and J.C. Ng. 2021. Assessing the human health risks of per- and polyfluoroalkyl substances: a need for greater focus on their interactions as mixtures. *Journal of Hazardous Materials* 407:124863. doi:10.1016/j.jhazmat.2020.124863.

Olmstead, A.W., and G.A. Leblanc. 2005. Toxicity assessment of environmentally relevant pollutant mixtures using a heurisitc model. *Integrated Environmental Assessment and Management* 1(2):114–122.

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.

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):343–375.

Richard, A.M., R.S. Judson, K.A. Houck, C.M. Grulke, P. Volarath, I. Thillainadarajah, C. Yang, J. Rathman, M.T. Martin, J.F. Wambaugh, T.B. Knudsen, J. Kancherla, K. Mansouri, G. Patlewicz, A.J. Williams, S.B. Little, K.M. Crofton, and R.S. Thomas. 2016. ToxCast Chemical Landscape: Paving the Road to 21st Century Toxicology. *Chemical Research in Toxicology* 29(8):1225–1251. doi:10.1021/acs.chemrestox.6b00135.

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., and G.A. LeBlanc. 2005. An integrated addition and interaction model for assessing toxicity of chemical mixtures. *Toxicological Sciences* 87(2):520–528. doi:10.1093/toxsci/kfi247.

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.

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.

Simmons, J.E., L.K. Teuschler, C. Gennings, T.F. Speth, S.D. Richardson, R.J. Miltner, M.G. Narotsky, K.D. Schenck, E.S. Hunter, R.C. Hertzberg, and G. Rice. 2004. Component-based and whole-mixture techniques for addressing the toxicity of drinking-water disinfection by-product mixtures. *Journal of Toxicology and Environmental Health, Part A* 67(8-10):741–754. doi:10.1080/15287390490428215.

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):223–241.

Van Der Ven, L.T.M., P.V. Ommeren, E.P. Zwart, E.R. Gremmer, H.M. Odemaekers, H.J. Heusinkveld, J.D. van Klaveren, and E. Rorije. 2022. Does addition in the induction of craniofacial malformations in zebrafish embryos exposed to a complex mixture of food-relevant chemicals with dissimilar modes of action. *Environmental Health Perspectives* 130(4).

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.

Wambaugh, J., M. Hughes, C. Ring, D. MacMillan, J. Ford, T. Fennell, S. Black, R. Snyder, N. Sipes, B. Wetmore, J. Westerhout, W. Setzer, R. Pearce, J. Simmons, and R. Thomas. 2018. Evaluating *in vitro-in vivo* extrapolation of toxicokinetics. *Toxicological Sciences* 163(1):152–169.

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.

Wetmore, B.A., J.F. Wambaugh, B. Allen, S.S. Ferguson, M.A. Sochaski, R.w. Setzer, K.A. Houck, C.L Strope, K. Cantwell, R.S. Judson, E. LeCluyse, H.J. Clewell, R.S. Thomas, and M.E. Andersen. 2015. Incorporating high-throughput exposure predictions with dosimetry-adjusted *in vitro* bioactivity to inform chemical toxicity testing. *Toxicological Sciences* 148(1)121–36.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* 117:1563–1570.

Wolf, C.J., J.E. Schmid, C. Lau and B.D. Abbott. 2012. Activation of mouse and human peroxisome proliferator-activated receptor-alpha (PPARalpha) by perfluoroalkyl acids (PFAAs): further investigation of C4-C12 compounds. *Reproductive Toxicology* 33(4):546-551. doi:10.1016/j.reprotox.2011.09.009.

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.

Zhao, Y., K. Zhang, J.P. Giesy, and J. Hu. 2015. Families of Nuclear Receptors in Vertebrate Models: Characteristic and Comparative Toxicological Perspective. *Scientific Reports* 5:8554. doi:10.1038/srep08554.