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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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Washington, DC

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## Notices

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

This document provides a framework for estimating noncancer human health risks associated with mixtures of per- and polyfluoroalkyl substances (PFAS), based on longstanding EPA mixtures guidelines. This document is not a regulation and does not impose legally binding requirements on the 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 guidelines on mixtures (e.g., USEPA, 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; Food Quality Protection Act; Comprehensive Environmental Response, Compensation, and Liability Act). The EPA may change certain aspects of this document in the future based on evolving availability of information relevant to human health risk assessment and increasing confidence in New Approach Methods.

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 the 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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## Abbreviations and Acronyms

| | |
|---|---|
| 6:2 FTS | 6:2 fluorotelomer sulfonic acid |
| ADONA | 4,8-dioxa-3H-perfluorononanoic acid |
| AED | administered equivalent dose |
| 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 |
| CF | carbon-fluorine |
| CAR | constitutive androstane receptor |
| CAS | Chemical Abstracts Service |
| CCL 5 | fifth Contaminant Candidate List |
| CERCLA | Comprehensive Environmental Response, Compensation, and Liability Act |
| CPSC CHAP | Consumer Product Safety Commission Chronic Hazard Advisory Panel |
| DA | dose additivity |
| DAF | dosimetric adjustment factor |
| DBP | di-n-butyl phthalate |
| DEHP | di(2-ethylhexyl)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 |
| EOF | extractable organofluorine |
| EPA | U.S. Environmental Protection Agency |
| EU | European Union |
| FT4 | free serum thyroxine |
| FQPA | Food Quality Protection Act |
| GenX chemicals | hexafluoropropylene oxide (HFPO) dimer acid and HFPO dimer acid ammonium salt |
| GD | gestational day |

| | |
|---|---|
| HBWC | health-based water concentration |
| HED | human equivalent dose |
| HepaRG | epoxide hydrolase endpoint in liver |
| HFPO | hexafluoropropylene oxide |
| HFPO-DA | hexafluoropropylene oxide dimer acid |
| HI | hazard index |
| HQ | hazard quotient |
| HQ-115 | lithium bis[(trifluoro-methyl)sulfonyl]azanide |
| 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 |
| $k_e$ | elimination rate constant |
| KE | key event |
| L | liter |
| LOAEL | lowest-observed-adverse-effect level |
| M-BMD | mixture benchmark dose |
| 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/kg/week | nanograms per kilogram per week |
| ng/L | nanograms per liter |
| NHANES | National Health and Nutrition Examination Survey |
| 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-dioxin |
| PCDF | polychlorinated dibenzofuran |
| PECO | Population, Exposure, Comparator, and Outcome |
| PFAA | perfluoroalkyl acid |
| 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 | perfluoroalkyl phosphonic acids |
| PFPeA | perfluoropentanoic acid |
| PFPeS | perfluoropentanesulfonic acid |
| PFPIA | perfluoroalkyl phosphinic acid |
| PFPrA | perfluoropropanoic acid |
| PFPS | perfluoropropane sulfonic acid |
| PFSA | perfluoroalkane sulfonic acid |
| PFSIA | perfluoroalkane sulfinic acid |
| PFTA | perfluorotetradecanoic acid |
| PFTrDA | perfluorotridecanoic acid |
| PFUnA | perfluoroundecanoic acid |
| PND | postnatal 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 |
| SD | standard deviation |
| T3 | triiodothyronine |
| T4 | serum thyroxine |
| TT4 | total serum thyroxine |
| TCDD | 2,3,7,8-tetrachlorodibenzo-p-dioxin |
| TD | toxicodynamic |
| TEF | toxicity equivalence factor |
| TEQ | toxic equivalent |
| TK | toxicokinetic |
| TOSHI | target-organ-specific hazard index |
| $TOSHI_{DEV}$ | target-organ-specific hazard index for developmental effects |
| TSCA | Toxic Substances Control Act |
| TSCATS | Toxic Substances Control Act Test Submissions |
| TTD | target-organ toxicity dose |
| UCMR | Unregulated Contaminant Monitoring Rule |
| UF | uncertainty factor |
| $UF_A$ | interspecies UF |
| $UF_C$ | composite UF |
| $UF_D$ | database UF |
| $UF_H$ | human interindividual variability UF |
| $UF_L$ | LOAEL-to-NOAEL uncertainty factor |
| $UF_S$ | extrapolation from subchronic to a chronic exposure duration UF |
| Vd | volume of distribution |

# EXECUTIVE SUMMARY

The U.S. Environmental Protection Agency is releasing the final *Framework for Estimating Noncancer Health Risks Associated with Mixtures of Per- and Polyfluoroalkyl Substances (PFAS)* ("PFAS Mixtures Framework" or "framework"). This document is designed to communicate and illustrate the practical application of existing EPA chemical mixtures assessment approaches and methods to assess noncancer human health hazards and risks associated with exposure to two or more PFAS co-occurring in environmental media, using hypothetical drinking water examples. In November 2021, the EPA released a draft version of this document for Science Advisory Board (SAB) review, and in March 2023, this document underwent public comment as part of the proposed National Primary Drinking Water Regulation for PFAS (USEPA, 2023b). The EPA has considered the SAB and public comments and revised the document accordingly.

In a chemical 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 the integration of available toxicity information for the individual component chemicals that co-occur in environmental media.

This PFAS Mixtures Framework describes flexible, data-driven approaches that facilitate practical component chemical-based mixtures evaluation of two or more PFAS based on dose additivity. Dose additivity (described in detail in Section 3.0) means that the combined effect of the component chemicals in the mixture is equal to the sum of the individual doses or concentrations scaled for potency. Several perfluoroalkyl acid species (PFAAs) of PFAS tested to date have been shown to elicit the same or similar profiles of adverse effects in several organs and systems (ATSDR, 2021; EFSA, 2018, 2020; USEPA, 2021a, 2021b). Studies with PFAS and other classes of chemicals (e.g., phthalates, polycyclic aromatic hydrocarbons, etc.) support the EPA's health-protective conclusion that chemicals that elicit similar adverse health effects following individual exposure will act in a dose-additive manner when present in a mixture (unless data demonstrate otherwise). Although similarities among some PFAS have been shown at the level of molecular and cellular perturbations, no conserved modes of action (MOAs) have been identified across PFAS for noncancer health effects assessed thus far. As such, in this framework, the evaluation of toxicological similarity among component PFAS in a mixture is proposed at the level of adverse health outcome. This concept and proposed application of dose additivity for PFAS mixtures assessment are consistent with the EPA's mixtures guidelines (USEPA, 1986, 2000b) and the EPA Risk Assessment Forum's *Advances in Dose Addition for Chemical Mixtures: A White Paper* (USEPA, 2023h).

Descriptions of dose additivity-based approaches such as the hazard index (HI), relative potency factor (RPF), and mixture benchmark dose (M-BMD) are presented here to demonstrate potential application to PFAS mixtures, but they are not intended to provide a comprehensive treatise on the methods themselves; EPA chemical mixtures guidelines (USEPA, 1986, 2000b) and the EPA Risk Assessment Forum's *Advances in Dose Addition for Chemical Mixtures: A White Paper* (USEPA, 2023h) exist for such a purpose. The 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.

PFAS are a large and structurally diverse 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 then, hundreds of PFAS have been identified in environmental media, including water, soil, and air.

Many PFAS and/or their precursors or degradants are environmentally persistent, bioaccumulative, and have long half-lives in humans, particularly the longer-chain perfluoroalkyl carboxylic acid (PFCA) and perfluoroalkane sulfonic acid (PFSA) species such as PFOA and PFOS, respectively. PFCAs/PFSAs with shorter carbon chain lengths, such as perfluorobutanesulfonic acid (PFBS) and hexafluoropropylene oxide dimer acid (HFPO-DA) (also known as GenX Chemicals[1]), were developed and integrated into various consumer products and industrial applications because they have the desired performance properties and characteristics associated with this class of compounds but are more rapidly 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 April 2024, final EPA human health assessments are available for PFBS (USEPA, 2021a), HFPO-DA (USEPA, 2021b), perfluorobutanoic acid (PFBA; USEPA, 2022e), perfluorohexanoic acid (PFHxA; USEPA, 2023c), PFOA (USEPA, 2024a), PFOS (USEPA, 2024b), perfluoropropanoic acid (PFPrA; USEPA, 2023d), and lithium bis[(trifluoromethyl)sulfonyl]azanide (HQ-115) (USEPA, 2023e). In addition, the EPA's Integrated Risk Information System (IRIS) program is developing PFAS human health assessments for perfluorohexanesulfonic acid (PFHxS), perfluorononanoic acid (PFNA), and perfluorodecanoic acid (PFDA), which are expected to be completed in 2024. In May 2021, the Agency for Toxic Substances and Disease Registry (ATSDR) published a *Toxicological Profile for Perfluoroalkyls* that included quantitative minimal risk levels (MRLs) for PFAS, including PFOA, PFOS, PFHxS, and PFNA (ATSDR, 2021).

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, the EPA and the National Institute of Environmental Health Sciences 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). Examples of this coordination include publishing systematic evidence maps for hundreds of PFAS (e.g., Carlson et al., 2022), generating new hazard and dose-response data (e.g., new approach methodologies or NAMs), applying read-across tools, and developing the EPA Transcriptomic Assessment Product, which entails the derivation of toxicity reference values using transcriptomic pathway-

[1] The 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 salts.

based data from 5-day *in vivo* rat studies (see: https://www.epa.gov/chemical-research/epa-transcriptomic-assessment-product-etap-and-value-information-voi-case-study). Until results from ongoing research and testing efforts are available, the evaluation of potential toxicity/risk associated with PFAS mixtures is primarily limited to existing hazard and dose-response data under the purview of human health assessments by federal, state, and/or international entities.

This framework describes component-based mixture assessment methods that can be used to assess noncancer human health hazards and risks associated with exposure to PFAS mixtures. It is not the intent of the framework to ignore potential carcinogenic effects associated with PFAS exposure(s); however, 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, the EPA would consider approaches for addressing joint carcinogenic effects. The EPA's *National PFAS Testing Strategy: Identification of Candidate Per- and Polyfluoroalkyl Substances (PFAS) for Testing* (USEPA, 2021e) is underway to develop and issue test orders on data-poor[2] PFAS. Testing requirements encompass physicochemical properties, environmental fate and transport, and human health hazards, including mechanistic information (e.g., genotoxicity).

It is anticipated that real-world practical application of the approaches communicated and demonstrated in this framework may entail collecting, evaluating, and integrating diverse hazard and dose-response information. For example, only a small fraction of the thousands of PFAS have existent human health noncancer toxicity reference values, dozens more PFAS have gradations of traditional *in vivo* bioassay data available, and dozens more have data only from NAM assays/platforms (e.g., *in vitro* cell bioactivity). As such, to facilitate the use of potentially disparate sources of PFAS toxicity information in a mixtures assessment context, the application of the component-based methods presented in this framework is demonstrated using a hypothetical example mixture of five PFAS:

> **PFAS 1** = comprehensively studied, most potent for effect(s), and has formal noncancer human health assessment value(s) (i.e., reference dose [RfD]) and a health-based water concentration (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** = *in vivo* animal toxicity data available but no formal human health assessment and no HBWC; and
>
> **PFAS 5** = data-poor; no *in vivo* animal toxicity data or human data available.

The hypothetical PFAS mixture is purposefully designed to demonstrate how this framework allows for flexible integration of information derived from health assessment data sources (e.g., federal, state, international), available human and/or experimental animal hazard and dose-

[2] In this framework document, "data-poor" refers to the lack or absence of hazard and dose-response data traditionally used to support noncancer and/or cancer human health assessment (e.g., chronic oral exposure studies in humans and/or animals).

response data (that have not yet been formally evaluated in an assessment product), and information from NAMs. Opportunities for integrating additional PFAS into the context of a mixture assessment are 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

Per- and polyfluoroalkyl substances (PFAS) are an urgent public health and environmental issue facing communities across the United States. In April 2021, Administrator Michael Regan established the Environmental Protection Agency's Council on PFAS and charged the Council to develop a whole-of-EPA strategy to protect public health and the environment from the impacts of PFAS. In October 2021, the EPA released the PFAS Strategic Roadmap[3] (the Roadmap), which lays out the 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 the protection of disadvantaged communities. In November 2022, the EPA released *EPA's PFAS Strategic Roadmap: A Year of Progress*, which underscores key actions taken by the agency during the first year of implementing the Roadmap (USEPA, 2022f).

Recognizing that PFAS tend to occur in mixtures in environmental media (see Section 1.5), the EPA has developed this data-driven framework for assessing 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 related to human health risk assessment for mixtures (USEPA, 1986, 1991, 2000b). Although the framework and illustrative hypothetical examples contained within focus on PFAS in drinking 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 approaches presented here are not intended to be used to assign groups or subclasses or otherwise classify PFAS (instead, see the EPA *National PFAS Testing Strategy: Identification of Candidate Per- and Polyfluoroalkyl Substances (PFAS) for Testing* for categorization efforts; USEPA, 2021e). Rather, the framework is designed for the practical application of the EPA's mixtures assessment approaches and methods to gain insight into the potential joint toxicity associated with mixtures of PFAS. The mixtures 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 risks associated with exposure to PFAS mixtures.

The framework and hypothetical examples presented here are intended to demonstrate data-driven application of EPA component-based mixture assessment methods based on gradations of data availability and completeness anticipated to occur in real-world scenarios for PFAS. Although the examples provided are focused on drinking water, the approaches described in this framework could also be applied to other environmental media with oral[4] exposure routes (e.g., soil, fish/shellfish, food). Due to the constantly evolving science related to PFAS, the approaches

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

[4] In general, the component-based approaches presented in this document may also be applicable in assessing health risks associated with inhalation exposures to 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.

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, and information from high(er)-throughput bioassays and other new approach methodologies (NAMs), including data submitted to the agency under the Toxic Substances Control Act (TSCA).

Experimental evidence supports dose-additive effects from combined exposure to multiple PFAS. Dose additivity, described in detail in Section 3.0, means that the combined effect of the component chemicals in the mixture is equal to the sum of the individual doses or concentrations scaled for potency. Several perfluoroalkyl acid species (PFAAs) of PFAS tested to date, including perfluorohexanesulfonic acid (PFHxS), perfluorononanoic acid (PFNA), hexafluoropropylene oxide dimer acid (HFPO-DA), and perfluorobutanesulfonic acid (PFBS), have been shown to elicit the same or similar profiles of adverse effects in mammalian biological systems including effects on thyroid hormone levels, lipid synthesis and metabolism, development, immune system function, and liver function (ATSDR, 2021; EFSA, 2018, 2020; USEPA, 2021a, 2021b). An increasing body of evidence also shows similarities in molecular and cellular perturbations (e.g., common receptor binding/activation) across some PFAS; however, no conserved noncancer or cancer mode(s) of action (MOA(s)) have been identified to date.

The framework is not a regulation and does not impose legally binding requirements on the EPA, states, Tribes, or the regulated community, and might not apply to a particular situation based on the circumstances.

## 1.2 The EPA Science Advisory Board Review

In November 2021, the EPA released the *Draft Framework for Estimating Noncancer Health Risks Associated with Mixtures of PFAS* ("draft framework;" USEPA, 2021d) for the Science Advisory Board (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 framework and three other technical documents supporting the EPA's development of a National Primary Drinking Water Regulation for PFAS under the Safe Drinking Water Act. The EPA sought SAB comment on whether the draft framework and illustrative examples provided within were scientifically supported, clearly described, and informative for assessing potential health risk(s) associated with exposure to mixtures of PFAS. The EPA asked specific charge questions on PFAS dose additivity and three component-based approaches: hazard index (HI), relative potency factor (RPF), and mixture benchmark dose (M-BMD). A draft of the written SAB recommendations was published on April 1, 2022, and the EPA received the final report from the SAB on August 22, 2022 (SAB, 2022).

The EPA received a generally favorable review from SAB (SAB, 2022) for its development of component-based mixture assessment approaches that rely on a health-protective conclusion of dose additivity based on the same or similar adverse health outcome(s) instead of a shared MOA to evaluate risks from exposure to PFAS mixtures in drinking water and other environmental media. The EPA 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 Substances (PFAS)*. The SAB's overarching consensus recommendations and the

EPA's responses are summarized below. To view the EPA's complete responses to SAB comments on the draft framework, please see USEPA (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" (SAB, 2022).
    - The EPA has added text in Section 3.0 (Dose Additivity for PFAS) to address the SAB's comments related to uncertainties associated with dose additivity as the default assumption for assessment of PFAS mixtures. The EPA has added further discussion on deviations from dose additivity, such as synergy or antagonism, but available evidence suggests that dose additivity should be considered 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" (SAB, 2022).
    - In response to this point of clarification, the 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" (SAB, 2022).
    - In response to this and other SAB comments, the EPA has eliminated the tiered approach and restructured the framework as a data-driven, flexible approach to facilitate PFAS mixtures 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, the EPA has included a 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 mixture assessment approach(es), and implementing 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" (SAB, 2022).

    - The EPA has added a section (Section 8.0) that describes similarities and differences among the different component-based mixtures assessment approaches. In addition, the EPA has revised the framework to use the same hypothetical example mixture of five PFAS (ranging from data-poor to well-studied) for all 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" (SAB, 2022).
    - Text has been added in several places to indicate that it is optimal to calculate and use HEDs rather than oral-administered doses in test animals when possible. This includes additional text that walks the reader through the EPA's logic flow for cross-species scaling (see new Subsection 5.2.1). Regarding the M-BMD approach, 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-similarly shaped 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 approach).

## 1.3. Public Review

On March 14, 2023, the EPA released the draft framework for public comment (revised in response to the SAB review, as summarized in Section 1.2) as part of the proposed National Primary Drinking Water Regulation for six PFAS (USEPA, 2023b). The public comment period ended on May 30, 2023. The public docket can be accessed at www.regulations.gov under Docket ID: EPA-HQ-OW-2022-0114. The EPA has developed responses to public comments to support the final National Primary Drinking Water Regulation, including responses to comments on PFAS dose additivity and regulation of PFAS mixtures in drinking water using an HI approach (USEPA, 2024d).

## 1.4 Background on PFAS

PFAS are a large group of structurally diverse anthropogenic chemicals that include perfluorooctanoic acid (PFOA), perfluorooctanesulfonic acid (PFOS), and thousands of other fully or partially fluorinated chemicals. There is no consensus definition of PFAS as a class of chemicals (OSTP, 2023). Based on three related structural definitions associated with the EPA's identification of PFAS to be included in the fifth Contaminant Candidate List (CCL 5; see below), the universe of environmentally relevant PFAS, including parent chemicals, metabolites, and degradants, is approximately 15,000 compounds.[5] 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,

[5] See the EPA List of PFAS Structures: https://comptox.epa.gov/dashboard/chemical-lists/PFASSTRUCT

the EPA has identified more than 1,300 PFAS on the TSCA Inventory, of which more than 600 are considered "active" in U.S. commerce.

PFAS have been manufactured and used in a wide variety of industries worldwide, including in the United States, since the 1940s. The chemical structures and physicochemical properties of some PFAS enable them to repel water and oil, remain chemically and thermally stable, and exhibit surfactant properties; these properties confer utility in commercial and industrial applications but are also, in part, what make some PFAS persistent in the human body and the environment (Calafat et al., 2007, 2019). In general, PFAAs studied to date have strong, stable carbon-fluorine (CF) 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 the EPA's *National PFAS Testing Strategy: Identification of Candidate Per- and Polyfluoroalkyl Substances (PFAS) for Testing*; USEPA, 2021e). Due to their widespread use, physicochemical properties, persistence, and bioaccumulation potential, many PFAS co-occur in exposure media (e.g., indoor air/house dust, 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; USEPA, 2021c). These PFAS families can be divided into two primary categories: nonpolymers and polymers. Nonpolymer PFAS include perfluoroalkyl and polyfluoroalkyl substances and encompasses parent structures, precursors, and some environmental degradation and transformation products. Polymer PFAS include fluoropolymers, perfluoropolyethers, and side-chain fluorinated polymers (Table 1-1). Several U.S. federal, state, and industry stakeholders and European entities have posited various definitions of what constitutes a PFAS. The OECD-led "Reconciling Terminology of the Universe of Per- and Polyfluoroalkyl Substances: Recommendations and Practical Guidance" workgroup provided an updated definition of PFAS (OECD, 2021), 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 is referred to OECD (2021) for review. However, for the purposes of development of the EPA's CCL 5, the structural definition of PFAS includes chemicals that have <u>at least one of the following three structures</u>:

1. R-(CF2)-CF(R′)R″, where both the CF2 and CF moieties are saturated carbons, and none of the R groups can be hydrogen.
2. R-CF2OCF2-R′, where both the CF2 moieties are saturated carbons, and none of the R groups can be hydrogen.
3. CF3C(CF3)RR′, where all the carbons are saturated, 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 nonpolymers | 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 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, perfluoroalkoxy 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 | Nonfluorinated polymer backbone with fluorinated side chains with variable composition | n:1 or n:2 fluorotelomer-based acrylates, urethanes, oxetanes, or silicones; perfluoroalkyl fluorides; perfluoroalkane sulfonyl fluorides |

*Note:*
[a] Amalgamation of information from Figure 9 in OECD (2021) and Buck et al. (2011).

PFOA and PFOS are PFAAs in the nonpolymer PFAS category and are among the most studied PFAS in terms of human health toxicity and biomonitoring (see USEPA, 2024a, 2024b; Podder et al., 2021). The PFAA family includes perfluoroalkyl carboxylic, phosphonic, and phosphinic acids and perfluoroalkane sulfonic and sulfinic acids (Table 1-2). Many PFAA are highly persistent and are frequently found in the environment (Ahrens, 2011; Brendel et al., 2018; Wang et al., 2017). Although the 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 Federal Register [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 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 (PFAAs), including perfluoroalkylether acids.[a]**

| Group | Functional group | Examples |
|---|---|---|
| Perfluoroalkyl carboxylic acids (PFCAs) | -COOH | Perfluorooctanoic acid (PFOA), C7F15COOH |
| 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 chemicals), 4,8-Dioxa-3H-perfluorononanoic acid (ADONA) |
| Perfluoroalkylether sulfonic acids (PFESAs) | -OCF2CF2SO3H | Nafion byproduct 2 (NBP2) |
| 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 |

*Note:*
[a] Modified from Figure 9 in OECD (2021).

**Table 1-3. Characterization system of short-chain and long-chain PFAAs.[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** | | |
| | PFPrA | PFBA | PFPeA | PFHxA | PFHpA | PFOA | PFNA | PFDA |
| **# of fluorinated carbons** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** |
| **PFSAs** | PFPS | PFBS | PFPeS | PFHxS | PFHpS | PFOS | PFNS | PFDS |
| | **Short-chain PFSAs** | | | **Long-chain PFSAs** | | | | |

*Notes:*
PFPrA = 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 in 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 goes beyond 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 studied PFAS to date and have been the primary focus of formal human health risk assessment activities in the federal and state sectors.

## 1.5 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.[6] PFOA and PFOS have historically been target analytes, but recent water monitoring studies have begun to focus on additional PFAS via advanced analytical instrumentation/methods and nontargeted analysis (De Silva et al., 2020; McCord and Strynar, 2019; McCord et al., 2020). The proposed framework for estimating the likelihood of noncancer human health risks associated with oral exposure to mixtures of

[6] 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 USEPA (2024f, 2024g).

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.

The EPA uses the Unregulated Contaminant Monitoring Rule (UCMR) to collect occurrence data nationwide for contaminants suspected to be present in drinking water. Between 2013 and 2015, the EPA's third UCMR (UCMR 3) required all large public water systems (PWSs) (each serving more than 10,000 people) and a statistically selected, representative national sample of 800 small PWSs (each serving 10,000 people or fewer) to monitor for 30 unregulated contaminants in drinking water, including six PFAS: PFOS, PFOA, PFNA, PFHxS, PFBS, and perfluoroheptanoic acid (PFHpA). 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 (Guelfo and Adamson, 2018; USEPA, 2019b). The EPA found that 4% of PWSs reported results for which one or more of these six PFAS were measured at or above their respective minimum reporting levels (USEPA, 2019b).[7] Under UCMR 5 (2023 to 2025), PWSs will monitor for 29 PFAS. A small subset of UCMR 5 data (24% of the total results that the EPA expects to receive) was released to the public in early 2024. Preliminary sampling results from UCMR 5 are available in the *PFAS Occurrence and Contaminant Background Support Document for the Final PFAS NPDWR* (USEPA, 2024c).

Outside of the UCMR data collection, many states have undertaken individual efforts to monitor for PFAS in both source and finished drinking water. These results show that occurrence in multiple geographic locations is consistent with what was observed during UCMR 3 monitoring, as well as the occurrence and co-occurrence of other PFAS not included in the UCMR 3. Additionally, these results show that PFAS are very likely to co-occur as mixtures in the environment. These data suggest that PWSs with high concentrations of one PFAS are likely to have high concentrations of other PFAS and that there is notable co-occurrence at elevated concentrations (Cadwallader et al., 2022; USEPA, 2024c).

PFAS mixtures have also been reported in U.S. ambient surface waters and aquatic biota (Ahrens, 2011; Benskin et al., 2012; Burkhard, 2021; McCord and Strynar, 2019; Nakayama et al., 2007; Remucal, 2019; Zareitalabad et al., 2013). Most environmental monitoring of PFAS in surface waters has focused on sites of historical manufacturing and known contamination (3M Company, 2000; Boulanger et al., 2004; Cochran, 2015; Hansen et al., 2002; Jarvis et al., 2021; Konwick et al., 2008; Nakayama et al., 2007). Simcik and Dorweiler (2005) consistently detected both PFOA and PFHpA in all 12 surface waters sampled across the U.S. Midwest and PFOS in all but two locations. Sinclair and Kannan (2006) detected PFOA and PFOS in all effluent-dominated samples collected across New York State; Sinclair and Kannan (2006) also detected PFHxS, but PFBS and perfluorooctane sulfonamide (PFOSA) were below detection limits in all samples. De Silva et al. (2011) detected PFOS and additional PFAS (i.e., perfluoropentanoic acid [PFPeA] [C5], perfluorohexanoic acid [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. Other PFAS, including PFNA (C9), perfluorodecanoic acid (PFDA) (C10), perfluoroundecanoic acid (PFUnA) (C11), PFBS (C4), PFHxS (C6),

[7] 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 (USEPA, 2019b).

perfluoroethylcyclohexane sulfonate (PFECHS) (C8), and perfluoromethylcyclohexane sulfonate (C7), 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 the direct application of manufactured products that contain a specific mixture of PFAS. For example, Anderson et al. (2016) quantified PFAS in ambient surface waters across 10 U.S. Air Force bases where there were known historical uses of aqueous film-forming foam, which is used in firefighting and training activities and can contain hundreds of polyfluoroalkyl precursors (Ruyle et al., 2021). PFOA and PFOS largely co-occurred and were detected in 88% and 96% of samples, respectively. Anderson et al. (2016) also detected PFBA, PFBS, PFPeA, PFHxA, PFHxS, and PFHpA in ≥ 80% of samples.

Environmental monitoring of PFAS in aquatic biota has primarily focused on fish. Generally, PFCAs are less bioaccumulative than PFSAs in aquatic systems, with longer-chain PFAS being more bioaccumulative than short-chain PFAS (Burkhard, 2021; Conder et al., 2008; Kannan et al., 2005). Within the United States, PFAS in aquatic biota have been measured in major rivers, in the Laurentian Great Lakes region, in several estuaries, and in targeted studies of sites of known contamination (e.g., industrial). A recent national probabilistic survey by the EPA measured up to 33 PFAS in fish samples collected in 2013–2014 and 2018–2019 from hundreds of river sites across the U.S. One or more PFAS were detected in 99.7% of fish fillet samples collected in 2013–2014 and in 95.2% of samples collected in 2018–2019. For both sampling periods, detection frequency was dominated by PFOS (91%–99%), PFUnA (85%–88%), PFDA (84%–88%), and PFDoA (69%–70%) (Stahl et al., 2023). 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 tissues of lake trout from across all the Great Lakes, with PFOA, PFECHS, and perfluorodecanesulfonic acid (PFDS) also being detected in lake trout from Lake Ontario (De Silva et al., 2011). 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 concentration of 3.9 nanograms (ng) per gram (g); geometric mean PFOSA concentration of 3.2 nanograms per gram [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. Charleston Harbor (the more developed of the two sites) had higher overall PFAS concentrations. PFOA, PFOS, PFNA, PFDA, PFUnA, PFDoDA, PFHxS, and PFOSA were all commonly detected in tissues of the six fish species from Charleston Harbor. PFOS and PFDoDA were the only two PFAS detected at elevated concentrations in the fish from Sarasota Bay (Houde et al., 2006). A study in New Jersey found co-occurrence of PFAS in ambient water, sediment, and fish at sites with historical 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 = 14.7 nanograms/liter [ng/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).

## 1.6 Evidence of PFAS Exposure in Humans

Humans can be exposed to PFAS through a variety of sources, including food packaged in PFAS-containing materials, processed with equipment that uses 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, aqueous film-forming foam; 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 via 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 (CDC, 2023). Results from this nationally representative biomonitoring study in which data were gathered from 1999–2000 through 2017–2018 documented measurable serum levels of PFOS, PFOA, PFHxS, and PFNA in more 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, from 1999 to 2018, blood levels of PFOA and PFOS declined by > 70% and > 85%, respectively, presumably due to restrictions on commercial use of PFOA and PFOS in the United States. Under the 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 to eliminate these substances in products by 2015 (USEPA, 2021c). However, since the voluntary phase-out of these longer-chain PFAS in the United States, manufacturers have been shifting to shorter-chain and alternative forms of PFAS, such as HFPO-DA. The most recent available NHANES survey (2017–2018) measured ADONA, HFPO-DA, perfluoroheptanesulfonic acid (PFHpS), 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate, and PFHxA in blood and found that PFHpS was detected in 78% of samples and 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate was detected in 12% of samples (the others were not detected or found in less than 1% of samples). The 2015–

2016 NHANES survey detected some other PFAS in more than 30% of samples, including PFDA, PFUA, and 2-(N-methylperfluoroctanesulfonamido)acetic acid (Me-PFOSA-AcOH). 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 (ATSDR, 2022; Table 17-6 in ITRC, 2022; Kotlarz et al., 2020; Yu et al., 2020). Compared to PFOA and PFOS, there is less publicly available information on the occurrence and health effects of replacements for PFOA and PFOS and other members of the carboxylic acid and sulfonate PFAS families.

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

In 2016, the EPA finalized drinking water Health Advisories of 70 parts per trillion (ppt or ng/L) for PFOA and PFOS, both individually and when present as a mixture (USEPA, 2016a, 2016b), because the reference doses (RfDs) were based on developmental effects and numerically identical. Subsequently, some states developed state-specific cleanup levels or drinking water or groundwater guidelines, advisories, or standards for PFOA, PFOS, and other PFAS. In some cases, the state values are the same as the EPA's 2016 drinking water Health Advisory; in other cases, states developed different values (see examples in Table 1-4).

In June 2022, the EPA issued interim updated drinking water Health Advisories for PFOA and PFOS and final Health Advisories for HFPO-DA and PFBS (USEPA, 2022a, 2022b, 2022c, 2022d). The 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 (see Section 5.0) to assess the potential noncancer human health risks of exposure to a mixture of PFAS (USEPA, 2022a, 2022b, 2022c, 2022d) consistent with the approach presented in this framework.

In March 2023, the EPA proposed and requested comment on a National Primary Drinking Water Regulation that included an HI public health goal (i.e., Maximum Contaminant Level Goal [MCLG]) and enforceable level (i.e., Maximum Contaminant Level [MCL]) to protect public health from exposure to mixtures of any combination of two or more of PFHxS, PFNA, HFPO-DA, and/or PFBS, four PFAS that individually can affect similar health endpoints/outcomes and co-occur in drinking water (USEPA, 2023b). After consideration of prior peer-review advice and public comment, and consistent with the provisions set forth under the Safe Drinking Water Act, the EPA finalized an HI MCLG and MCL for mixtures of these four PFAS. In consideration of their known toxic effects, dose additivity health concerns, and occurrence and likely co-occurrence in drinking water, the EPA finalized an HI of 1 (unitless) as the MCLG and MCL for any mixture containing two or more of PFHxS, PFNA, HFPO-DA, and PFBS (USEPA, 2024e).

International approaches to addressing multiple PFAS in drinking water have resulted in a range of proposed and promulgated standards and guideline values, as well as a variety of grouping methods (Table 1-4). Canada proposed a drinking water objective of 30 ng/L as a summed total of all PFAS measured in drinking water (using EPA Method 533 or EPA Method 537.1 or both). Australia has established a combined level of 70 ppt for PFOS and PFHxS, 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. 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 organofluorine (EOF) or adsorbable organofluorine (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 this framework (Borg et al., 2013; RIVM, 2018). Although not specifically related to drinking water, the European Food Safety Authority has also taken PFAS mixture toxicity into consideration in its development of a Tolerable Weekly Intake for the sum of PFOA, PFNA, PFHxS, and PFOS (4.4 nanograms per kilogram per week [ng/kg/week]) (EFSA, 2020).

**Table 1-4. 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 (USEPA, 2024e, 2022a, 2022b, 2022c, 2022, 2016a, 2016b) | 2024 | 10 PFHxS<br>10 PFNA<br>10 HFPO-DA<br>2000 PFBS | HI MCLG and MCL = 1 for any combination of two or more of four PFAS | Final PFAS National Primary Drinking Water Regulation Rulemaking. |
| | 2022 | 0.004 PFOA<br>0.02 PFOS<br>10 HFPO-DA<br>2000 PFBS | Example HI for four PFAS | Interim Updated Drinking Water Health Advisories (HAs) for PFOA and PFOS; Final HAs for PFBS and HFPO-DA. |
| | 2016 | 70 | PFOA and PFOS | Drinking Water Health Advisory. |
| Alaska (USA) (Alaska DEC, 2019) | 2019 | 70 | PFOA and PFOS | Application of the EPA 2016 Health Advisory. |
| Colorado (USA) (CDPHE, 2020) | 2020 | 70 | PFOA and PFOS | Application of the 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 the EPA 2016 Health Advisory. |

| Entity | Date | Conc. (ng/L) | Sum of PFAS | Background |
|---|---|---|---|---|
| Maine (USA) (Maine DEP, 2021) | 2021 | 20 | PFOA, PFOS, PFNA, PFHxS, PFHpA, and PFDA | Based on similarities in chemical structure and toxicities of four PFAS to PFOS and PFOA. Same approach as the 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 four PFAS to PFOS and PFOA. Same approach as the EPA 2016 Health Advisory but includes an additional uncertainty factor. |
| Montana (USA) (MT DEQ, 2021) | 2019 | 70 | PFOA and PFOS | Application of the EPA 2016 Health Advisory. |
| Ohio (USA) (Ohio EPA, 2019) | 2019 | 70 | PFOA and PFOS | Application of the EPA 2016 Health Advisory. |
| Rhode Island (USA) (RIDEM, 2017) | 2019 | 70 | PFOA and PFOS | Application of the EPA 2016 Health Advisory. |
| Vermont (USA) (Levine, 2018; VT DEC, 2021) | 2019 | 20 | PFOA, PFOS, PFNA, PFHxS, and PFHpA | PFHxS, PFHpA, and PFNA are considered sufficiently similar to PFOA and PFOS. Difference from the EPA 2016 Health Advisory is due to Vermont's calculation being based on infant consumption rates. |
| Wisconsin (USA) (WI DHS, 2022) | 2022 | 70 | PFOA and PFOS | Application of the EPA 2016 Health Advisory. |
| European Union (EU, 2020) | 2020 | 100<br>500 | 100 ng/L for sum of 20 PFAS (C4–C13 PFSAs and C4–C13 PFCAs)<br>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 on PFAS other than PFOS and PFOA are limited. |

| Entity | Date | Conc. (ng/L) | Sum of PFAS | Background |
|---|---|---|---|---|
| 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 on PFAS other than PFOS and PFOA are limited. |
| 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 on PFAS other than PFOS and PFOA are limited. |
| Canada (Health Canada, 2023) | 2023 | 30 | Total all PFAS measured using EPA Method 533, 537.1 or both | Technology-based. |

*Notes:*
[a] Modified from Cousins et al. (2020).
[b] As of July 2021, several states have passed or proposed compound-specific MCLs or Health Advisories (e.g., California, Illinois, Michigan, Minnesota, New Jersey, New York, Pennsylvania, Texas, and Washington). Some states have applied the 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 MCL of 21 ppt and PFOS MCL of 14 ppt.

## 1.8 Overview of Proposed Framework for Estimating Noncancer Health Risks for PFAS Mixtures

This document describes a framework of component-based options with different levels of data requirements and objectives for estimating the noncancer human health risks associated with exposure to mixtures of PFAS based on longstanding EPA chemical mixtures guidelines. To address concerns over health risks from multichemical exposures, the EPA issued the *Guidelines for the Health Risk Assessment of Chemical Mixtures* in 1986 (USEPA, 1986). The 1986 guidelines were followed in 2000 by the *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (USEPA, 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" (USEPA, 1986, 2000b); this definition is used in this framework document.

Several laws direct the 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 of 1986, and amendments in 2002 (CERCLA, 2002; SARA, 2002) (commonly referred to as Superfund); the Clean Air Act Amendments of 1990 (CAA, 1990); the Safe Drinking Water Act Amendments of 1996 (SDWA, 1996); and the Food Quality Protection Act (FQPA) of 1996 (FQPA, 1996). Both the 1986 *Guidelines for the Health Risk Assessment of Chemical Mixtures* (USEPA, 1986) and the 2000 *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (USEPA, 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

the EPA's programs currently implement environmental laws through regulations that rely on the methods articulated in these two chemical mixtures guidelines documents. This framework does not supersede previously published EPA guidelines 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; FQPA; 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 based on 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 general HI (based on overall RfDs [or similar noncancer toxicity value such as an ATSDR minimal risk level (MRL)] irrespective of similarity in target organ or system) and TOSHI (based on RfDs in same target organ) for each component chemical provide an indication of noncancer risk associated with 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 dose additivity (DA) model-based equation (similar to the Berenbaum equation; Section 4.2.6 in USEPA, 2000b) to calculate a BMD (e.g., $BMD_{X\text{-}HED}$) for the mixture (Section 7).

The HI facilitates the estimation of potential combined toxicity associated with the co-occurrence of chemicals in environmental media (e.g., water, soil) (USEPA, 1991, 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[8] 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 exhibit similar shape and slope over the exposure ranges most relevant to the decision context (USEPA, 2000b). The RPF method is illustrated in Section 6 using the same target organs/systems, including liver, thyroid, and developmental. An 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 similar dose-response functions (i.e., same/similar shape or slope) across component chemicals. This approach provides 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 interest for toxicological evaluations and human health risk assessment, data pertaining

[8] Mode of action is a sequence of key events and processes, starting with interaction between an agent and a cell, proceeding through operational and anatomical changes, and resulting in a noncancer effect or cancer formation (modification of footnote 2 in USEPA, 2005).

to biological pathway perturbations are limited or not available for many PFAS; so, while there is an evolving landscape of evidence demonstrating shared molecular and cellular effects by some PFAS, no conserved noncancer or cancer MOA(s) have been identified across PFAS to date. 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 chemical mixtures guidelines (USEPA, 1986, 1991, 2000b), the EPA Risk Assessment Forum's *Advances in Dose Addition for Chemical Mixtures: A White Paper* (USEPA, 2023h), and expert opinion from the National Academy of Sciences, National Research Council (NRC, 2008).

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 ~15,000 PFAS estimated at the time of this writing, 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-additivity-based component chemical mixture approaches using hypothetical human health-relevant toxicity and exposure information.

## 2.0 Background on EPA Mixtures Additivity Guidelines

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 the evaluation of potential health risks associated with chemical mixtures, the EPA developed the 1986 *Guidelines for the Health Risk Assessment of Chemical Mixtures* and, subsequently, the 2000 *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (USEPA, 1986, 2000b). In those guideline documents, the EPA proposed a hierarchy of mixtures approaches where the preferred approach is to evaluate health risk 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 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., a limited number of component chemicals at fixed proportions/ratios). As such, the EPA also developed multiple component chemical-based mixtures assessment approaches. Component-based methods are used more frequently than whole-mixture methods. These component-based methods are based on assumptions about how the chemicals behave biologically 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." The basic tenets of the EPA mixtures additivity theory and practice are:

- 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 documents, the development of component-based mixture approaches was 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 mixture component 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 demonstrates or suggests 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 mixture component 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 demonstrates or suggests 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 the 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, although all based on dose addition, involve different assumptions, data requirements and objectives for evaluating the joint toxicity of component chemicals in a mixture. Each method is introduced and detailed in Sections 5–7 and includes a demonstration of the 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 example mixture of five PFAS:

**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;

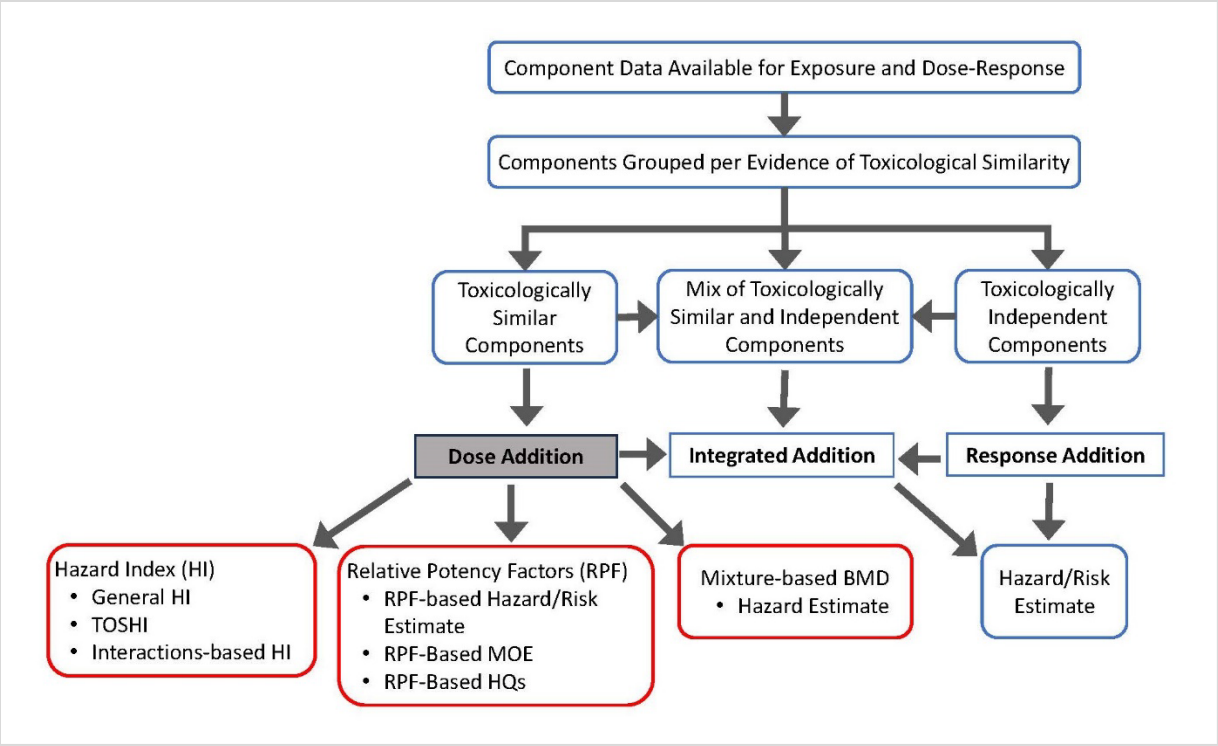
**PFAS 4** = *in vivo* animal toxicity data available but no formal human health assessment and no HBWC; and

**PFAS 5** = data-poor; no *in vivo* animal toxicity data or human data available.

This hypothetical PFAS mixture is purposefully designed to demonstrate how the framework allows for the flexible integration of information derived from diverse data types and sources. Opportunities for integrating PFAS into a mixture assessment are 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 the effects of a mixture even when all the individual component chemical exposures are at or below their individual no-observed-adverse-effect levels (NOAELs; i.e., "something from nothing"). For example, in the hypothetical PFAS mixture applications (Sections 5–7), the HI in general indicates a point (i.e., 1) above which a hazard might be anticipated for the mixture, or below which adverse effects are not expected (see Section 5). In the RPF approach, the sum of the scaled IC[9] equivalent doses/concentrations across component chemicals is compared to the equivalent threshold dose of the IC (Jonker et al., 1996; Silva et al., 2002). In the context of water-specific application, a mixture IC equivalent dose/concentration may be compared to a health-based water concentration (HBWC) for the IC to indicate if the selected health effect may or may not be expected (see Section 6). Finally, no IC is required in the M-BMD approach, and

[9] An IC is that mixture component that is typically the most toxicologically well-studied. The qualitative and quantitative hazard and dose-response data for an IC 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 IC.




*Notes:*
Modification of Figure 4-3b (USEPA, 2007).
BMD = benchmark dose; HI = hazard index; HQ = hazard quotient; MOE = margin of exposure; RPF = relative potency factor; TOSHI = target organ-specific hazard index.
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.

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

dose-response shape(s) and slope(s) do not have to be similar among components. The aggregated M-BMD is converted to a noncancer RfV and corresponding HBWC and then compared directly to the total measured mixture PFAS concentration to indicate if the selected health effect may or may not be expected following exposure to the combinations and proportions for that specific mixture (see Section 7).

### *2.1.1 Application of Dose Addition as the EPA's Default Approach*

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; 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; USEPA, 2007; 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 (NRC, 2008; Rider et al., 2009; Van Der Ven et al., 2022). In general, the results of such studies listed here, and many

others, support the continued application of DA as the EPA's default component-based mixture assessment approach. Further discussion and examples of the basis for the use of dose additivity for component-based evaluation of PFAS mixtures are 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 and cellular perturbations interact. Section 3.2 discusses the evidence demonstrating that mixtures of chemicals disrupting common pathway events 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 pathway events, 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 DA as the default model for estimating mixture effects, even when the mixtures included chemicals with diverse biological signal transduction pathways (but common target organs/effects). 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 conclusion that a mixture of chemicals with similar apical effect profiles should be assumed to also act in a dose-additive manner unless data demonstrate otherwise. Further, experimental data demonstrate that PFOS, PFOA, and other PFAS disrupt signaling in multiple biological pathways, resulting in common adverse effects on several of the same biological systems and functions, including thyroid hormone signaling, lipid synthesis and metabolism, developmental toxicity, and immune and liver function, and are reviewed in Section 3.4. Finally, Section 3.4 summarizes several EPA Office of Research and Development (ORD) PFAS developmental toxicity mixture studies that provide robust evidence that PFAS behave in a dose-additive manner.

## 3.1 Overview of Assessment Approaches for Chemical Mixtures

Over 30 years ago, scientists developed quantitative dose metrics and methods to assess the joint toxicity of mixtures of large classes of chemicals that disrupt a common biological pathway (NATO, 1988). For example, 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 on their potency relative to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Many of the lessons learned from assessing the effects of mixtures of dioxin-like chemicals (DLCs) are also generally applicable to assessing the effects of PFAS mixtures. Since that time, TEF-like approaches (e.g., RPFs) have been used to evaluate mixtures of other chemical classes. However, the evolving picture since early applications of RPFs or TEFs is that some chemicals, regardless of similarities or dissimilarities in molecular initiating events (MIEs) or early key events (KEs), produce mixture effects on common apical endpoints that generally are well predicted using DA models. This has, in part, been explained by the concept of pathway convergence; that is, across mixture component chemicals, some pathway perturbations may qualitatively look dissimilar at the level of MIEs or early KEs but ultimately converge upon shared or common events nearer to the terminus of a pathway leading to health effects (for further details, see the EPA Risk Assessment Forum's *Advances in Dose Addition for Chemical Mixtures: A White Paper* [USEPA, 2023h]).

The general applicability of DA models is based on reviews of studies specifically designed to evaluate how well different mixture models predict how 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 dose-additive 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 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 its 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 dose-additive manner if the individual mixture components 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 Toxicity Equivalence Factors*

In 2010, the EPA published guidelines for using TEFs for human health risk assessment of DLCs, which produce many of their adverse effects by acting as aryl hydrocarbon receptor (AhR) agonists (USEPA, 2010). It should be noted that the TEF approach is a specialized application under the RPF umbrella that 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, the EPA and the World Health Organization have recommended the use of the TEF methodology to evaluate risks associated with exposure to mixtures of TCDD and DLCs for human health (USEPA, 1987, 1989, 2003) and ecological risk assessments (USEPA, 2008). TEFs can be calculated for each DLC based on dietary dose or internal whole-body toxic equivalent concentrations.

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 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:

- Chemical mixture components interact in a dose-additive manner;
- They all act via a common AhR-mediated pathway, 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.

The EPA's TEFs have undergone several revisions (Van den Berg et al., 2006). In 2010, the EPA published guidelines for the use of TEFs for human health risk assessment of DLCs (USEPA, 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). The EPA stated that the TEQ methodology was appropriate for evaluating risks to fish, birds, and mammals associated with AhR agonists (USEPA, 2008).

Studies of AhR agonists in various species indicate:

- Species and tissues differ in sensitivity to the effects of a DLC 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 at 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 single oral gavage dose of a mixture of 11 pyrethroid pesticides to adult male rats 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.

The 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) a shared ability to interact with voltage-gated sodium channels, resulting in disruption of membrane excitability in the nervous system; and (3) neurotoxicity characterized by two different toxicity syndromes. In 2011, after establishing a common mechanism grouping for the pyrethroids and pyrethrins, the EPA conducted a cumulative risk assessment using an RPF approach (USEPA, 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 administered simultaneously. Subsequently, all organophosphate (OP) pesticides in use were evaluated in binary mixture studies to determine if deviation from additivity (e.g., synergism or antagonism) 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 four pairs showed greater-than-additive effects on lethality (Dubois, 1961). Moser et al. (2005, 2006) reported a range of responses with mixtures of four or five 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, the EPA determined that the OPs form a common mechanism group based on their shared ability to bind to and phosphorylate the enzyme acetylcholinesterase (AchE), leading to the accumulation of acetylcholine and, ultimately, cholinergic neurotoxicity (USEPA, 1999). As such, the cumulative risk to OPs has been assessed using AchE inhibition as the effect on which dose-response data are integrated under the assumption of dose addition. The most recent OP cumulative assessment was conducted in 2006 and employed an RPF approach (USEPA, 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 uterotrophic assay, an EPA Endocrine Disruptor Screening Program Test Guideline that is a sensitive *in vivo* test for estrogenicity (USEPA, 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 measured. Exposures can be oral or through subcutaneous injection. 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*

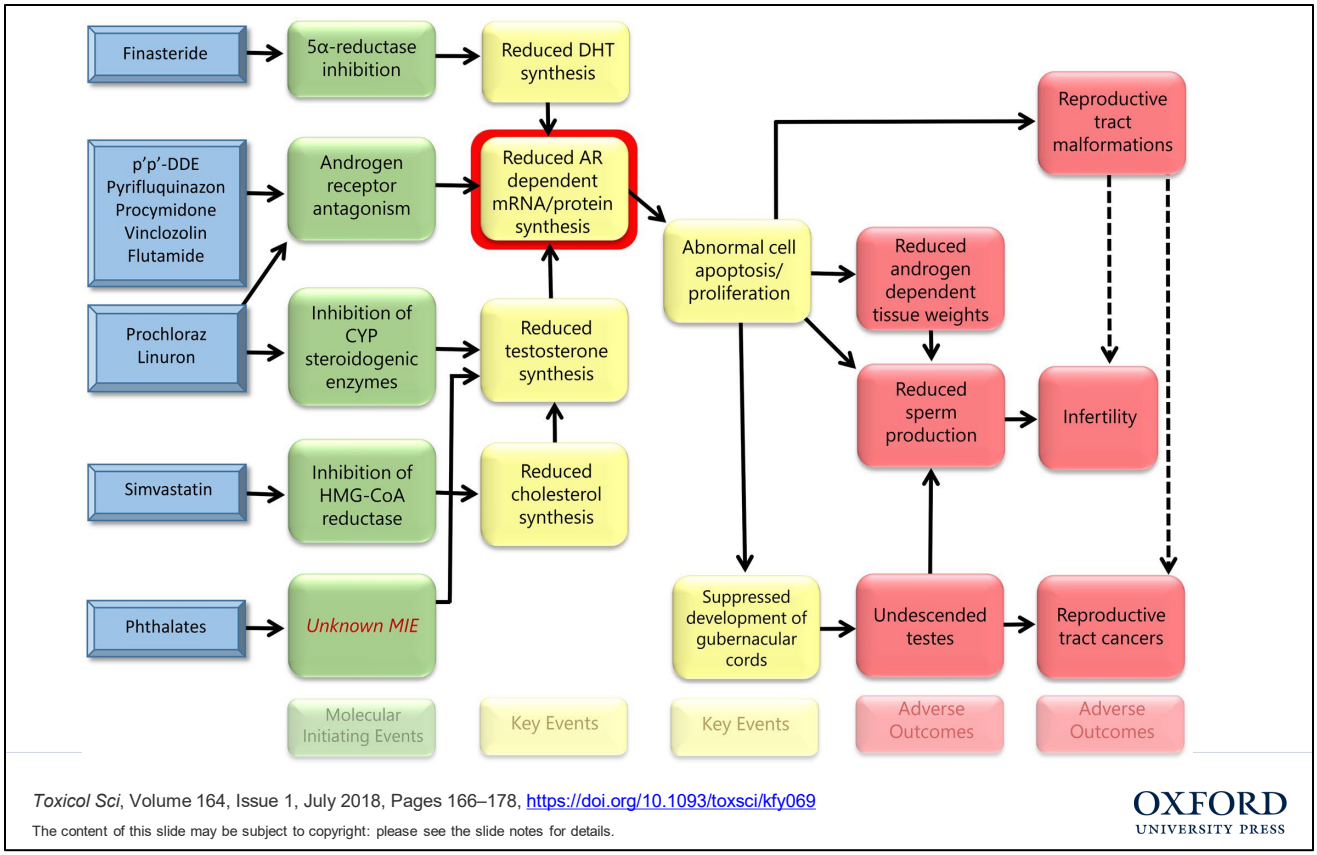
Hannas et al. (2013) reported that administration of a mixture of five phthalates (> 520 mg total phthalate) to pregnant rats from gestational days (GDs) 8 to 13 induced reproductive tract malformations in female rat offspring. These malformations included complete to partial uterine

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 an 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 the NRC (2008). Studies on the effects of mixture exposures on male reproductive development provide one of the larger databases supporting the use of DA 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 adverse outcome pathways (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 number of chemicals used in these studies range from 2 to 18, administered at a range of doses, enabling one to discriminate additive from antagonistic or synergistic interactions. In all these studies, the DA model predicted the effects of the mixture on the male reproductive tract more accurately than IA or RA. Likewise, Metzdorff et al. (2007) concluded that the "Effects of a mixture of similarly acting anti-androgens can be predicted fairly accurately based on the potency of the individual mixture components by using the DA concept. Exposure to anti-androgens, which individually 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 its review, no published studies 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 three 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 in estimating mixture effect. For example, 60% of male offspring were found to have penile malformations that resulted in infertility; DA accurately predicted this effect, 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 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 involved in the development of androgen-dependent tissues. Each of the identified chemicals/classes reduces 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 with a 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 dose-additive manner.

In summary, an examination of the literature on the effects of mixtures on male reproductive tract development demonstrates that common effects are most accurately modeled by DA; the chemicals discussed above acted in a dose-additive manner even when including chemicals with different MIEs, and IA and RA models consistently underestimated the hazard of a mixture of chemicals acting on common molecular or cellular (more downstream) events resulting in a common profile of apical effects.

## 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, six 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 published between 2007 and 2017. Martin et al. (2021) identified 1,220 mixture studies, ~65% of which did not incorporate more than 2 component chemicals. 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 based on an assumption of 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, 20 years of research have 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 alterations in metabolic activity leading to synergistic or antagonistic dose-response among chemicals in mixture, there are other examples of deviations from DA that do not include interactions across different levels of biological organization but instead entail physical-chemical interactions. For example, although melamine and its derivatives, including cyanuric acid, individually present low toxicity, together the compounds can physically interact to 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 in infants and young children in China from contaminated infant formula and related dairy products (WHO, 2008).

## 3.4 PFAS Dose Additivity

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 elicit similar toxicological effects across different levels of biological organization, tissues/organs, 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 pathway perturbations or KEs (for those chemicals with established MOAs) 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 identification and characterization of MOA(s) for most PFAS, it is considered a health-protective conclusion that PFAS that can be demonstrated to share one or more molecular/cellular pathway events and/or adverse health outcomes will produce dose-additive effects from co-exposure. The EPA's SAB supported this approach in its review of a draft version of this document (SAB, 2022). 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, intermediate pathway events, and adverse outcomes that have been reported for those PFAS studied to date and experimental evidence that supports dose-additive effects from combined exposure to multiple PFAS. This overview highlights study results from, among others, the National Institute of Environmental Health Sciences' 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 effects/endpoints spanning MIEs, intermediate pathway events, and adverse outcomes. More comprehensive reviews of PFAS toxicity endpoints in experimental animal studies and observational human studies can be found elsewhere (e.g., ATSDR, 2021; EFSA et al., 2018, 2020).

Mechanistically, *in vitro* and *in vivo* studies have demonstrated the activation of multiple nuclear receptors associated with exposure to many structurally diverse PFAS, indicating several potential MIEs for PFAS-relevant toxicity pathways. The most commonly reported MIE associated with many PFAS is the 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 (NTP, 2019a, 2019b). 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 the activation of the constitutive androstane receptor (CAR) for both PFOA and PFOS, among other PFAS, due to the upregulation of CAR-dependent genes (Rosen et al., 2017). All PFAA carboxylates and sulfonates that were tested in the NTP 28-day studies and displayed upregulation of the PPARα target genes also displayed upregulation of the CAR-inducible genes Cyp2b1 and Cyp2b2 in adult male and female rat livers (NTP, 2019a, 2019b). Additional *in vitro* data indicate potential involvement from several other nuclear receptors following PFAS exposures 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 data-driven basis for positing shared or overlapping pathway perturbations across PFAAs.

In addition to the cell- and/or tissue-specific gene expression changes described above, multiple pathway events or markers of toxicity downstream of the above-mentioned potential MIEs are also shared between PFOA, PFOS, and other PFAAs. In both rodent and nonhuman primate studies, serum lipids (cholesterol, triglycerides) are altered, 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 in rats following exposure to PFHxA, PFOA, PFNA, PFDA, PFBS, or PFOS (NTP, 2019a, 2019b). Further, circulating thyroid hormone concentrations were reduced following 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, 2019b). Ether-linked PFAAs have also been shown to reduce circulating thyroid hormone concentrations (Conley et al., 2019, 2022a). In combination with a common profile of nuclear receptor activity and gene expression, there is a pronounced similarity in the landscape of serum clinical chemistry and thyroid hormone-based markers of altered physiology for PFOA, PFOS, and several other studied PFAS. Identification and characterization of PFAS-relevant pathway events as formal Kes in established MOA(s) is currently an area of high research activity, as additional associations across different levels of biological organization with the reported MIEs continue to be investigated across various life stages and species.

Similar adverse health 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; an emerging polyfluoroethersulfonic acid compound recently detected in human serum) 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, 2005b; Thibodeaux et al., 2003). PFAS studied by NTP (2019a, 2019b) 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 are numerous adverse effects that occur in laboratory animals that are shared across PFAS such as PFOA, PFOS and other PFAAs. These adverse effects are consistent with the molecular and cellular pathway perturbations highlighted above. However, it is important to recognize that while there are the 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 exposure did not result in changes in 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, indicative of hepatocellular injury.

The specific molecular mechanisms or precise MOAs for a given adverse health outcome may be disparate across some PFAS. For example, 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 the 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, and more importantly the lack of formal MOA(s), in PFAS-related adverse health outcomes, it is a reasonable health-protective conclusion that effects shared across PFAS in a given mixture will be dose additive.

Some studies have evaluated other models for quantifying potential joint toxicity (e.g., dose addition, synergism, antagonism) associated with combined exposure to PFAS in experimental systems either *in vitro* or *in vivo*. *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 effects 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. (2022) reported both synergistic and antagonistic effects compared to a dose-addition model for binary, ternary, and multicomponent mixtures of PFAAs for cytotoxicity in HepG2 cells. Nielsen et al. (2022) demonstrated the utility of generalized dose 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 dose addition is applicable for prediction of *in vivo* mixture effects. Most recently, Addicks et al. (2023) conducted *in vitro* exposures of primary human liver spheroids to seven different PFAS mixtures, including combinations of PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFBS, PFHxS, PFOS, PFOSA, 6-2 FtS, and 8-2 FtS. Liver spheroids were evaluated for mRNA transcriptomic points of departure, and all mixtures produced effects that were within 2- to 3-fold of mixture effects predicted using dose addition. Similarly, 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 dose additivity (Martin et al., 2021). 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, an evaluation of chemical dose-response and comparison to mixture models was not conducted. Regarding zebrafish PFAS effects, it is notable that fish PPARγ has a 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 human 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 dose-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), or a mixture of PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFBS, PFHxS, and PFOS in rabbits (Crute et al., 2022) 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 impossible to ascertain if the mixtures behaved in a DA or RA manner or if interactions occurred (i.e., deviations from DA).

Recent and ongoing work at the EPA includes developmental toxicity studies of PFAS mixtures in rats. 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 to evaluate 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 body weight, pup serum triiodothyronine (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 body weight at term and gestational weight gain demonstrated departures from dose additivity, and these effects were slightly 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 conclusion for predicting mixture effects of co-occurring PFAS.

A second PFAS mixture study by Conley et al. (2023) reported data on a mixture of PFOS, HFPO-DA, and NBP2 (an emerging polyfluoroethersulfonic acid compound recently detected in human serum [Kotlarz et al., 2020]). Multiple endpoints, including maternal serum cholesterol, triglyceride, and thyroid hormone concentrations (total T3 and T4), pup birthweight and body weight at 2 days of age, and pup mortality, all conformed to dose additivity. Similar to the PFOA + PFOS mixture above, effects on maternal weight gain were slightly less than additive, and no endpoints demonstrated synergy. Further, the mixture shifted the dose-response curves for increased maternal and pup liver weights towards effects at lower doses when comparing the HFPO-DA responses in the mixture to the dose responses from HFPO-DA exposure alone. Finally, preliminary data from a third *in vivo* study of a mixture of six PFAS (PFOA, HFPO-DA, PFMOAA, PFOS, PFHxS, and NBP2) further indicate dose-additive effects on maternal and neonatal endpoints including pup body weight and pup survival. The published and preliminary 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. Similarly, the joint toxicity of a mixture of PFOS and PFOA on Japanese quail chick 10-day survival is accurately predicted by DA but not RA (Gray et al., 2023).

In summary, 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. Most notably, 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 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. The EPA will continue to review how mixtures of PFAS and other chemicals interact. Dose additivity is proposed as the "default" model for PFAS mixtures assessment, 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 combinations 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 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 combinations and proportions across time and space in environmental media. In the context of PFAS, increasing environmental evidence (e.g., ambient and drinking water, fish, air, and soil sampling results) suggests that the complexities briefly summarized above with regard to the diversity of chemicals co-occurring in different component 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(s) to mixtures of PFAS (see Sections 5–7).

The EPA's *Supplemental Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (USEPA, 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 determining 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 profiles. 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 mixture 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 are most appropriate for localized, site-specific assessments with stable mixtures (i.e., low/no temporal and spatial variability) and should be considered before 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 the 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 the evaluation of PFAS mixtures in other exposure media as well (e.g.,

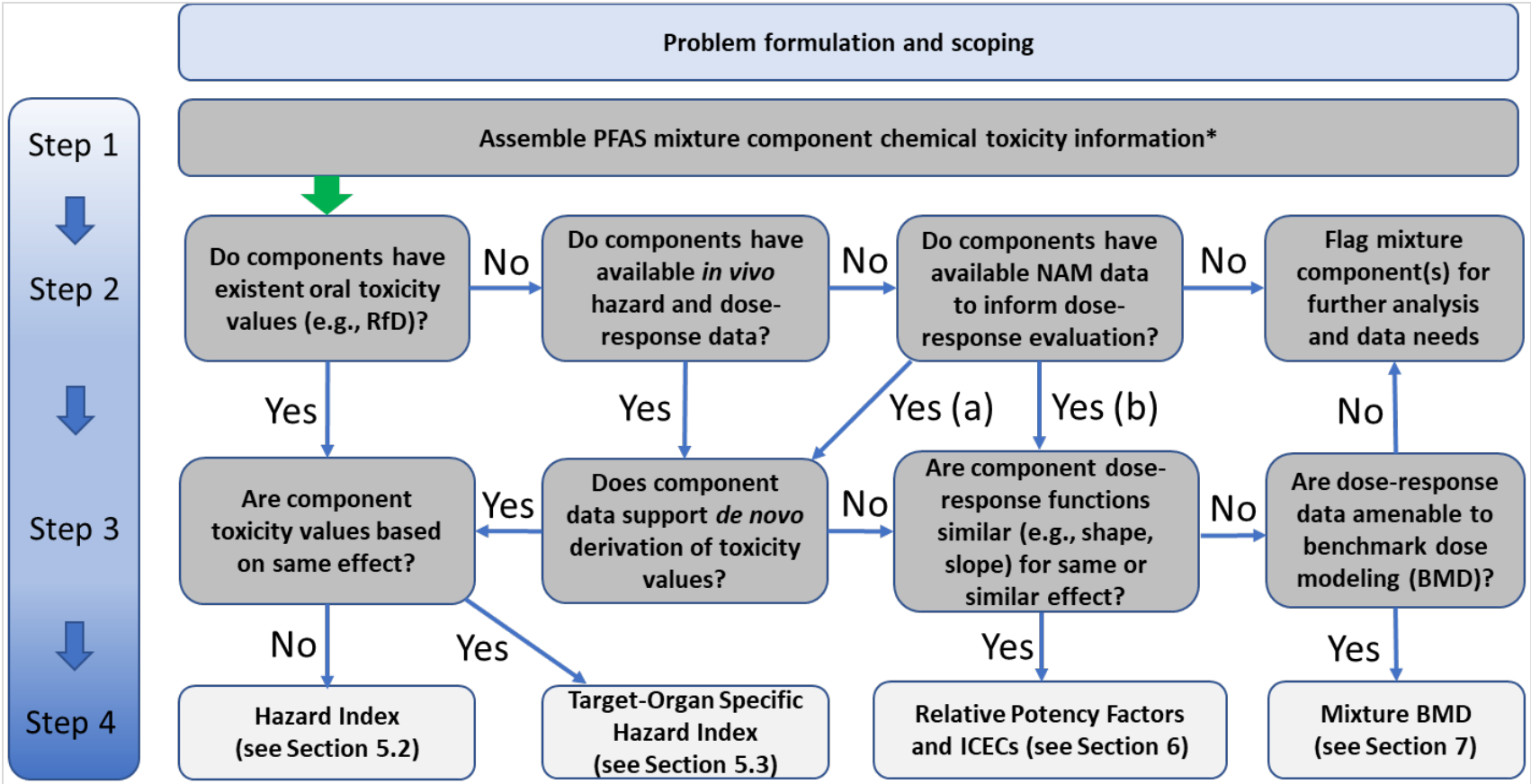



**Figure 4-1. Framework for data-driven application of component-based assessment approaches for mixtures of PFAS based on dose additivity.**

soil, air). As outlined in earlier sections of this framework, while EPA component-based methods and approaches are available for an evaluation of mixtures of chemicals under different assumptions of additivity (USEPA, 2000b), the currently available hazard evidence on PFAS, and several other classes of environmental chemicals, support 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 guidelines, and actively used by the EPA. These two approaches are discussed below and include illustrative examples 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 the EPA mixtures guidelines [USEPA, 2000b]), is also a dose-additive approach that is described and illustrated (see Section 7). The primary difference between the RPF and M-BMD approaches is that RPF assumes component chemical dose-response curves are similarly shaped, while the M-BMD approach is more applicable for mixture component chemicals with dissimilar dose-response curve shapes/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 use of this framework document in guiding formal component-based assessment of PFAS mixtures. The M-BMD approach was indirectly described within the context of a mixture RfD in the EPA's mixtures guidelines (USEPA, 2000b) and by the National Research Council (NRC) (NRC, 2008); further, laboratory studies have provided empirical evidence in support of this approach (Gray et al., 2022; see 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. The general steps of the component-based approach, as shown in Figure 4-1, are:

**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 developing a conceptual model and analysis plan and engaging with potentially affected stakeholders (e.g., 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 the 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 toxicity data for the whole 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 is conducted. Although the optimal approach would be to use formal systematic literature search and review principles as set forth by the EPA (please see the Integrated Risk Information System [IRIS] systematic review protocol for PFBA, PFHxA, PFHxS, PFNA, and PFDA as an example[10]), 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

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

landscape used in steps 2–4 of the framework approach are transparent. It should be noted that while this step is primarily intended for the identification and collation of human epidemiological and/or traditional experimental animal toxicity data, it is ideal to also assemble information such as toxicokinetic (TK) 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 collected, 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; it should be noted that human health assessment values may be available from different sources utilizing different levels of analytical rigor and/or peer-review. The practitioner is advised to consider the confidence associated with noncancer assessment values located across mixture components and integrate accordingly into subsequent steps of this workflow; and (b) Development of health effect domain profiles (see example literature inventory in Figure 4-2 excerpted from the IRIS systematic review protocol for PFBA, PFHxA, PFHxS, PFNA, and PFDA[10]), and associated dose-response information sorted based on exposure duration (e.g., acute or short[er]-term, longer-term [i.e., subchronic, chronic]; developmental/reproductive), across component PFAS supported by the assembled (i.e., human epidemiological and/or experimental animal) toxicity data from Step 1.

Users of this framework may find that many component 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 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 (Lizarraga et al., 2023; 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., PODs) are then adopted from a selected (single-best) analog as a 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 undergo BMD modeling and be included in the calculation of an overall M-BMD. The 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 of the approach, please see Appendix A of the Provisional Peer-Reviewed Toxicity

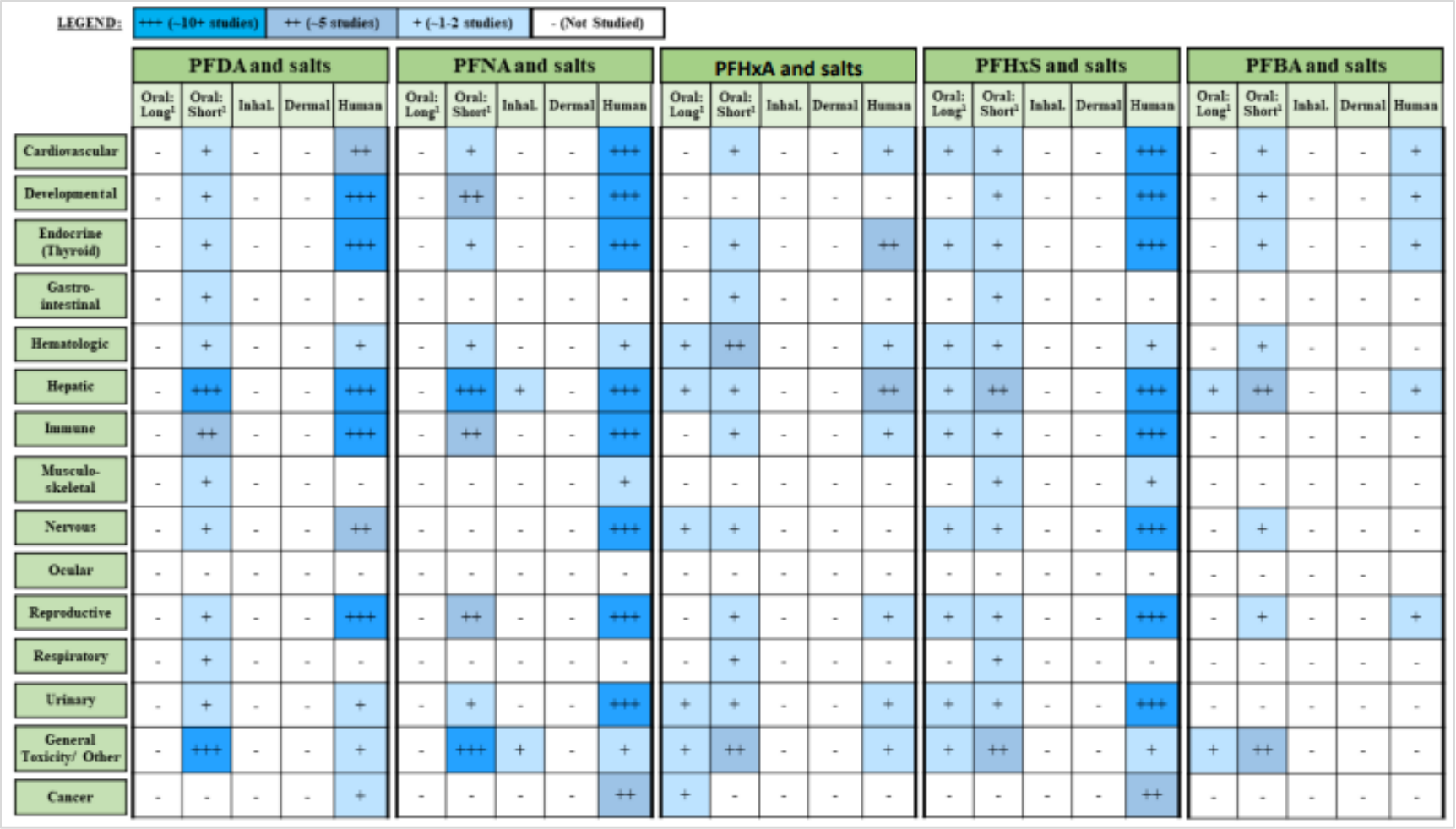



**Figure 4-2. Example literature inventory heatmap for epidemiological or traditional experimental animal studies for five PFAS currently under development/review in the EPA/ORD's IRIS program (heat map circa 2018). Health effects are based on groupings from the IRIS website (https://ordspub.epa.gov/ords/eims/eimscomm.getfile?p_download_id=542033).**

Value (PPRTV) document for 2,3-toluenediamine at https://cfpub.epa.gov/ncea/pprtv/recordisplay.cfm?deid=352932 and/or for perylene at https://cfpub.epa.gov/ncea/pprtv/recordisplay.cfm?deid=357451.

Another opportunity for integration of NAMs into the proposed mixture approaches involves cell bioactivity (e.g., ToxCast/Tox21), including pathway-based transcriptomic and metabolomic data from experimental animals and/or *in vitro* cell cultures. For over a decade, the EPA and the National Institute of Environmental Health Sciences 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 cell bioactivity 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). However, inherent complexities and challenges are associated with study designs and data interpretation using NAM assays/platforms, such as *in vitro* cell culture. For example, there is no *a priori* assumption that molecular/cellular perturbations observed in cells *in vitro* have any direct qualitative relationship to phenotypic health effect(s). Further, considerations such as metabolic capacity, shorter-term exposure durations (e.g., hours to days), and specificity or sensitivity of *in vitro* effect(s), and how these factors influence precision, accuracy and/or reproducibility of quantitative concentration-response relationship within an assay, within a lab, across labs, etc., continue to present challenges for integration into decision-making foci.

Recent investigation has demonstrated that quantitative data (e.g., PODs) from *in vitro* cell bioactivity and corresponding traditional *in vivo* toxicity assay-based PODs differ by approximately 100-fold (median of range); however, the NAM-based PODs were found to be lower than *in vivo* PODs for 89% of chemicals evaluated (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., 2011, 2013). For many chemicals, bioactivity assays may also provide information on the potential to disrupt specific MIEs and KEs of known or postulated MOAs or AOPs and could potentially inform the relevance of specific pathways to humans. While *in vitro* assays can be informative, they are not without limitations. For example, to use the cell bioactivity data in the component-based mixture approaches discussed in this framework, the *in vitro* concentration-based metrics should be first converted to administered equivalent doses (AEDs) in humans. Converting *in vitro* bioactivity concentrations to estimated human *in vivo* doses (i.e., AEDs) requires the application of *in vitro*-to-*in vivo* extrapolation (IVIVE) and reverse toxicokinetics (rTK), which introduces additional uncertainty and might 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

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

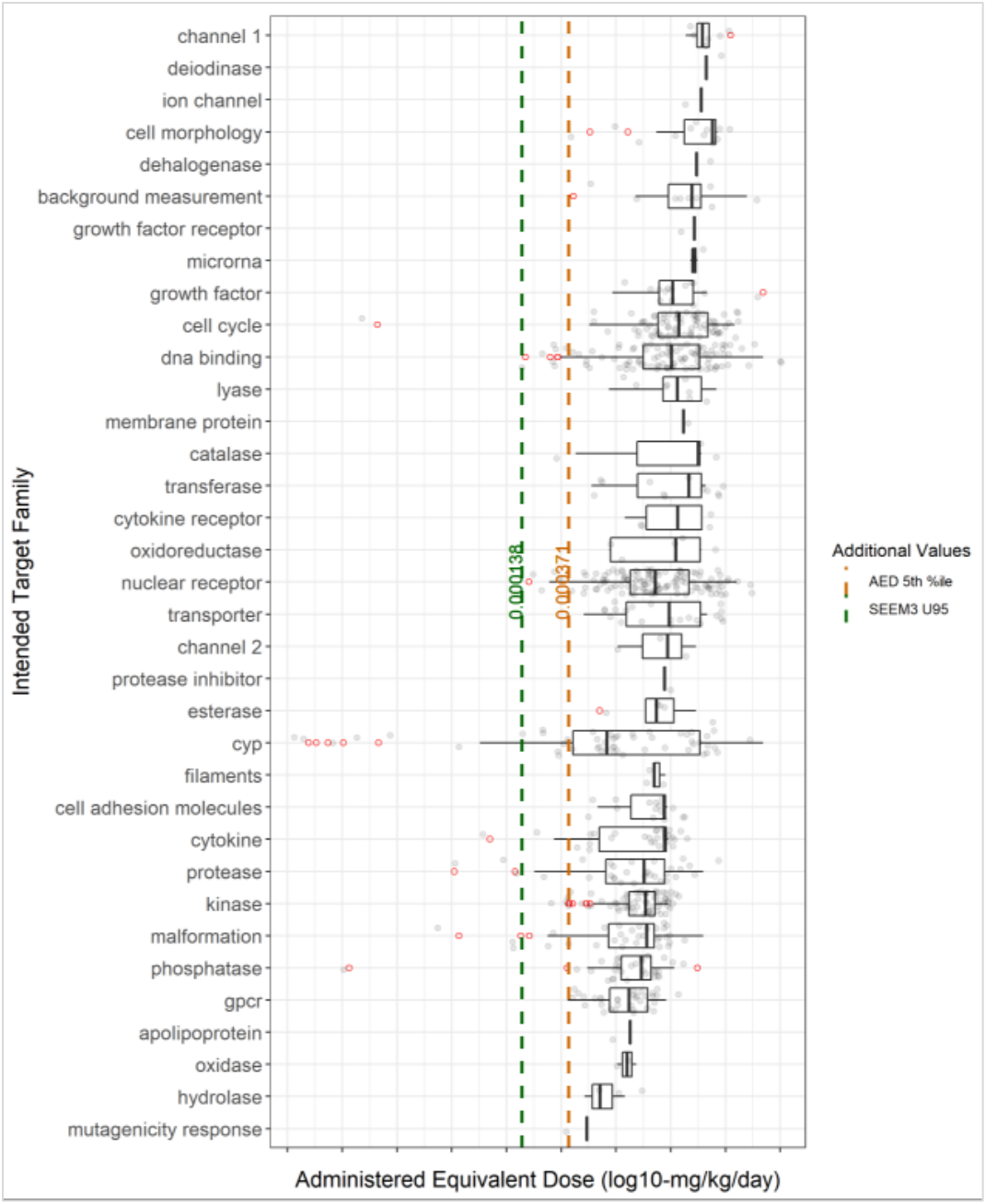



**Figure 4-3. Example plot illustrating *in vitro* cell bioactivity expressed in AEDs. An AED is an estimated oral exposure dose that results in an internal steady-state concentration consistent with the *in vitro* concentration associated with a biological perturbation or activity. The example shows 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 the EPA's Systematic Empirical Evaluation of Models (SEEM3): https://www.epa.gov/chemical-research/computational-toxicology-communities-practice-systematic-empirical-evaluation).**

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.

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

Considering 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 apply 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 similar, 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 "same" health outcomes 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 component-based mixture approaches, it is optimal to calculate and use HEDs (for PODs, Edx, etc.) rather than orally 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 when/where supported; however, study data types, design (e.g., exposure duration, route), and confidence will likely dictate the optimal selection of component-based approach for PFAS on a case-by-case basis. In most cases, a user would likely 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 depending on characteristics and confidence in available dose-response data across mixture components.

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

**a. HI and TOSHI.** For component PFAS that have human health risk assessment value(s), the user in Step 3 should have determined if the critical effect(s) for 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 the same/similar effect, then the general HI approach is recommended. In brief, the HI approach entails the 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 the health outcome domain. Because each mixture component HQ is calculated using a corresponding RfV (protective of all effects), the mixture HI may represent a health-protective indicator of potential mixture risk. The component PFAS HQs are then summed to generate a mixture HI (see Equation 5-1). A mixture HI exceeding one (1) 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., ≤ 0.0X) might be deprioritized as they may not have a 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 RfVs across PFAS mixture components are based on the same effect, then a 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 only those mixture components with a reference value for the specified effect*. As such, while more consistent with the concept of toxicological similarity, a potential limitation of the TOSHI approach might entail the loss or exclusion of one or more mixture components for which a toxicity value for the specific health effect does not exist. A further advancement under the TOSHI is the development of 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 component PFAS, where/when hazard and dose-response data support. This may facilitate the 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.

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 USEPA, 2000b). However, in this framework, based on 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 nonexistent for PFAS co-occurring in the 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 for a selected health effect when specific media concentrations of the component PFAS are available (see Section 6). In the RPF approach, potency for an effect across each component 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 the 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., the total dose of IC) is compared directly to an HBWC (e.g., Health Advisory, 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, health risk is not anticipated if the mixture ICECs for all effect domains are below the corresponding IC HBWC. Additionally, component 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 the calculation of an M-BMD (see Section 7) and is applicable even when PFAS in the mixture have dissimilarly shaped dose-response curves for the same or similar effect. In contrast to the RPF approach, there is no need for identification of mixture ICs, calculation of RPFs or ICECs, or existence of HBWCs. The final determination of risk is based on a comparison of the observed total mixture water concentration with an HBWC derived from the most sensitive effect-based M-BMD. Similar to the RPF approach, dose-response data across one or more health effect domains for each PFAS in the mixture are needed to determine the corresponding dose at the benchmark response (BMR) for each PFAS component (i.e., each component PFAS BMD). 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 similarly shaped dose-response functions (i.e., slopes). The resulting M-BMD (i.e., mixture POD) could be converted into a mixture RfD, using expert judgment in 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). Consistent with the RPF approach, Section 7.2 presents an illustrative example for liver, thyroid, and developmental effects associated with the hypothetical five component PFAS mixture. If the total measured PFAS mixture water concentration exceeds the mixture-specific HBWC, derived using the mixture toxicity value based on the M-BMD (and appropriate composite UF application), 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 mixture-specific HBWCs calculated, then a 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 a demonstration of practical application to a hypothetical five-component mixture of PFAS. As a reminder, PFAS 1–5 are:

**PFAS 1** = comprehensively studied, most potent for effect(s) among PFAS 1–3, 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** = *in vivo* animal toxicity data available but no formal human health assessment and no HBWC; and

**PFAS 5** = data-poor; no *in vivo* animal toxicity data or human available.

To help introduce the illustrative case study, the hypothetical drinking water scenario is as follows: Periodic 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 the hypothetical analytical quantitation limits[12] (Table 4-2).

**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 identifying 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 modifying circumstances or factors.

**Table 4-1. Hypothetical drinking water concentrations for five hypothetical PFAS.**

| PFAS exposure estimates (measured in drinking water) (ng/L) | | | | | |
|---|---|---|---|---|---|
| | **PFAS 1** | **PFAS 2** | **PFAS 3** | **PFAS 4** | **PFAS 5** |
| Median | 4.8 | 55 | 172 | 58 | 120 |

*Note:*
The values represent the median of a distribution of sampling data collected across a community over time.

**Table 4-2. Hypothetical 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 |

**Step 1: Assemble information.**

The structured literature search for the hypothetical mixture of PFAS 1–5 included comprehensive Boolean search strings applied across information databases such as PubMed,

[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, the 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.

Web of Science, Toxline, and the Toxic Substances Control Act Test Submissions (TSCATS) (Figure 4-4). Please note that the specific PFAS names, synonyms, and Chemical Abstracts Service (CAS) registry numbers in Figure 4-4 are for illustrative purposes only. In an application, the search string(s) would need to be scoped and developed to optimize the literature search for component PFAS on a case-by-case basis.

The assembled literature inventory was then screened at the title and abstract level 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 as the basis of the illustrative PFAS mixture example in subsequent sections.

- PubMed (National Library of Medicine)
- Web of Science (Thomson Reuters)
- Toxline (National Library of Medicine)

| 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. Hypothetical PFAS-specific literature search string applied to toxicity information databases such as PubMed, Web of Science, Toxline, and TSCATS.**

| PECO element | Description |
|---|---|
| Population | Human: Any population and lifestage (occupational or general population, including children and other sensitive populations).<br><br>Animal: Nonhuman mammalian animal species (whole organism) of any lifestage (including fetal, early postnatal, adolescents and adults). |
| Exposures | Relevant forms:<br>[chemical X] (CAS number)<br>Other forms of [chemical X] that readily dissociate (e.g., list any salts, etc.)<br>Metabolites of interest, including metabolites used to estimate exposures to [chemical X]<br>Occupations that may be considered surrogates of exposure<br>Human: Any exposure to [chemical X] [via [oral or inhalation] route[s] if applicable]. Studies will also be included if biomarkers of exposure are evaluated (e.g., measured chemical or metabolite levels in tissues or bodily fluids) but the exposure route is unclear or likely from multiple routes. Other exposure routes, such as those that are clearly dermal, will be tracked during title and abstract screening and tagged as "potentially relevant supplemental material."<br><br>Animal: Any exposure to [chemical X] via [oral or inhalation] route[s] of >1 day duration, or any duration assessing exposure during reproduction or development. Studies involving exposures to mixtures will be included only if they include an experimental arm with exposure to [chemical X] alone. Other exposure routes, including [dermal or injection], will be tracked during title and abstract as "potentially relevant supplemental material." |
| Comparators | Human: A comparison or referent population exposed to lower levels (or no exposure/exposure below detection limits), or exposure for shorter periods of time, or cases versus controls, or a repeated measures design. However, worker surveillance studies are considered to meet PECO criteria even if no statistical analyses using a referent group is presented. Case reports or case series of > 3 people will be considered to meet PECO criteria, while case reports describing findings in 1–3 people will be tracked as "potentially relevant supplemental material."<br><br>Animal: A concurrent control group exposed to vehicle-only treatment and/or untreated control (control could be a baseline measurement, e.g., acute toxicity studies of mortality, or a repeated measure design). |
| Outcomes | All health outcomes (cancer and noncancer). In general, endpoints related to clinical diagnostic criteria, disease outcomes, biochemical, histopathological examination, or other apical/phenotypic outcomes are considered to meet PECO criteria. |

**Figure 4-5. Hypothetical 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 the removal of duplicate references and systematic screening of the initial inventory using the defined PECO, nonrelevant 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., do not meet PECO criteria but are potentially relevant to the specific aims of the assessment); and (3) studies or reports that upon further review were excluded as not PECO-relevant (bottom of Figure 4-6).

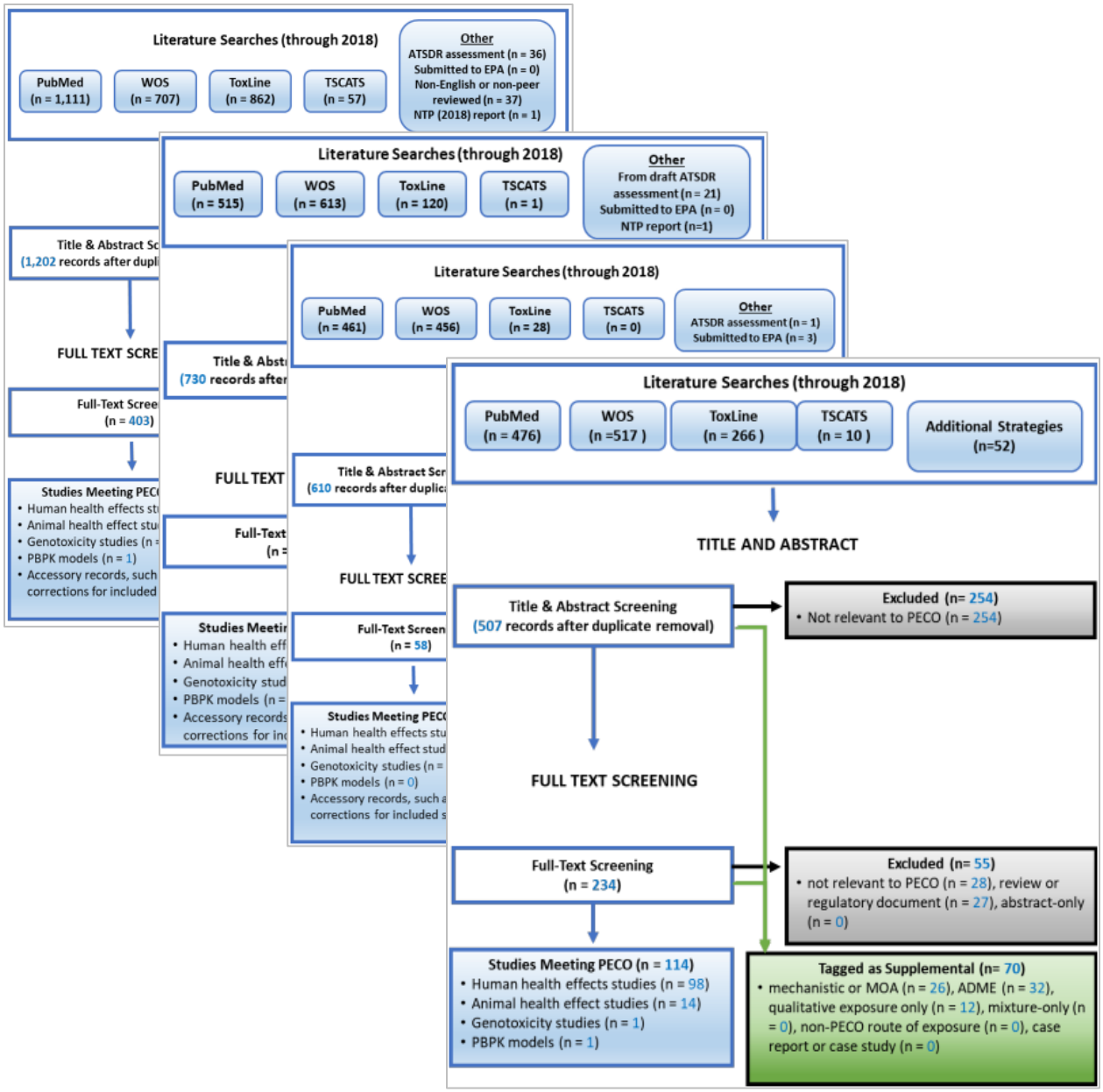



**Figure 4-6. Example literature screening logic flow for hypothetical PFAS using an EPA systematic review approach. The figure depicts example PECO-dependent development of evidence bases to support human health assessment application(s). Note: Only four hypothetical PFAS (i.e., PFAS 1–4) are represented,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

### Step 2: Evaluate data objectives.

For the hypothetical example, the systematic literature search and screening resulted in studies meeting the PECO criteria for only PFAS 1–4. PFAS 5 was identified as data-poor (no *in vivo* animal toxicity data or human data available) and further interrogated in the EPA's Computational Toxicology Dashboard (https://comptox.epa.gov/dashboard/) for the 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 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 assessment values for the oral route of exposure (Figure 4-7); (3) PFAS 4 has existing hazard and potentially useful 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 (identified from the systematic literature search/publications tagged as supplemental and/or searching of the EPA's Computational Toxicology Dashboard). In addition, across the five hypothetical PFAS, different levels/types of TK data were identified. Specifically, clearance[13] values for experimental rats and humans were located, for example, for 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. For PFAS 5, only rat serum half-life data were located from publications tagged as supplemental during the literature search. This type of data is important because it may inform data-driven cross-species extrapolation of TKs between experimental animals and humans (i.e., kinetic adjustment of PODs and corresponding reduction of the animal-to-human uncertainty factor [$UF_A$]).

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 studies meeting the PECO criteria 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).

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

As illustrated in the hypothetical 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 the single best study/dataset for a critical effect to represent the PFAS in a given effect domain (Table 4-3). "Single best" study/dataset will be subjective and user-dependent; however, considerations such as the methodological strengths 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 amenability to BMD modeling are just a few factors for which a study might be selected. For PFAS with an existing health assessment (e.g., in the hypothetical example, PFAS 1–3), the publishing authors have already made such decisions. In practice, if the user of this framework deviates from use of existing health assessment dose-response metrics, a clear rationale must be provided in the mixtures assessment. The dose-response data/metrics (e.g., PODs, dose-response curves) selected across component PFAS should be clearly presented. It should be noted that for NAM-based data (such as *in vitro* cell bioactivity in the hypothetical

[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 fraction of chemical eliminated from the body per unit of time, commonly expressed in units of hour(s) or day(s).

[14] The Vd represents the degree to which a chemical is distributed in body tissues. For example, chemicals highly bound to plasma proteins and not broadly distributed in tissues have a low Vd; conversely, chemicals with 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).

PFAS mixture example), the "POD" should be clearly described consistent with the datastream from which it was derived and expressed in terms of an HED. This facilitates comparisons to other PODs (i.e., human epidemiological or experimental animal-based) for other mixture components.

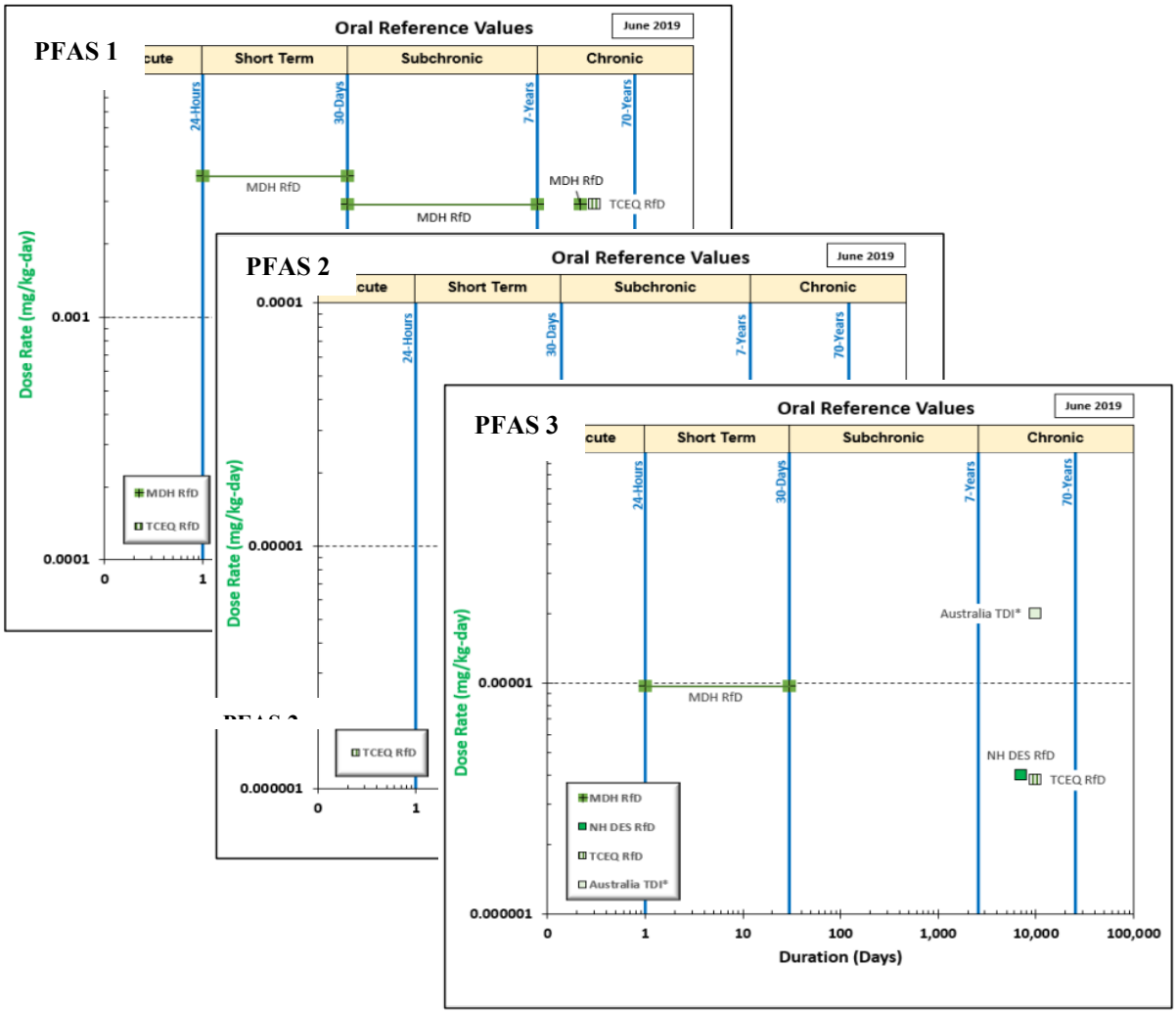
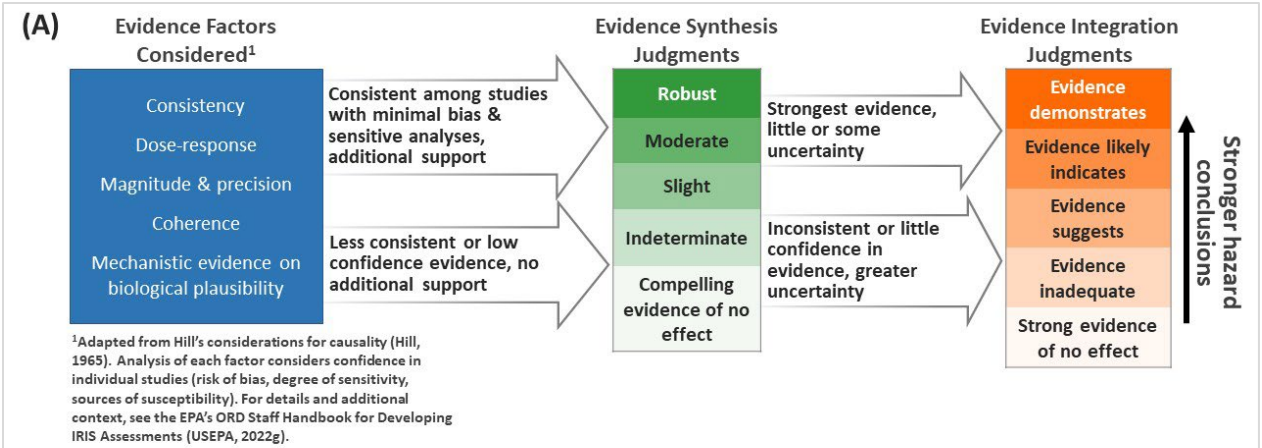



**Figure 4-7. Example exposure-response arrays for the hypothetical example PFAS 1–3 identified as having existing human health risk assessment values for one or more exposure durations.**

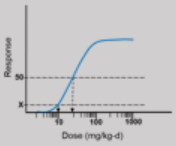
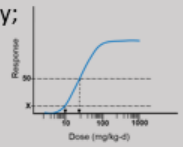
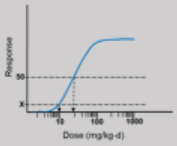
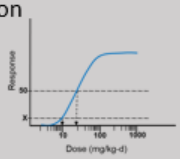



(B)

| Effect Domain | PFAS 1 | PFAS 2 | PFAS 3 | PFAS 4 | PFAS 5 |
|---|---|---|---|---|---|
| Liver | ++ | +++ | — | ++ | —* |
| Thyroid | ++ | ++ | +++ | — | — |
| Developmental | +++ | ++ | ++ | +++ | — |

**Figure 4-8. Evidence synthesis and integration across three target health effects domains for a mixture of five hypothetical PFAS. (A) Figure depicting evidence synthesis and integration considerations and judgments, based on USEPA (2022g). (B) The heat map indicates the strength of evidence supporting an effect of each hypothetical PFAS in each of three target health effects domains. (+++) evidence likely indicates an effect; (++) evidence suggests an effect; (—) evidence is inadequate to determine an effect. *Although PFAS 5 has no applicable human epidemiological or traditional experimental animal assay data available, *in vitro* cell bioactivity data are available from assays performed predominately in hepatocyte cell lines.**

**Table 4-3. Example 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<br>$POD_{HED}$: 0.00001<br>$UF_C$: 300<br>Critical effect: Delayed growth and development in offspring (ABC et al. 2022) | S-D rat; single generation repro/dev study;<br>Daily gavage GD 1-20<br>(ABC et al. 2022)  | – |
| **PFAS 2** | RfD: 1 E-5<br>$POD_{HED}$: 0.0013<br>$UF_C$: 100<br>Critical effect: Liver necrosis (DEF et al. 2022) | S-D rat; 2-year bioassay;<br>DW ad libitum<br>(DEF et al. 2022)  | Bioactivity profile in ToxCast; Biological perturbation at AED (2.8 E-4 mg/kg-day) = ↓ epoxide hydrolase in HepaRG cells; ↑ oxidative stress |
| **PFAS 3** | RfD: 7 E-4<br>$POD_{HED}$: 0.21<br>$UF_C$: 300<br>Critical effect: Decreased thyroid hormones (T4/T3) (GHI et al. 2022) | C57BL6 mouse;<br>90-day gavage;<br>(GHI et al. 2022)  | – |
| **PFAS 4** | – | F344 Rat; two generation repro/dev study;<br>Multiple dev outcomes in offspring;<br>Feed ad libitum<br>(JKL et al. 2022)  | – |
| **PFAS 5** | – | – | Bioactivity profile in ToxCast; Biological perturbation at AED (8 E-5 mg/kg-day) = ↓ epoxide hydrolase in HepaRG cells; ↑ oxidative 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 a 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 hypothetical 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.

# 5.0 Hazard Index Approach

## 5.1 Background on the HI Approach

The HI is the EPA's most commonly used component-based mixture risk assessment method. Because the HI employs a population-level human exposure and human health assessment value, such as an oral RfD, this ratio indicates potential health risk(s). The HI is based on an assumption of DA among the mixture components (USEPA, 2000b; Svendsgaard and Hertzberg, 1994). In the HI approach, an HQ is calculated as the ratio of human exposure I to a health-based RfV for each mixture component chemical (USEPA, 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), 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 such as an EPA lifetime drinking water Health Advisory (e.g., USEPA, 2022a, 2022b) 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 an 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 an 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 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 an HED ($POD_{HED}$) 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 (i.e., 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 general HI, the RfV for each mixture component chemical is used in the calculation of an 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 the most sensitive health effects are often used as the basis for each respective chemical RfV and

corresponding HQ. Conversely, the TOSHI entails derivation of HQs for each mixture component chemical based on a toxicity value for the "same" effect, which may or may not be the most sensitive or potent effect across the landscape of identified hazards. For example, in the case of a liver-specific HI, for some mixture components, 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.

In some cases, the liver may not be identified as a hazard for a given component chemical; for example, the available toxicity data may be insufficient or lacking to support the derivation of a toxicity value. To use this TOSHI approach more fully, organ-specific reference values (osRfVs) or target-organ toxicity doses (TTDs) are needed (note: these are the same type of noncancer values, just with different naming conventions) for each mixture component of potential concern. Under the TOSHI approach, for chemicals lacking hazard and dose-response data from traditional or NAM-based data streams for the selected health effect, it may not be possible to determine their potential contribution to joint toxicity of the mixture, which might result in an underestimation of the overall mixture risk.

An HI greater than one (1) is generally regarded as an indicator of potential adverse health risks associated with exposure to the mixture. An HI less than or equal to 1 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 (USEPA, 1986, 1991, 2000b). However, in some circumstances, the user may want to consider an HI less than 1, 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 15,000 environmentally relevant PFAS (e.g., see the summary of the EPA and ATSDR PFAS assessments in Table 5-1). The EPA's primary source of peer-reviewed human health 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], the EPA Office of Water toxicity assessments[18], TSCA risk evaluations[19], and ATSDR's ToxProfiles[20], undergo rigorous peer and public review processes (note: PPRTV assessments do not include public review); as a result, they are considered to be of high scientific quality. The chronic RfDs for PFOA (USEPA, 2024a), PFOS (USEPA, 2024b), PFHxA (USEPA, 2023c), PFPrA (USEPA, 2023d), HQ-115 (USEPA, 2023e), PFBA (USEPA, 2022e), PFBS (USEPA, 2021a), and HFPO-DA (USEPA, 2021b) represent the only final EPA toxicity values for PFAS available at the time of drafting of this document. Several more PFAS assessments are under development in the EPA/ORD (e.g., PFHxS, PFNA, and PFDA; see Table 5-1 below) that can be considered in the future. Also, the use of this approach could consider other PFAS toxicity values (e.g., ATSDR MRLs).

[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

**Table 5-1. EPA and ATSDR peer-reviewed human health assessments containing noncancer toxicity values (RfDs or MRLs) for PFAS that are final or under development.**

| Chemical | EPA chronic oral RfD | ATSDR intermediate oral MRL[a] |
|---|---|---|
| PFOA | **Final 2024 RfD = $3 \times 10^{-8}$ mg/kg/day** (USEPA, 2024a) | **Final 2021 MRL = $3 \times 10^{-6}$ mg/kg/day** (ATSDR, 2021) |
| PFOS | **Final 2024 RfD = $1 \times 10^{-7}$ mg/kg/day** (USEPA, 2024b) | **Final 2021 MRL = $2 \times 10^{-6}$ mg/kg/day** (ATSDR, 2021) |
| PFNA | Under development in the EPA IRIS program | **Final 2021 MRL = $3 \times 10^{-6}$ mg/kg/day** (ATSDR, 2021) |
| PFDA | ***Draft*** **2023 RfD = $4 \times 10^{-10}$ mg/kg/day** (USEPA, 2023f) | N/A |
| PFBA | **Final 2022 RfD = $1 \times 10^{-3}$ mg/kg/day** (USEPA, 2022e) | N/A |
| PFBS | **Final 2021 RfD = $3 \times 10^{-4}$ mg/kg/day** (USEPA, 2021a) | N/A |
| PFHxA | **Final 2023 RfD = $5 \times 10^{-4}$ mg/kg/day** (USEPA, 2023c) | N/A |
| PFHxS | ***Draft*** **2023 RfD = $4 \times 10^{-10}$ mg/kg/day** (USEPA, 2023g) | **Final 2021 MRL = $2 \times 10^{-5}$ mg/kg/day** (ATSDR, 2021) |
| HFPO-DA | **Final 2021 RfD = $3 \times 10^{-6}$ mg/kg/day** (USEPA, 2021b) | N/A |
| PFPrA | **Final 2023 RfD = $5 \times 10^{-4}$ mg/kg/day** (USEPA, 2023d) | N/A |
| HQ-115[b] | **Final 2023 RfD = $3 \times 10^{-4}$ mg/kg/day** (USEPA, 2023e) | 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; the EPA and ATSDR may apply different uncertainty/modifying factors) and are developed for different purposes.

[b] HQ-115 is the trade name for lithium bis[(trifluoromethyl)sulfonyl]azanide (CASRN 90076-65-6).

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 the EPA or ATSDR assessments.

There may be scenarios where a final peer-reviewed toxicity assessment for one or more component chemicals in a mixture is not available. In these cases, evaluating available hazard and dose-response information for PFAS in the mixture may be necessary under an 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 need to develop a targeted, fit-for-purpose assessment, if possible (i.e., based on the 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 an underestimation of the potential health risk(s) 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. The EPA has published several peer-reviewed 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 the 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 (e.g., transcriptomics; macromolecular/cellular bioactivity), *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 experimental animal bioassay and human epidemiological information, NAMs could potentially play a pivotal and transformational role in human health 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., quantitative structure-activity models) might inform the 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 an estimated human *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 an 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. Alternatively, particularly for RPF application, NAM-based dose-response data expressed in HEDs could be leveraged to calculate/model BMD values (e.g., $BMD_{X\text{-}AED}$) for expertly selected bioactivity (e.g., same/similar transcriptional pathway(s) and/or cellular bioactivity) to compare to that of a more data-informed member of the mixture with a similar

bioactivity profile (i.e., mixture IC). This might facilitate the derivation of RPFs for data-poor mixture components.

A critical consideration in using NAM-based hazard and concentration/dose-response data is recognizing that for some platforms or bioassays, perturbations of underlying biological pathways may not be readily identifiable as being directly related to specific apical toxic effects or even the hazard domain of interest. 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 a health 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 quantitative approximation for dose-response (e.g., POD) associated with traditional apical effects that are protective for the majority of chemicals evaluated (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 if the accuracy of the predicted PODs can demonstrated (e.g., HI, RPF, M-BMD).

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 the 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 contextualize the applicability of results appropriately 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 (see 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 (see 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 noncancer health RfVs 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 chemical health effects-based values (e.g., 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 (see Table 4-3). However, the critical effect on which each corresponding RfD was derived is 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, applying the general HI is optimal in this scenario and will entail using the overall RfD (or ATSDR MRL), regardless of the underlying critical health effect. If a subchronic RfD or an MRL is only available for an intermediate duration (akin to subchronic duration for EPA purposes), the user may consider the available evidence base. 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 an RfV are available, but traditional hazard and dose-response (e.g., traditional experimental animal study) data were judged adequate to support derivation. Systematic review and evaluation of the animal study data led to the identification of a single best study (e.g., hypothetical 2-Gen repro/dev rat study; see Table 4-3) and multiple developmental health outcomes as candidate critical effects such as delayed growth and development at postnatal day 1 (PND 1) and decreased neonatal viability and thyroid hormone levels at PND 4. Thus, the user may choose to calculate an RfV using appropriate dose-response metrics (i.e., $POD_{HED}$) and the 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 rat offspring provided the most robust endpoint and confidence in dose-response for PFAS 4. Following BMD modeling (as per the EPA BMD guidelines [USEPA, 2012]), a lower statistical bound on a BMD (BMDL) for developmental effects of 1.06 mg/kg-day was calculated and used as the POD.

As the candidate POD for RfD derivation is identified from rats, available TK data for PFAS 4 in rats and humans should be considered for a data-informed adjustment approach for cross-species extrapolation (i.e., estimating the dosimetric adjustment factor [DAF]; Equation 5-2). In *Recommended Use of Body Weight$^{3/4}$ as the Default Method in Derivation of the Oral Reference Dose* (USEPA, 2011b), the 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, the EPA endorses $BW^{3/4}$ as a default to extrapolate toxicologically equivalent doses of orally administered agents from laboratory animals to humans to derive an RfD under certain exposure conditions. In this illustrative hypothetical mixture example, it was determined that: (1) clearance values for

experimental animals and humans were available and included in the dosimetric adjustment of PODs used in the derivation of noncancer 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 PND 1 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.

Having made this assumption, 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 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 hypothetical DAF to the rat POD results in a $POD_{HED}$ of 0.0011 mg/kg-day. This $POD_{HED}$ was then divided by a hypothetical composite UF of 100, resulting in an 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 the development of a POD and, potentially, derivation of a NAM-based RfV using the application of UFs consistent with the data scenario (Judson et al., 2011; Parish et al., 2020). It is recommended that NAM data be systematically evaluated for suitability in supporting the derivation of RfVs using accepted approaches and practice. Unfortunately, no formal EPA technical guidelines currently exist to guide the approach for the use of NAM-based PODs in quantitative human health risk assessment applications. However, for the purposes of demonstrating the 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.

**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 |

*Note:*
* The value of 1.0 was used for volume of distribution (Vd) strictly for the purpose of calculating an estimated clearance value; the Vd of 1.0 is not based on empirical evidence for PFAS 4.

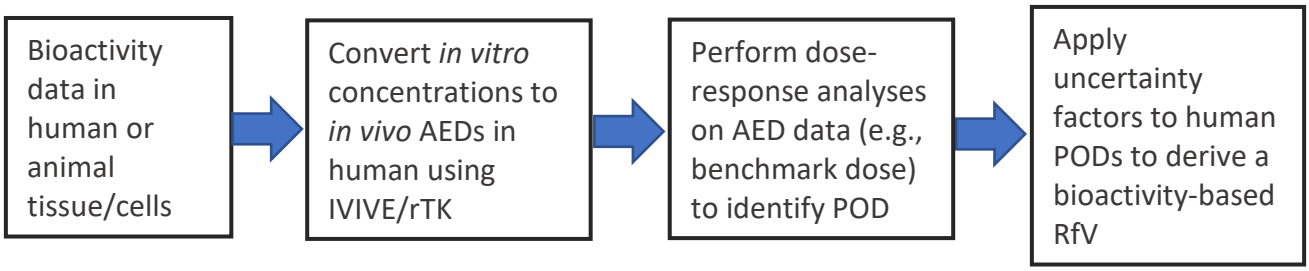



**Figure 5-1. General steps to derive bioactivity-based RfV using bioactivity data in human or animal tissue/cells.**

The detailed steps and mechanics of the bioactivity > IVIVE/rTK > AED process outlined in Figure 5-1 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. In this hypothetical example, 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; in practice, there are no *a priori* data-driven or default BMRs suggested for NAM data. BMR identification will be assay/platform/data-specific and should be contextualized by expert-driven analysis of the available data. As such, due to this flexibility in NAM-based data application, in the hypothetical PFAS mixture example, the $BMD_{X\text{-}AED}$ = 0.004 mg/kg-day. This human equivalent POD was then divided by a hypothetical composite UF of 100. 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 the NAM data leveraged in POD identification and corresponding 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 for data-poor PFAS.

### Summary of RfDs

In summary, as shown in Table 5-3, RfDs for PFAS 1–5 range from $10^{-4}$ 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 different 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) | Develop-mental $POD_{HED}$ (mg/kg-day) | RfD (mg/kg-day) | Basis |
|---|---|---|---|---|---|
| **PFAS 1** | 0.044 ($BMDL_{X\text{-}HED}$) | 0.24 ($BMDL_{Y\text{-}HED}$) | **0.00001 ($BMDL_{Z\text{-}HED}$)** | 3 E−8 | formal toxicity assessment |
| **PFAS 2** | **0.0013 ($BMDL_{X\text{-}HED}$)** | 0.23 ($BMDL_{Y\text{-}HED}$) | 0.0051 ($BMDL_{Z\text{-}HED}$) | 1 E−5 | formal toxicity assessment |
| **PFAS 3** | N/A | **0.21 ($BMDL_{Y\text{-}HED}$)** | 2.1 ($BMDL_{Z\text{-}HED}$) | 7 E−4 | formal toxicity assessment |
| **PFAS 4** | 50 ($BMDL_{X\text{-}HED}$) | N/A | **0.0011 ($BMDL_{Z\text{-}HED}$)** | 1 E−5 | high quality *in vivo* data |
| **PFAS 5** | **0.004 ($BMD_{X\text{-}AED}$)**[a] | N/A | N/A | 4 E−5 | bioactivity-based |

*Note:*
Bold values indicate the lowest (most-sensitive) POD for the corresponding RfD derivation.
[a] Represents the NAM-based POD for *in vitro* cell bioactivity (e.g., for PFAS 5 = ↓ epoxide hydrolase activity).

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

Depending on the problem formulation, the user can either use the oral RfVs calculated for mixture components or 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 an 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 for sensitive populations and life stages, and is typically based on data from chronic experimental animal toxicity and/or human epidemiological studies. The calculation of an HBWC includes an oral RfV such as an 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 exposure attributed to drinking water sources (USEPA, 2000a), with the remainder 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, 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 (USEPA, 2019a) to derive the HBWC. In practice, when multiple populations or life stages are identified based on the critical study design and critical effect or other health effects data (from animal or human studies), the 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., nondevelopmental critical effect as for PFAS 2 and 3 or NAM-based RfD as for PFAS 5), the DWI-BW corresponding to all ages of the general population may be selected (Table 5-4).

Table 5-4 shows the 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.

To account for potential aggregate risk from exposures and exposure pathways other than oral ingestion of drinking water, the 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 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 (USEPA, 2000a). The purpose of the RSC is to ensure that the level of a contaminant (e.g., HBWC), when combined with other identified potential sources of exposure for the population of concern, will not result in exposures that exceed the RfD (USEPA, 2000a).

**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 | 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 (USEPA, 2019a) |
| Infants | 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 (USEPA, 2019a) |
| Children | 0.0343 | 90th percentile direct and indirect consumption of community water, consumer-only two-day average, birth to < 21 years. | 2019 Exposure Factors Handbook Chapter 3, Table 3-21, NHANES 2005–2010 (USEPA, 2019a)[a] |
| 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 (USEPA, 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 (USEPA, 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[b] (USEPA, 2019a) |

*Notes:*
CSFII = continuing survey of food intake by individuals; L/kg bw-day = liter per kilogram body weight per day.
[a] DWI-BWs are based on NHANES 2005−2010 data, also reported in the Exposure Factors Handbook. DWI-BWs for this population or life stage were calculated using the EPA's Food Commodity Intake Database, Commodity Consumption Calculator (https://fcid.foodrisk.org/percentiles).
[b] Estimates are less statistically reliable based on guidance published in NCHS (1993).

To determine the RSC, the EPA follows the Exposure Decision Tree for Defining Proposed RfD (or POD/UF) Apportionment in the EPA's *Methodology for Deriving Ambient Water Quality Criteria for the Protection of Human Health* (USEPA, 2000a). The 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 the EPA's methods, in the absence of adequate data to quantitatively characterize exposure to a contaminant, the 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 (USEPA, 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 is presented in Table 5-5.

### *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 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 (see Table 4-1) and the calculated HBWCs for PFAS 1–5 (see Table 5-5), individual component HQs are derived as shown in Table 5-6. Component HQs are expressed to two decimal places (hundredths place) and then summed across the PFAS mixture to yield the HI. The HI is rounded to one (1) significant digit.

**Table 5-5. Calculation of hypothetical HBWCs for example 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 | 40 |
| **PFAS 5** | 4 E−5 | 0.0338 | 0.2 | 200 |

**Table 5-6. Calculation of individual component HQs for the hypothetical PFAS mixture.**

| | Hypothetical drinking water exposure estimate (ng/L) | Hypothetical HBWC (ng/L) | Hypothetical general HQ |
|---|---|---|---|
| **PFAS 1** | 4.8 | 0.2 | 24.00 |
| **PFAS 2** | 55 | 60 | 0.92 |
| **PFAS 3** | 172 | 4,000 | 0.04 |
| **PFAS 4** | 58 | 40 | 1.45 |
| **PFAS 5** | 120 | 200 | 0.60 |
| **Mixture general HI** | | | **27.01 (rounded to 30)** |

*Note:*
HQ is the DW exposure estimate / HBWC; HI is the sum of individual HQs.

### *5.2.5 General HI Step 5: Interpret the PFAS mixture HI*

The HI (30) in the hypothetical example is significantly greater than 1, indicating potential health risks resulting from exposure 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; PFAS 2 and 5 also appear to be contributors with HQs of 0.92 and 0.60, respectively. Assessment of PFAS 2 and 5 in isolation (individually) would indicate no/low health risk (i.e., individual HQs < 1.00), but the assessment of the binary mixture of PFAS 2 and 5 would indicate appreciable risk (HI = 1.52, rounded to 2). Conversely, with an HQ of 0.04, PFAS 3 is less influential than 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 example PFAS mixture, the hypothetical HBWC for PFAS 1 (0.2 ng/L) is lower than its corresponding hypothetical drinking water analytical quantitation limit of 3 ng/L (see Table 4-2) 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 for the whole mixture.

## 5.3 Illustrative Example Application of the Target-Organ-Specific Hazard Index 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 the use of human health/toxicity values across mixture components is effect/endpoint-specific. For some PFAS, this might be the overall RfD or MRL; for other PFAS, this may involve TTDs (i.e., an RfD for a specific health effect that may differ from the overall RfD for a given component chemical). In the TOSHI approach, 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 ATSDR MRL) has been derived, TTDs could potentially be derived *de novo* 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 a 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 the availability of data and confidence in the evidence conclusions.

### *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). Each DWI-BW is the 90th percentile direct and indirect consumption of community water, consumer-only two-day average and was selected based on sensitive populations or life stages as identified by evaluating of each of the critical studies, including the exposure intervals. For this hypothetical example, the DWI-BW for PFAS 1, 2, and 3 is for women of childbearing age (13 to < 50 years), and the DWI-BW for PFAS 4 is for lactating women (see Table 5-4).

**Table 5-7. Hypothetical TTDs for the hypothetical component 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.

**Table 5-8. Calculation of hypothetical 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 | 40 |
| **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 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 (see Table 4-1) and the calculated HBWCs for PFAS 1–4 derived from TTDs for the developmental effect domain (see Table 5-8), individual component HQs are derived as shown in Table 5-9.

**Table 5-9. Calculation of hypothetical individual component HQs specifically for developmental effects associated with the hypothetical PFAS mixture.**

| Chemical | Hypothetical drinking water exposure estimate (ng/L) | Hypothetical $TOSHI_{DEV}$ HBWC (ng/L) | Hypothetical $TOSHI_{DEV}$ HQ |
|---|---|---|---|
| **PFAS 1** | 4.8 | 0.2 | 24.00 |
| **PFAS 2** | 55 | 50,000 | 0.0011 |
| **PFAS 3** | 172 | 10,000 | 0.02 |
| **PFAS 4** | 58 | 40 | 1.45 |
| **PFAS 5** | 120 | ND | ND |
| **Mixture $TOSHI_{DEV}$** | | | **25.47 (rounded to 30)** |

*Notes:*
HQ is the DW exposure estimate / HBWC; HI is the sum of individual HQs; ND = not determined.
The HBWCs in this TOSHI application are derived from TTDs for the developmental effect domain.

### 5.3.5 TOSHI Step 5: Interpret the PFAS mixture HI

The $TOSHI_{DEV}$ of 30 in the hypothetical example indicates concern for developmental effects associated with exposure to the hypothetical PFAS mixture at the measured drinking water concentrations (see 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.

## 5.4 Advantages and Challenges of the General HI and TOSHI Approaches

The general 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 the 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 for which the HQs have greater contribution to an HI > 1, relative to other PFAS mixture components).

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 general HI approach can be used where the individual HQ calculated for each mixture component PFAS is based on the most well-characterized, and often the most sensitive, toxic effect and corresponding noncancer RfV (e.g., oral RfD). As such, a general HI will typically represent the most health-protective indicator of mixture risk, as each component HQ is based on each mixture component's overall RfV.

Alternatively, 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). Although more closely aligned to the concept of DA, 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. Another limitation is that so many PFAS lack human epidemiological or experimental animal hazard and dose-response information across a broad(er) health effect range, thus limiting the potential scope or landscape of derived TTD values. As with the general HI, a TOSHI approach might benefit from consideration of NAM data and approaches that can inform organ/tissue-specific dose-response.

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 routes/scenarios (e.g., oral versus inhalation; comparing drinking water His to soil ingestion His) can be misleading and challenging to interpret. 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 health endpoints, and similar life stage), the two exposure scenarios could be judged to have the same potential for causing toxic effects. That interpretation has the strongest scientific foundation when there are only minor differences in the

component exposures (thus, same exposure route, same chemicals, and similar exposure duration for specific receptors) between the two scenarios. In addition, 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. The practical interpretation is that both mixture His would indicate an appreciable risk of health effects in exposed populations.

Another challenge of the application of the general HI and TOSHI approaches 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, TTD). 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 possible PFAS values that might exist or could be derived. 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 and associated with lower levels of qualitative and quantitative uncertainty than other values.

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 NAMs, dose-response metrics obtained from bioactivity-based assays/platforms (or read-across) may be assigned some level of *a priori* uncertainty simply because of a lack of confidence by end-users in the 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 or platform 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 (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 NAMs when and where possible while 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 POD identification, RfV derivation, and subsequent HQ and HI calculations. The disadvantage of 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.

# 6.0 Relative Potency Factor Approach

## 6.1 Background on RPF Approach

RPF approaches comprise another basic dose-addition method used most commonly by the 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 such as polycyclic aromatic hydrocarbons 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 (USEPA, 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, the 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 the 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 (USEPA, 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, nonhuman 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 calculating RPFs across component PFAS, BMDs (e.g., the central tendency estimate) would be optimal. BMDs incorporate the totality of a given dose-response and facilitate the identification of a dose at a predefined BMR level (e.g., 0.5 standard deviation (SD) or 1 SD over control; 10% change in some effect/endpoint). BMD modeling would optimize the comparison of "same" as a function of dose across component PFAS for a given health effect or endpoint. It is recognized that dose-response data for chemicals are sometimes not amenable to BMD modeling. Isoeffective 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:

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

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, the 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) (USEPA, 2000b) and (2) using effect-specific HBWCs for the IC 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 the magnitude of health effect concern and identification of potential component chemical drivers of an ICEC.

The EPA's supplementary guidelines (USEPA, 2000b) state: "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 the 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, the 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 (USEPA, 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, 2023), due to the 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 approach is taken in the illustrative RPF examples below; it is consistent with previous NAS recommendations for evaluating 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 hypothetical example focuses on the 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 (ATSDR, 2021; EFSA et al., 2020; Section 7.1 in ITRC, 2022; USEPA, 2021a, 2021b). The approach here is to use a construct that allows for a combination of PFAS with a shared, common health outcome (e.g., delayed growth and development in offspring), as opposed to a stringent requirement of the same MOA, to calculate RPFs across one or more health effect domains. Including 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 the identification of those effects shared among the PFAS in the assessed mixture. 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 Index Chemicals, $POD_{HEDs}$)

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 an HBWC. This is necessary so that the ICEC for the mixture ($ICEC_{MIX}$) can be compared to the IC's corresponding HBWC to determine the 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 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.

The dose-response metrics for this RPF example are the same as those used above in the HI example (see Table 5-3) with the addition of a NAM-based POD for PFAS 2 (the IC for the liver effect domain). 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 hypothetical $POD_{HEDs}$ 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 ($BMDL_{X\text{-}HED}$) | 0.24 ($BMDL_{Y\text{-}HED}$) | **0.00001 ($BMDL_{Z\text{-}HED}$)** |
| **PFAS 2** | **0.0013 ($BMDL_{X\text{-}HED}$)**; 0.0052 ($BMD_{X\text{-}AED}$)[a] | 0.23 ($BMDL_{Y\text{-}HED}$) | 0.0051($BMDL_{Z\text{-}HED}$) |
| **PFAS 3** | N/A | **0.21 ($BMDL_{Y\text{-}HED}$)** | 2.1 ($BMDL_{Z\text{-}HED}$) |
| **PFAS 4** | 50 ($BMDL_{X\text{-}HED}$) | N/A | **0.0011 ($BMDL_{Z\text{-}HED}$)** |
| **PFAS 5** | **0.004 ($BMD_{X\text{-}AED}$)**[a] | N/A | N/A |

*Notes:*
Bold indicates lowest POD for the corresponding RfD derivation.
[a] Represents the NAM-based POD for same *in vitro* cell bioactivity event between PFAS 2 and PFAS 5 (e.g., ↓ epoxide hydrolase activity).

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

For this illustrative RPF example, the hypothetical HBWCs are the same as those used in the General HI example (see 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 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 only has bioactivity data; however, the molecular and cellular perturbations were observed primarily in hepatocyte cell cultures (e.g., HepaRG). As such, there is increased confidence in the 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 a significantly *increased incidence of hepatocellular death* was common across studies. In this hypothetical example, the identified common liver effect was the effect used as the basis for deriving an oral RfD and corresponding HBWC for PFAS 2 (e.g., this liver RfD was interpreted with the highest confidence across PFAS 1, 2, and 4). 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*.

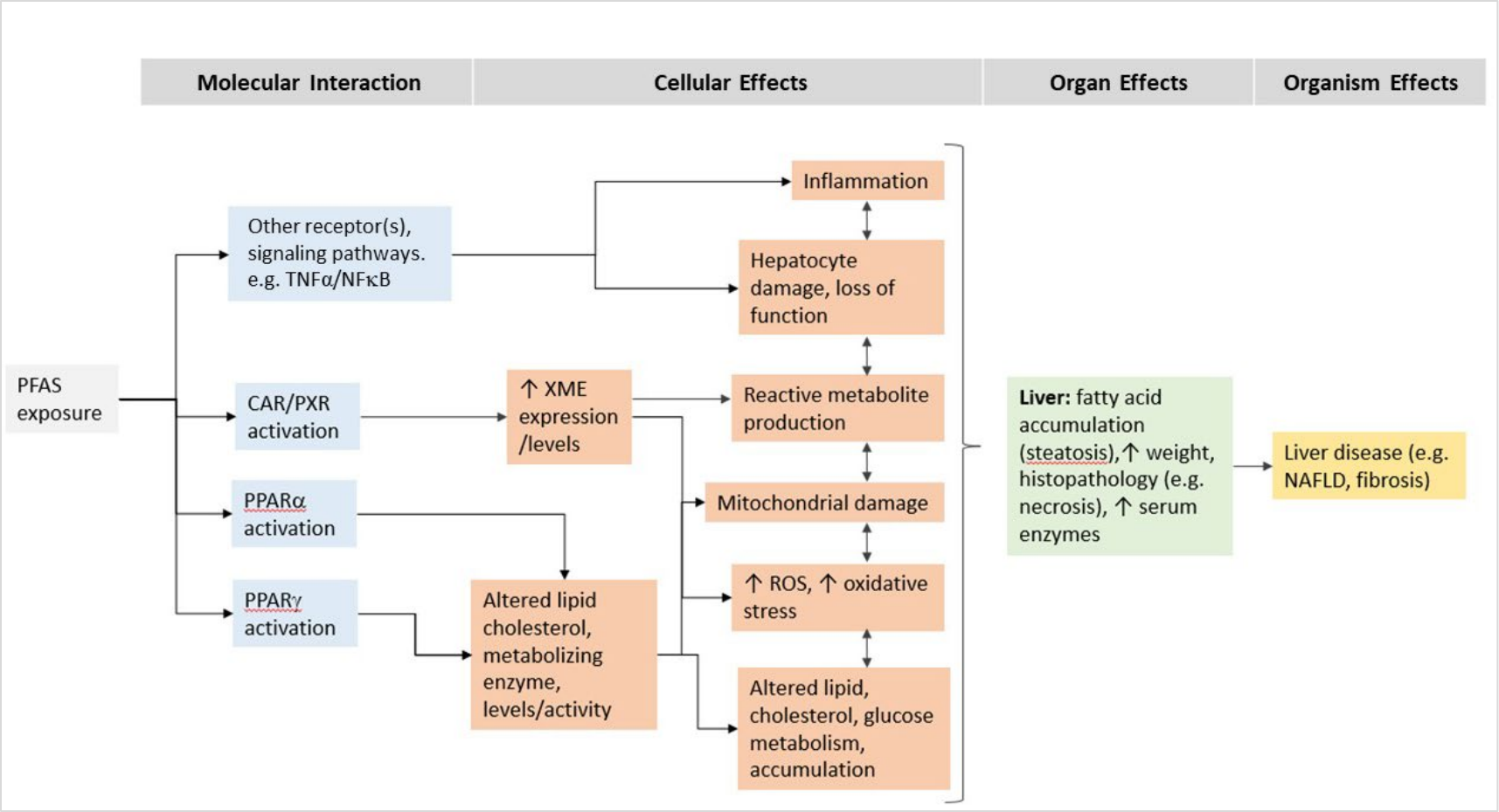



**Figure 6-1. General cell signaling pathways associated with PFAS-induced liver injury. Figure sourced from Appendix A of the EPA's 2021 Systematic Review Protocol for the PFBA, PFHxA, PFHxS, PFNA, and PFDA IRIS Assessments (USEPA, 2021f).**

**Table 6-2. Example liver effect RPFs and ICECs for a hypothetical mixture of five PFAS.**

| Mixture component | Hypothetical $POD_{HED}$ (mg/kg-day); *Increased incidence of hepatocellular death* | Hypothetical example RPF | Hypothetical exposure estimate (ng/L) | Hypothetical PFAS 2 ICEC (ng/L) |
|---|---|---|---|---|
| PFAS 1 | 0.044 ($BMDL_{X\text{-}HED}$) | 0.03 | 4.8 | 0.14 |
| PFAS 2 (IC) | 0.0013 ($BMDL_{X\text{-}HED}$); 0.0052 ($BMD_{X\text{-}AED}$)[a] | 1 | 55 | 55 |
| PFAS 3 | N/A | N/A | 172 | — |
| PFAS 4 | 50 ($BMDL_{X\text{-}HED}$) | 0.00003 | 58 | 0.0017 |
| PFAS 5 | 0.004 ($BMD_{X\text{-}AED}$)[a] | 1.3 ($RPF_{NAM}$)[b] | 120 | 39[c] |
| **Mixture total PFAS 2 ICEC (ppt)** | | | | **94** |

*Notes:*

[a] NAM-based BMD modeled from the AED-based dose-response for the selected bioactivity event (e.g., decreased epoxide hydrolase activity, denoted as "hydrolase" in the example plots). This selected event is based on identifying the lowest (i.e., most sensitive) *common* bioactivity between the cell assay profiles for the IC (PFAS 2) and PFAS 5.

[b] $RPF_{NAM}$ for PFAS 5 was calculated as the ratio of the $BMD_{X\text{-}AED}$ for PFAS 2 (IC) / $BMD_{X\text{-}AED}$ for PFAS 5 for the selected bioactivity event; in this example application, 0.0052 mg/kg-day / 0.004 mg/kg-day = 1.3.

[c] The ICEC for PFAS 5 was calculated by first deriving the $ICEC_{NAM}$ as follows: $RPF_{NAM}$ × Exposure estimate for PFAS 5 = 1.3 × 120 (ng/L) = 156 ng/L; the $ICEC_{NAM}$ was then converted to an ICEC by multiplying by the ratio of the $BMDL_{X\text{-}HED}$ for PFAS 2 / $BMD_{X\text{-}AED}$ for PFAS 2 = 156 ng/L × (0.0013 / 0.0052) = 39 ng/L.

For PFAS 5, the dose-response data used in this hypothetical example RPF application, obtained from IVIVE/rTK of the *in vitro* cell bioactivity data, is identified based on the lowest bioactivity event(s)[21] common with the IC; that is, noncancer bioactivity at the lower end of the distribution for the IC is the driver for identification of "same" effect for the data-poor mixture component PFAS. In a perfect scenario, the "same" bioactivity event(s) would be shared at the level of NAM-based PODs between the IC and one or more data-poor components. However, while two or more mixture components may share a qualitatively similar profile of biological perturbations, the relative quantitative potency or dose-response at which various bioactivity events occur may be diverse. For simplification of the hypothetical PFAS mixture application, the selected bioactivity for the IC (PFAS 2) and PFAS 5 in HepaRG cells *in vitro* was identified as the same event, decreased epoxide hydrolase activity, with a $BMD_{X\text{-}AED}$ of 0.0052 mg/kg-day and $BMD_{X\text{-}AED}$ of 0.004 mg/kg-day, respectively (see Table 6-1). Importantly, epoxide hydrolases are a key component in the metabolism and detoxification of xenobiotics, particularly structures with reactive epoxide moieties; decreased hydrolase activity has been associated with increased oxidative stress, cellular/tissue inflammation, and cell death.

This NAM-based approach aims 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 $BMD_{X\text{-}AED}$ for the

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

selected bioactivity event of the IC to the $BMD_{X\text{-}AED}$ for the same event associated with the data-poor mixture component chemical (see Table 6-2). 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 $BMD_{X\text{-}HED}$ for *increased incidence of hepatocellular death*) to the $BMD_{X\text{-}AED}$ 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; however, it is 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.

**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. Applying the same approach outlined for the liver, the selected common effect was identified and hypothetically best represented the thyroid RfD for PFAS 3. Thus, the hypothetical 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 total serum thyroxine (TT4) and free serum thyroxine (FT4). The calculation of the thyroid-specific RPFs and corresponding ICECs are presented in Table 6-3.

**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. Based on the approaches above, the common effect was identified and hypothetically best represented by the oral RfD from 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).

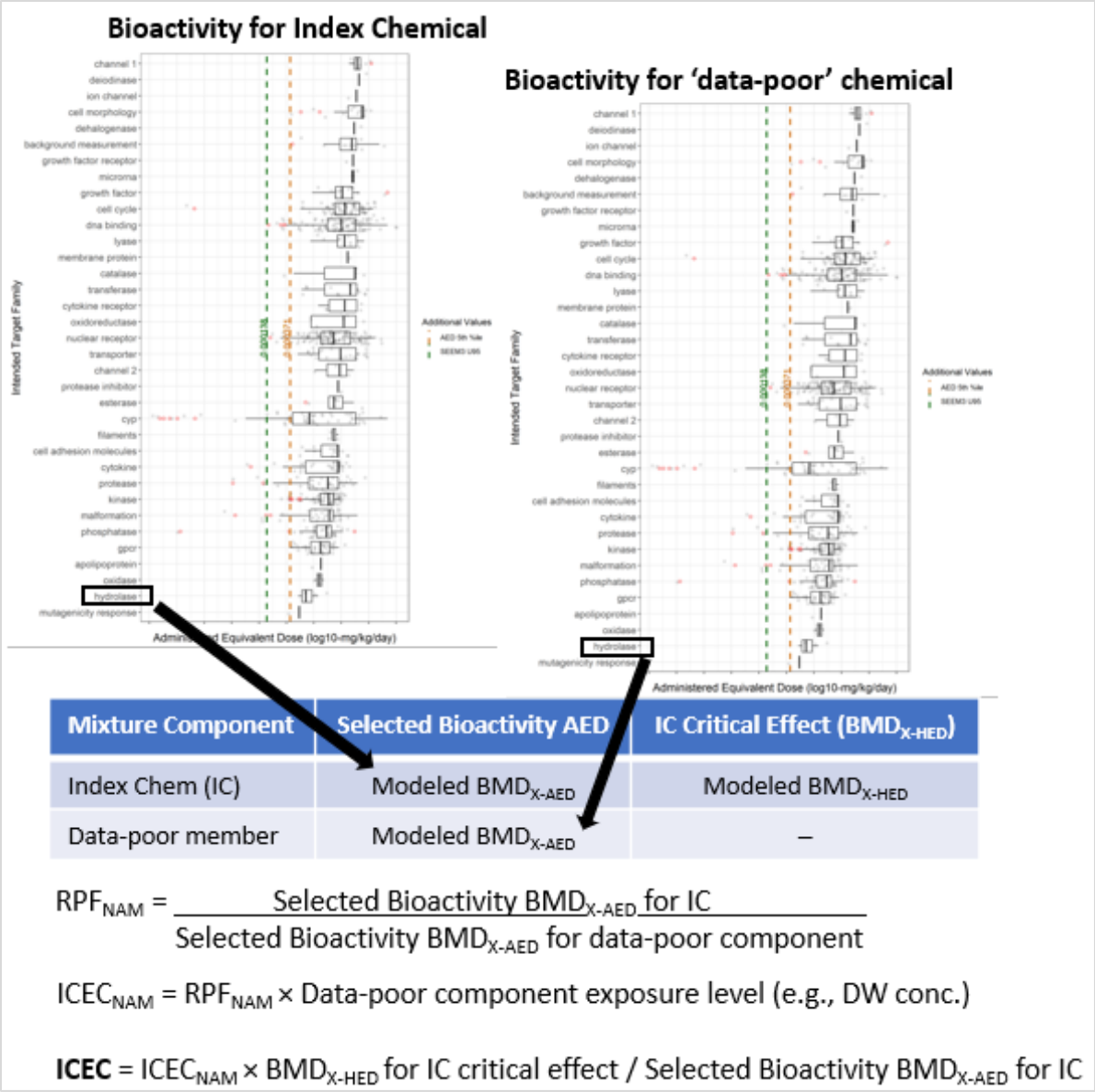



**Figure 6-2. Example hypothetical process for integrating NAM-based RPFs and ICECs into mixtures assessment. Black boxes = "same" bioactive event for RPF approach.**

**Table 6-3. Hypothetical example thyroid effect RPFs and ICECs for a hypothetical mixture of five PFAS.**

| Mixture component | Hypothetical $POD_{HED}$ (mg/kg-day); *Decreased TT4 and FT4* | Example RPF | Hypothetical exposure estimate (ng/L) | Hypothetical PFAS 3 ICEC (ng/L) |
|---|---|---|---|---|
| PFAS 1 | 0.24 ($BMDL_{Y\text{-}HED}$) | 0.9 | 4.8 | 4.3 |
| PFAS 2 | 0.23 ($BMDL_{Y\text{-}HED}$) | 0.9 | 55 | 50 |
| PFAS 3 (IC) | 0.21 ($BMDL_{Y\text{-}HED}$) | 1 | 172 | 172 |
| PFAS 4 | N/A | N/A | 58 | — |
| PFAS 5 | N/A | N/A | 120 | — |
| **Mixture total PFAS 3 ICEC (ppt)** | | | | **226 (230)** |

**Table 6-4. Hypothetical example developmental effect RPFs and ICECs for a hypothetical mixture of five PFAS.**

| Mixture component | Hypothetical $POD_{HED}$ (mg/kg-day); *Decreased Body Weight in Offspring* | Hypothetical example RPF | Hypothetical exposure estimate (ng/L) | Hypothetical PFAS 1 ICEC (ng/L) |
|---|---|---|---|---|
| PFAS 1 (IC) | 0.000010 ($BMDL_{Z\text{-}HED}$) | 1 | 4.8 | 4.8 |
| PFAS 2 | 0.0051 ($BMDL_{Z\text{-}HED}$) | 0.002 | 55 | 0.11 |
| PFAS 3 | 2.1 ($BMDL_{Z\text{-}HED}$) | 5 E−6 | 172 | 0.00086 |
| PFAS 4 | 0.0011 ($BMDL_{Z\text{-}HED}$) | 0.009 | 58 | 0.52 |
| PFAS 5 | N/A | N/A | 120 | — |
| **Mixture total PFAS 1 ICEC (ppt)** | | | | **5.4** |

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

In the liver-specific RPF application (see 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 94 ppt exceeds the PFAS 2 HBWC of 60 ppt, indicating the 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 (see 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 (e.g., for this example, decreased TT4 and FT4). In this hypothetical example, the PFAS 3 $ICEC_{MIX}$ of 226 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 (see 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. In this hypothetical example, the PFAS 1 $ICEC_{MIX}$ of 5.4 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 the differences in TK and TD, PFAS may exhibit complex gradations of potency for different effects, which 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, the 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 depending 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, limitations for PFAS are the availability of human health assessment grade toxicity data, and as with many environmental mixture chemicals, dissimilarity in dose-response shapes and slopes; 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 unnecessary 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 the same effect/endpoint using the same dose metrics from the same study design/durations, calculation of RPFs across PFAS may, in practical application, entail or necessitate the 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. When sufficiently supported through evaluation of the available component-specific studies, such adjustments can provide 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 of this is being transparent about 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 commonly presents a problem as it pertains to the practical application of RPF methodology in that a vast majority of environmental chemicals, including PFAS, have limited-to-no MOA data available. The EPA mixtures guidelines provide flexibility in using 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 a greater probability of identifying effect/endpoint and associated dose-response data (e.g., effect-specific PODs) for mixture components than for MOA-type data. However, as the data for PFAS evolve, the toxicity profiles, including the 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 the calculation of an actual mixture toxicity dose or concentration estimate, as opposed to the HI, which is considered an indicator of potential health risk/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 an HBWC for an IC).

A clear challenge, not uniquely associated with the RPF approach, is the use of potentially disparate hazard and dose-response data (both in terms of type and confidence) 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 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 LOAELs are acceptable but ideally evaluated at isoeffective dose [$ED_X$], which may not be practicable), 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 hypothetical RPF examples shown above, the risk is indicated based on the liver and developmental RPFs but not for the thyroid effect domain. To use the RPF approach effectively, 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 may present an opportunity to advance the science of mixtures risk assessment is the use of NAM data. The constantly evolving information coming from alternative toxicity testing assays and platforms is important to human health assessment of environmental chemicals in general (not just for mixtures applications); however, there are inherent challenges associated with the 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 the selection of "effect" data, a key requirement is that the "effect" on which RPFs are based be the same. For example, one component 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. Using an IC in the RPF approach assumes component chemicals have similarly shaped dose-response slopes for the 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 similarly shaped 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 the EPA's supplementary guidelines (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 response-equivalent single point estimate (e.g., BMDx) or derive a full dose-response curve for the PFAS mixture of interest (i.e., by using multiple BMDx response levels for each compound in the mixture).

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 similarly shaped dose-response curves for all component chemicals for the given effect. When the response curves are dissimilar in shape, 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.

The following discussion compares the predictions of two DA models. One assumes that the individual chemicals in the mixture have similarly shaped 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 in a laboratory study to predict the full dose-response curve of a mixture using the $ED_{50}$s and slopes of each component chemical in the mixture (Gray et al., 2022). The first model, similar to the RPF method (discussed above), assumes that the chemicals in the mixture have similar dose-response 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 similarly shaped dose-response curves for the chemicals in the mixture, may 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 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 $i$th 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 similar shape among slopes is violated (NRC, 2008). The following example compares the accuracy of a DA model that assumes similar dose-response shapes (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, four-parameter logistic regression (Chemical A slope = 40, Chemical B slope = 5 using linear Y axis and log10 X axis). The mixture effect described in Figure 7-1 is the percent reduction in reproductive organ weight, ranging from 0% reduction in the control (0 dose of the mixture) to complete agenesis (100% reduced).

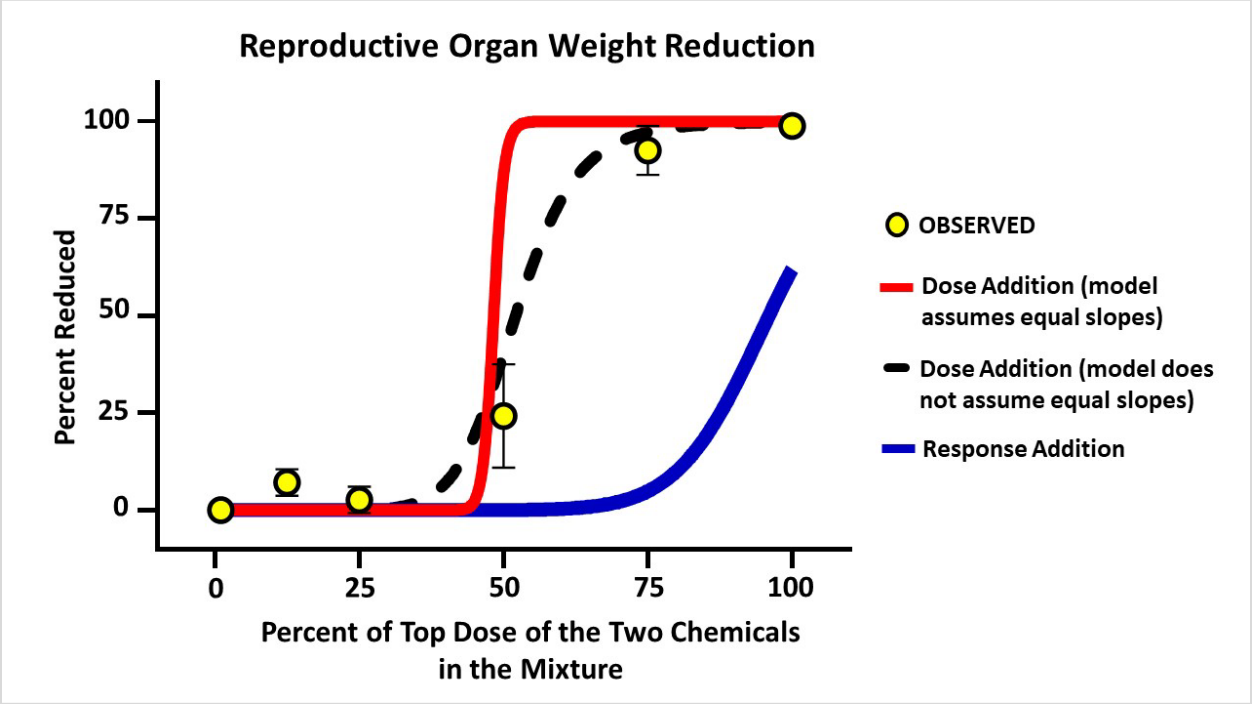



**Figure 7-1. Example comparison of observed data with model predictions using two dose addition-based mixture models and an RA model for a binary mixture study (adapted from Gray et al., 2022). The two chemicals displayed individual dose-response curves with widely disparate slopes for the endpoint (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-similarly shaped dose-response curve slopes (black dashed line). For these chemicals with disparate slopes, the dose addition model that does not assume similar slopes provided a better fit of the observed data (see Table 7-1).**

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 on 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 similar, then it is preferable to model the data with the M-BMD equation (Equation 7-2) that does not assume similarly shaped curves, 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 common 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, nonhuman primates, and humans, it is strongly recommended to convert experimental animal dose-response data to human equivalents (i.e., HEDs). Lastly, of the options for dose-response metrics to use across component PFAS, risk assessment-based PODs (e.g., $BMDL_{xHED}$) would be optimal. BMDs incorporate the totality of a given dose-response and facilitate the identification of a dose at a predefined BMR level (e.g., 0.5 SD or 1 SD over control; 10% change in the common effect/endpoint). BMD modeling would optimize the comparison of "same" as a function of dose across component PFAS for a given health effect or endpoint and identify a human health-relevant POD for M-BMD derivation. It is recognized that dose-response data for chemicals is sometimes not amenable to BMD modeling.

Importantly, the response level (i.e., BMR) for the common endpoint should be the same for all PFAS included in the calculation, for example, $BMDL_x$ for a common liver effect. In this case, the equation will produce an equivalent response metric (i.e., $BMDL_x$) for the total mixture with the given proportions of component PFAS being evaluated. In the illustrative example below, the BMDLs associated with hypothetical response levels (i.e., $BMDL_x$, $BMDL_Y$, $BMDL_Z$) estimated for each chemical in a mixture are used to determine an "M-BMD" that represents an equivalent POD for the mixture that was identified for each component PFAS. The choice of BMRs is based on expert judgment and data availability. As stated above, it is preferable to calculate the M-BMDs using HED doses rather than oral mg/kg doses administered to test animals. The equation explanation and example below will reference $BMDL_i$ 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 mixture-specific M-BMD 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 is compared to the estimated M-BMD-HBWC from the M-BMD equation. 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 the risk of health effects is not expected. Further, the calculation can be repeated at multiple BMR levels to allow for the modeling of a full mixture dose-response curve, if needed. 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.

For consistency and comparison with the RPF illustrative example above, in the illustrative example below, the effect levels for each chemical in the hypothetical mixture of five PFAS from Tables 6-2, 6-3, and 6-4 are used to determine an "M-BMD." The previously described (Section 6.2) hypothetical response-equivalent POD 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 POD 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 $BMDL_{10}$ values, the DA calculation derives a $BMDL_{10}$ 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., human equivalent of 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). In practice, the lowest mixture-specific endpoint indicates the most sensitive effect domain for the mixture; this endpoint can then be used to derive an equivalent M-BMD-HBWC and estimation of risk. The derived M-BMD-HBWC can then be compared to the actual (measured) mixture concentration; if the actual mixture concentration exceeds the M-BMD-HBWC, there is a risk of the specific effect from exposure to that mixture at the measured concentrations.

## 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)*

Hypothetical data for the five PFAS in the illustrative example here are detailed in section 6.2 above (Note: Table 7-2 is a compilation of Hypothetical $POD_{HED}$s from Tables 6-2, 6-3, and 6-4 as used in the RPF example).

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

| | Hypothetical liver $POD_{HED}$ (mg/kg-day) | Hypothetical thyroid $POD_{HED}$ (mg/kg-day) | Hypothetical developmental $POD_{HED}$ (mg/kg-day) |
|---|---|---|---|
| **PFAS 1** | 0.044 ($BMDL_{X\text{-}HED}$) | 0.24 ($BMDL_{Y\text{-}HED}$) | 0.00001 ($BMDL_{Z\text{-}HED}$) |
| **PFAS 2** | 0.0013 ($BMDL_{X\text{-}HED}$)<br>0.0052 ($BMD_{X\text{-}AED}$)[a] | 0.23 ($BMDL_{Y\text{-}HED}$) | 0.0051 ($BMDL_{Z\text{-}HED}$) |
| **PFAS 3** | N/A | 0.21 ($BMDL_{Y\text{-}HED}$) | 2.1 ($BMDL_{Z\text{-}HED}$) |
| **PFAS 4** | 50 ($BMDL_{X\text{-}HED}$) | N/A | 0.0011 ($BMDL_{Z\text{-}HED}$) |
| **PFAS 5** | 0.004 ($BMD_{X\text{-}AED}$)[a]<br>0.001 ($BMDL_{X\text{-}HED}$)[b] | N/A | N/A |

*Notes:*

[a] NAM-based BMD modeled from the AED-based dose-response for the selected bioactivity event (e.g., decreased epoxide hydrolase activity, denoted as "hydrolase" in the example plots). This selected event is based on identifying the lowest (i.e., most sensitive) *common* bioactivity between the cell assay profiles for the IC (PFAS 2) and PFAS 5.

[b] Hypothetical NAM-derived Liver POD based on first calculating NAM-based relative potency for PFAS 5 = 0.0052 $BMD_{X\text{-}AED}$ / 0.004 $BMD_{X\text{-}AED}$ = 1.3 then estimating a PFAS 5 *in vivo* $POD_{HED}$ using the $POD_{HED}$ for PFAS 2 divided by the relative potency of PFAS 5, 0.0013 $BMDL_{X\text{-}HED}$ / 1.3 = 0.001 $BMDL_{X\text{-}HED}$.

### 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. The 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 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, as shown in Equation 7-4. This example is for the developmental domain, as it was the lowest M-BMD of the three effect domains.

(Eqn. 7-4)

$$Mixture\ BMD = \left(\sum_{i=1}^{4} \frac{a_i}{BMD_i}\right)^{-1} = \left(\frac{0.01}{0.00001} + \frac{0.13}{0.0051} + \frac{0.42}{2.1} + \frac{0.14}{0.0011} + \frac{0.29}{N/A}\right)^{-1} = 0.00087 \text{ mg/kg-day}$$

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

| | Median measured water concentration (ng/L) | Mixing ratio (proportion) | Liver $POD_{HED}$ (mg/kg/day) | Thyroid $POD_{HED}$ (mg/kg/day) | Develop-mental $POD_{HED}$ (mg/kg/day) |
|---|---|---|---|---|---|
| **PFAS 1** | 4.8 | 0.01 | 0.044 | 0.24 | 0.00001 |
| **PFAS 2** | 55 | 0.13 | 0.0013 | 0.23 | 0.0051 |
| **PFAS 3** | 172 | 0.42 | N/A | 0.21 | 2.1 |
| **PFAS 4** | 58 | 0.14 | 50 | N/A | 0.0011 |
| **PFAS 5** | 120 | 0.29 | 0.0022 | N/A | N/A |
| **Mixture total** | **409.8** | 1.0 | | | |
| **M-BMD calculation** | | | 0.0025 | 0.38 | **0.00087**[a] |

*Notes:*
N/A = data not available.
[a] The lowest M-BMD is converted to a mixture-HBWC using Eqn. 7-3 for comparison to the measured concentration (i.e., 409.8 ng/L).

The developmental-effect produced the lowest M-BMD (i.e., 0.00087 mg/kg-day), representing the most sensitive effect domain; this value is selected for the calculation of the M-BMD HBWC. The developmental-based M-BMD is first converted to an RfD by applying UFs that are consistent with the data being used. The 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 standard application of quantitative uncertainty for a mixture of components, although it is suggested that a user of this approach consider the composite uncertainty ($UF_C$) 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 the application of uncertainty to an 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 used in the hazard domain-specific calculation of the M-BMD selected for use in deriving the RfD. In the example illustrated in Equation 7-5, the POD from PFAS 4 is the most data-poor of the PFAS used to calculate the M-BMD for developmental effects selected for the RfD. Hypothetically, the $UF_C$ for this PFAS 4 POD was 300. Thus:

$$\text{RfD} = \left(\frac{\text{BMD}}{\text{UF}_\text{C}}\right) = \left(\frac{0.00087\frac{\text{mg}}{\text{kg}}/\text{d}}{300}\right) = 0.000003 \text{ mg/kg-day} \qquad \text{(Eqn. 7-5)}$$

An HBWC can then be derived using Equation 7-3. In the example shown in Equation 7-6, 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:

$$\text{HBWC} = \left(\frac{\text{RfD}}{\text{DWI-BW}}\right) * \text{RSC} = \left(\frac{0.000003\frac{\text{mg}}{\text{kg}}/\text{d}}{0.0354\frac{L}{kg}/d}\right) * 0.2 = 0.00002 \text{ mg/L} = 20 \text{ ng/L} \quad \text{(Eqn. 7-6)}$$

### 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's total PFAS concentration (409.8 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 of 409.8 ng/L exceeds the M-BMD HBWC 20 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, the M-BMD HBWC would be 48 ng/L (hypothetically assuming the same $UF_C$ and DWI-BW were used), below the measured PFAS concentration (409.8 ng/L), indicating there is also potential for liver effects in populations exposed to the hypothetical mixture. Alternatively, if the M-BMD was based on the thyroid effect domain (again assuming the same $UF_C$ and DWI-BW were applied to the M-BMD), the resulting M-BMD HBWC would be 7,177 ng/L, well above the measured total PFAS concentration (409.8 ng/L), indicating unlikely risk for thyroid effects among the exposed population. In practice, the composite $UF_C$ for each health-effect domain should be estimated based on the expert judgment of the most data-poor component chemical used to derive the M-BMD. Further, depending on the specific shared health effect within a given domain, the appropriate life stage DWI-BW should be used to convert the M-BMD to an M-BMD-HBWC.

## 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 response-equivalent effect endpoints (e.g., BMDLs) for each 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 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 the 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 similarly shaped 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 BMRs (e.g., $BMD_x$, PODx), 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 the 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. Similar to 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 the most sensitive—if not the most sensitive—effects across PFAS in the mixture of interest 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 to derive component chemical BMDs to calculate the M-BMD. Another limitation is the absence of standard guidelines for selecting uncertainty factors for a mixture of components, as opposed to the procedures used to apply uncertainty factors to individual chemicals in a risk assessment. The present document provides a hypothetical example of using a composite UF for the mixture based on the composite UF of the most data-poor component in the M-BMD calculation.

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 it is the most sensitive of the three assessed effect domains and, thus, is protective of the other effects (i.e., liver and 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 guidelines (USEPA, 1986, 2000b) and/or supported by NRC (2008). Although the approaches and illustrative hypothetical 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, foods). Each of the approaches included requires varying levels of data input, has relatively subtle but substantive differences in assumptions, and ultimately produces risk indications/estimations that may differ slightly based on those assumptions. Importantly, the 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 component. 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 from NAMs such as *in vitro* cell bioactivity, toxicogenomic platforms, and/or structure-activity/read-across 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 the 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 the 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/IC (such as in the RPF) or across each mixture component (such as in the 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); 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 the 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; the magnitude of HI is not an optimal comparator. On the other hand, 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 similarly shaped dose-response curves, whereas the M-BMD does not. If the mixture component chemicals have similarly shaped dose-response curves for a common 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 dose-response curve shapes, 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; thus, the Total ICEC to IC RfV or HBWC comparison or M-BMD HBWC to measured concentration may be affected. Therefore, 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 the 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 using the approaches in this framework document is that it may be prudent to apply each approach to the same mixture where data are available. The purpose of the comparison is not necessarily to determine which approach provides the most conservative estimate of mixture risk but rather which reflects the greatest level of confidence in the data underlying the component PFAS.

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