

Evaluation of Approaches for Assessing PFAS Mixtures

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ToxStrategies

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PREPARED FOR:
Hogan Lovells, LLP

PREPARED BY:
ToxStrategies, Inc.
23501 Cinco Ranch Blvd
Suite B226
Katy, TX 77494

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Acronyms

6:2 FTS	6:2 fluorotelomer sulfonic acid
AC50	50% maximum activity concentration
ATSDR	Agency for Toxic Substances and Disease Registry
C20	20% maximum response concentration
CAR	constitutive androstane receptor
EC50	50% maximum effect concentration
ECHA	European Chemicals Agency
EFSA	European Food Safety Agency
EQS	environmental quality standard
EU	European Union
hABP	human liver fatty acid binding protein
IC50	50% maximum activity inhibition concentration
IPCS	International Programme on Chemical Safety
ITRC	Interstate Technology and Regulatory Council
Log P	octanol-water partition coefficient
Log K _{OA}	octanol-air partition coefficient
MPC	maximum permissible concentration
NOAEL	no observed adverse effect level
PAH	polycyclic aromatic hydrocarbon
PFAS	per- and polyfluoroalkyl substances
PFHxS	perfluorohexane sulfonic acid
PFNA	perfluorononanoic acid
PFOA	perfluorooctanoic acid
PFOS	perfluorooctane sulfonic acid
PFPrA	perfluoropropanoic acid
PNEC	predicted no effect concentration
PPAR α	peroxisome proliferator-activated receptor alpha
PXR	human pregnane X receptor
QS	quality standard
RIVM	The Netherlands' National Institute for Public Health and the Environment
RPF	relative potency factor
SCHEER	Scientific Committee on Health, Environmental and Emerging Risks
TFA	trifluoroacetic acid
TFMS	trifluoromethanesulfonic acid
TR	thyroid receptor
TWI	tolerable weekly intake
USEPA	U.S. Environmental Protection Agency
WHO	World Health Organization

Executive Summary

ToxStrategies, Inc. evaluated the suitability of various exposure guidelines and regulatory approaches for assessing mixtures of per- and polyfluoroalkyl substances (PFAS). Approaches based on toxicity include dose additivity, use of relative potency factors (RPFs), or a hazard index. Each of those approaches is based at least in part on the assumption of similar toxicological profile. However, this assumption is not supported at this point in time for broad groups of PFAS based on information available in the scientific literature. Other methods include non-toxicity-based approaches such as grouping all PFAS together as a simple and precautionary approach under the assumption that all PFAS are persistent or have measurable total organofluorine. However, there are substantial limitations and criticisms of such non-toxicity-based approaches, and an overall lack of consensus in the scientific literature about how to characterize and manage mixtures of PFAS.

More specifically, with respect to approaches under consideration in the European Union (EU), the Netherlands' National Institute for Public Health and the Environment (RIVM) has adopted a RPF approach using increased liver weight relative to PFOA for protection of human health from exposure to PFAS mixtures. Despite limitations to the RPF approach, EU Commission (2021) has proposed to adopt this approach, which has been suggested as applicable to both human and ecological protection.

Based on our evaluation of the scientific literature concerning approaches for addressing mixtures of PFAS, we have concluded that there is no single suitable approach for handling PFAS mixtures. Mixtures of PFAS are complex and their complexities require situation-dependent approaches.

1 Introduction

ToxStrategies, Inc. evaluated the suitability of various exposure guidelines and regulatory approaches for PFAS mixtures. PFAS with various chain lengths and functional groups are often found together in the environment. Several approaches have been proposed or adopted to limit PFAS concentrations in mixtures. Some researchers and agencies have advocated for using simple dose additivity. However, additive effects between compounds may be assumed only when there are common toxicological effects and modes of action among the individual constituents in a mixture. In fact, known interactions among PFAS range from antagonistic to additive to synergistic with varying toxicological profiles and target organs. Some proposed approaches include the use of RPFs based on an index compound as well as use of a hazard index. However, both methods assume some degree of dose additivity, which may be an incorrect basis given the complexity of interactions among PFAS mixtures. RPF and hazard index methods have also been extended to applications for ecological protection, though the underlying bases for these approaches may not be suitable for non-human receptors.

A more precautionary approach considers the universal assumption that all PFAS are persistent, in which case all PFAS could be grouped together and managed based on their

persistence alone. However, many experts disagree with this approach. In addition, there is widespread disagreement that short- and ultrashort-chain PFAS should be grouped with long-chain compounds given their vast differences in toxicity and elimination profiles. There is also significant criticism of managing risk of all PFAS by total organofluorine measurements. Rather, the complexity of PFAS mixtures and their health effects requires combinations of approaches that are selected for specific purposes.

This evaluation considered general approaches to characterizing and managing PFAS mixtures. In particular, discussions include approaches undertaken in Europe and RIVM.

2 Toxicity Based Approaches

Conventional methods for assessing mixtures consider toxicological principles, such as interactions among mixture constituents. Dose additivity is applicable only when toxicity profiles among individual compounds are sufficiently similar. Related summation approaches, such as use of relative potency factors and hazard indices, also should be prudently used so as to not inappropriately apply underlying principles of toxicology.

2.1 Dose Additivity

EFSA (2019) guidance for combined exposure to multiple chemicals recommends that only the effects of compounds with common physicochemical properties, toxicity mechanisms of action, and target organ/system toxicity in a mixture can be summed (EFSA 2019). Similarly, an independent panel of experts who are credentialed in human health toxicology and risk assessment, recently agreed that human health risk assessment must be based on the principles of hazard and exposure; grouping compounds ought to relate to similar modes of action and dose additivity (Anderson et al. 2022). As the evidence base concerning the comparative toxicity across PFAS compounds continues to expand, there is increasing evidence that the toxicity profiles across PFAS are highly variable. As an example, Rericha et al. (2022) conducted experiments in zebrafish and concluded that the functional head group of the numerous PFAS compounds tested in their system drives developmental toxicity, suggesting different modes of action between structure groups.

There's also substantial variability concerning PFAS interactions reported in the scientific literature. While some researchers have reported dose additivity in limited toxicity studies, others have reported alternate effects ranging from antagonistic to synergistic. Some studies claimed additive effects between compounds with common toxicological effects and modes of action (Ojo et al. 2021a, Bil et al. 2021, Gray et al. 2020, Zhou et al. 2017), while other researchers have concluded that additivity is limited to low doses (USEPA Science Advisory Board 2022, Nielsen et al. 2022, Wolf et al. 2014). PFAS interactions have also been reported as synergistic (Jiang et al. 2022, Liu et al. 2022) or antagonistic (Menger et al. 2020, Rodea-Palomares et al. 2012). Many studies have also reported a combination of interactions depending on the dose, compound, and receptor organism (Fey et al. 2022, Marques et al. 2021, Ojo et al. 2020, 2021b, 2022, McCarthy et al. 2021, Preston et al. 2020, Hu et al. 2014, Ding et al. 2013, Carr et al. 2013). For example, Fey et al. (2022) demonstrated that the relative potencies of perfluorooctane sulfonic acid (PFOS)

and 6:2 fluorotelomer sulfonic acid (6:2 FTS) were not constant, and instead varied as a function of dose with less-than-additive interactions at low doses. Wolf et al. (2014) and Nielsen et al. (2022) observed that binary combinations of perfluoroalkyl acids behaved additively only at low doses, although Ding et al. (2013) reported complex interactions between PFOS and perfluorooctanoic acid (PFOA) that changed from additive to synergistic to antagonistic depending on their molar ratios. In an *in vivo* mouse study, Marques et al. (2021) determined that a mixture of three PFAS compounds had additive, synergistic, or antagonistic effects depending on endpoint. Preston et al. (2020) likewise reported in their epidemiological study that the direction and magnitude of the combined effects of prenatal exposure to multiple PFAS may vary across individual PFAS.

Anderson et al. (2022) reported that panel experts agreed that “all PFAS” should not be grouped together as it is inappropriate to assume equal toxicity and potency across the diverse class of PFAS. Health effects of PFAS vary in both humans and animals, and there is a lack of consensus for health risk even among well-studied PFAS (ATSDR 2021, Fenton et al. 2021, Steenland et al. 2020, Zodrow et al. 2022). The expert panel was evenly split as to whether an assumption of dose additivity is justified as a conservative and pragmatic approach to grouping PFAS compounds with less understood toxicological profiles. While there is some limited support for this approach in the scientific literature, half of the expert panelists asserted that there is too much uncertainty about the toxicological effects and potential modes of action to warrant an assumption of dose additivity (Anderson et al. 2022). Conversely, the USEPA (2021) draft framework for estimating noncancer health risks associated with mixtures of PFAS has proposed an assumption of dose additivity based on common outcomes rather than a common mode of action; however, the USEPA Science Advisory Board (2022) acknowledged uncertainties in USEPA’s draft PFAS mixtures approach that need to be clearly explained.

Several regulatory agencies throughout the world (e.g., Australia, Germany, Sweden, United States) have restricted their summing approach to a limited list of specific PFAS compounds (ITRC 2022). Many of these agencies have cited similarities in health effects as rationale for a precautionary dose additive approach. However, few, if any, have demonstrated common mechanisms of action and target organ/system toxicity in humans among the specific PFAS.

EFSA (2020) established a tolerable weekly intake (TWI) of 4.4 ng/kg-bw/week for four PFAS compounds, PFOA, PFOS, perfluorononanoic acid (PFNA) and perfluorohexane sulfonic acid (PFHxS) based on the assumption of dose additivity. This proposed limit is based on observations from a small cross-sectional study of 101 one-year-old breastfed children in Germany (Abraham et al. 2020). Children in this study were reported to show a significant association between serum PFOA levels and decreased vaccine antibody titers against diphtheria, *Hemophilus influenzae* type b and tetanus. No significant associations were found for the individual analyses of PFOS, PFNA or PFHxS serum levels. More importantly, no influence of PFOA and PFOS on infections during the first year of life was reported in these children (Abraham et al. 2020). Nonetheless, EFSA summed serum concentrations of the four PFAS reported in this study to demonstrate a significant association of these PFAS with reduced antibody titers. EFSA also assumed equal potencies for the effects of these PFAS on immune outcomes despite an absence of

association of PFOS, PFNA, and PFHxS individually to decreased antibody titers. Furthermore, the available literature does not provide empirical data demonstrating PFOA, PFOS, PFHxS and PFNA are in fact dose additive for immunotoxicity-related endpoints. Thus, EFSA (2020) did not rely on empirical evidence when making conclusions about the suitability of applying dose additivity, which is in contrast with EFSA (2019) guidance.

2.2 Relative Potency Factor Approach

A relative potency factor (RPF) approach assigns potency factors for a specific toxicity endpoint to different substances relative to an index substance. RIVM (2018) developed a RPF method for specific mixtures of PFAS. This method was later published by Bil et al. (2021) for select PFAS compounds. The European Union Commission's Draft EQS Dossier on PFAS (EU Commission 2021) proposed a similar method based on Bil et al. (2021). Using this approach, PFOA serves as the index compound and is assigned an RPF=1; potency factors are assigned to the other select PFAS based on potency for induction of increased relative liver weight in male rats relative to that of PFOA. PFOA-equivalents of a PFAS mixture are calculated by multiplying RPFs by the concentrations of the individual compounds in a specific medium (e.g., soil, water, food) and then taking the sum of the products. The overall PFOA equivalency is then compared to the group TWI established by EFSA in 2020 of 4.4 ng/kg-bw/week. Bil et al. (2022) subsequently derived internal RPFs for nine PFAS, using toxicokinetic models to estimate serum PFAS levels in male rats relative to liver weight increase. Given that the EFSA TWI was based on immunosuppressive effects in humans, Bil et al. (2023) also attempted to develop internal RPFs for eight PFAS using modeled serum PFAS concentrations and immunotoxicity endpoints in rats including thymus weight, spleen weight, and globulin concentration. There are several significant limitations of these RPF approaches as outlined below.

First, potential RPFs for PFAS compounds vary by endpoint. As shown in **Table 1**, there are a number of datasets that have been used to estimate PFAS RPFs, and PFAS compound rank ordering varies based on test model, target organ and/or health endpoint of concern. **Table 1** illustrates that the RIVM RPF estimates based on read-across are not very predictive of the empirical RPF estimates reported by Behnisch et al. (2021) or Goodrum et al. (2021). Furthermore, there may be variability among RPFs even for the same endpoint depending on the datasets chosen as evidenced by studies from Bil et al. (2021, 2022, 2023). There are a number of reasons why these RPF estimates vary, including that the *in vitro*-based RPFs do not account for the ADME differences observed *in vivo*, different sensitivities between cell lines, different physicochemical properties among PFAS that affect exposure and ADME, and different mechanisms by which PFAS elicit effects, etc. Dose-response relationships for *in vivo* data are expected to better predict potential effects and exposure-response relationships in humans; however, the variability between models suggests that the RIVM RPFs are not necessarily applicable to all PFAS-related health endpoints. Troung et al. (2022) similarly reported weak correlations between zebrafish bioactivity and certain PFAS physicochemical properties (i.e., compound mass, number of fluorinated carbons, predicted octanol-water partition coefficient (Log P), predicted octanol-air partition coefficient (Log K_{OA})), making read-across toxicity predictions problematic. Despite the Bil et al. (2021) RPF approach covering only a relatively small

subgroup of the larger PFAS chemical class, the specific PFAS compounds included in the RPF approach are themselves a diverse group of compounds for which empirical evidence demonstrates varying relative potencies for different health outcomes. For example, Goodrum et al. (2021) demonstrated that an RPF approach to grouping perfluoroalkyl carboxylic acids and perfluoroalkyl sulfonic acids was inappropriate given their differences in dose-response. Bil et al. (2023) further demonstrated variance in RPFs across different endpoints for a specific PFAS.

Table 1. Relative potency and rank order differences for various PFAS RPF estimates

PFAS Compound	Number of Carbons	RIVM (2021) and Bil et al. (2021) - in vivo liver hypertrophy		Bil et al. (2022) - in vivo liver hypertrophy		Bil et al. (2023) - in vivo liver hypertrophy		Bil et al. (2023) - Globulin		Behnisch et al. (2021) - in vitro TR		Goodrum et al. (2021) - PPAR- α , C20 max		Goodrum et al. (2021) - PPAR- α , AC50 max		Goodrum et al. (2021) - FABP, IC50		Goodrum et al. (2021) - PXR, EC50		Goodrum et al. (2021) - TR, IC50	
		RPF	Rank	RPF	Rank	RPF	Rank	RPF	Rank	RPF	Rank	RPF	Rank	RPF	Rank	RPF	Rank	RPF	Rank	RPF	Rank
C6O4	6	--		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PFNA	9	10	1	5	4	4	5	1	2	0.32	6	1.45	1	1.52	2	3.10	3	1.24	2	1.17	4
PFDA	10	10*	1	-	-	7	2	2	1	0.12	8	-	-	0.70	7	3.91	2	2.79	1	7.00	1
PFUnDA	11	4	3	-	-	-	-	-	-	0.052	11	0.19	6	0.88	6	4.74	1	-	-	2.10	3
PFDoDA	12	3	4	10	1	-	-	-	-	0.01	14	-	-	-	-	-	-	-	-	-	-
PFTTrDA	13	3*	4	-	-	-	-	-	-	0.075	10	-	-	-	-	-	-	-	-	-	-
PFHpS	7	2*	6	-	-	-	-	-	-	1	4	-	-	-	-	-	-	-	-	-	-
PFOS	8	2	6	3	5	5	4	0.2	6	2	1	0.06	8	1.27	4	2.73	4	1.16	3	2.63	2
PFDS	10	2*	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PFHpA	7	1*	9	-	-	-	-	-	-	1.4	3	1.07	2	1.37	3	0.15	7	0.49	6	0.19	7
PFOA	8	1	9	1	7	1	7	1	2	1	4	1.00	3	1.00	5	1.00	5	1.00	4	1.00	5
PFPeS	5	0.6*	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PFHxS	6	0.6	11	0.6	8	0.6	9	0.03	8	1.6	2	0.20	5	1.95	1	0.59	6	-	-	0.22	6
PFTeDA	14	0.3	13	-	-	-	-	-	-	0.019	13	-	-	-	-	-	-	-	-	-	-
GenX (HFPO-DA, FRD-902/-903)	6	0.06	14	9	3	25	1	1	2	-	-	-	-	-	-	-	-	-	-	-	-
PFBA	4	0.05	15	2	6	1	7	-	-	0.0018	15	-	-	-	-	-	-	-	-	-	-
PFPeA	5	0.05*	15	-	-	-	-	-	-	0.08	9	-	-	-	-	-	-	-	-	-	-
8:2 FTOH	10	0.04	17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ADONA	7	0.03	18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PFHxDA	16	0.02	19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PFODA	18	0.02	19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6:2 FTOH	8	0.02	19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.93	5	-	-
PFHxA	6	0.01	22	10	1	6	3	0.9	5	0.19	7	0.34	4	0.60	8	-	-	0.46	7	0.08	8
PFBS	4	0.001	23	0.2	9	2	6	0.1	7	0.052	11	0.08	7	-	-	0.05	8	-	-	0.04	9

Abbreviations: RPF, relative potency factor; TR, human thyroid receptor; PPAR α , human peroxisome proliferator-activated receptor alpha; hABP, human liver fatty acid binding protein; PXR, human pregnane X receptor; C20, 20% maximum response concentration; AC50, 50% maximum activity concentration; IC50, 50% maximum activity inhibition concentration; EC50, 50% maximum effect concentration.

Color Scheme: light blue, blue, and dark blue = upper third, middle third, and lower third rank ordering, respectively, of each RPF.

*RIVM and Bil et al. (2021) RPF estimates based on read across, not empirical data.

Second, there is insufficient evidence that the effects of the selected PFAS incorporated into the RPF approach proposed by Bil et al. (2021) can be assumed to be dose additive, which is a foundational assumption for any RPF approach. Instead of performing an assessment to confirm the dose-addition concept, Bil et al. (2021) selectively cited two examples of PFAS studies where additivity was reported (Wolf et al. 2014, Zhou et al. 2017). As noted above, antagonistic and synergistic effects have also been reported, but were not acknowledged in Bil et al. (2021). The limited number of published whole mixtures studies available directly testing for mixtures dose response report a variety of inconsistent outcomes for additivity, as well as the absence of an additive effect, antagonism, and/or synergism, with differences associated with choice of assay model, outcomes examined, PFAS dose levels tested, etc. (reviewed by Goodrum et al. 2021). Nielsen et al. (2022) similarly remarked that an RPF approach can deviate from empirical data, even at low concentrations, when there are varying efficacies among mixture components. These observations have led many regulatory agencies across the globe to consider the current state of the PFAS science on dose additivity to be insufficient for the development of RPFs applicable in risk assessment. For example, EFSA (2020) concluded that, "...the available data are insufficient to derive potency factors for the different PFASs, although such differences in potency are likely to exist..." In addition, ATSDR (2021) cited Peters and Gonzalez (2011) in noting that, "Although there is some evidence of similar health outcomes for some compounds, there is evidence of qualitative and mechanistic differences."

Third, the RIVM PFAS RPF approach is predicated on increased relative liver weight in rats from subacute/subchronic studies and is toxicologically distinct from that of EFSA's TWI. As described above, the EFSA TWI is derived from an epidemiological study in children that reported a significant association between serum PFOA levels and decreased vaccine antibody titers against diphtheria, *Hemophilus influenzae* type b, and tetanus. Applying RPFs based on empirical dose response data for one specific endpoint to a TWI derived from a completely different outcome in a different species is only appropriate if 1) there is clear empirical evidence that the relative potencies of the PFAS compounds are equivalent across endpoints and species, or 2) absent such information, there is evidence that the two endpoints are moderated by the same mode of action such that analogous relative potencies could be reasonably assumed across endpoints. No such empirical link has been identified between the dose response relationships for these two outcomes that could be used to inform and support the relative potencies of the PFAS in the RIVM RPF approach. Although Bil et al. (2023) attempted to demonstrate similarities in RPFs derived using liver and immunotoxicity endpoints, only two compounds (PFHxA and PFBS) shared the same RPF for relative liver weight and one of the four total immunotoxicity endpoints investigated.

Lastly, as mentioned above, several regulatory agencies from other countries have developed risk assessment approaches to address PFAS mixtures to some extent, typically limited to sums of specific PFAS compound concentrations (ITRC 2022). However, besides the EU Commission and RIVM, to date, no other regulatory agency has considered

the PFAS toxicology and epidemiology database to be sufficiently robust for the development of RPFs.

2.3 Hazard Index Approach

A hazard index approach sums hazard quotients for different constituents in a mixture. A hazard quotient is the ratio of a substance concentration to an allowable exposure standard. A hazard index of less than one typically indicates an acceptable risk. EFSA (2019), USEPA (2000), and WHO/IPCS (Meek et al. 2011) proposed a tiered approach according to the level of available toxicity information and level of uncertainty in assumptions. Under this guidance, a Tier 0 mixtures assessment uses a hazard index approach as a preliminary and initial screen. USEPA (2007), among others, acknowledge that a Tier 0 hazard index approach without regard to similarity in target organ toxicity likely overestimates risk. If a hazard index is greater than one, then more refined approaches may be considered. Tier 1 and Tier 2 approaches evaluate target organ-specific hazard indices or a target organ toxicity dose to accommodate mixtures with toxic effects in common target organs. USEPA (2021) likewise proposed a hazard index approach as a Tier 1 approach to evaluating PFAS mixtures. Goodrum et al. (2021) proposed an expanded decision tree for determining when to assess PFAS as a group based on the likelihood of co-exposure, toxicological similarities, and data sufficiency. However, most experts agreed that using a hazard index approach in which dose additivity is assumed may be a useful initial screening tool of *potential risk* but not an indication of *immediate risk* given the high degree of uncertainty (Anderson et al. 2022, Mumtaz et al. 2021).

2.4 Mixture Approaches for Ecological Protection

Although the draft EU dossier on PFAS (EU Commission 2021) claims that “the RPF approach is suitable for evaluating the cumulative risk of oral exposure to mixtures of PFAS and of PFAS concentrations occurring in various matrices to which humans or wildlife may be orally exposed”, the Scientific Committee on Health, Environmental and Emerging Risks (SCHEER 2022) indicated that EU Commission (2021) lacked reliable RPFs for ecotoxicity. The draft document itself presents ecotoxicity values only for individual compounds. In 2014, the EU Water Framework Directive (EU Commission 2014) established an environmental quality standard for biota (EQS_{biota}) for PFOS only of 9.1 µg/kg ww in fish fillet in order to protect human health and 33 µg/kg ww in whole fish to protect secondary poisoning of wildlife. RIVM (2010) derived PFOS water standards for direct ecotoxicity, secondary poisoning, and human consumption of fish. Human consumption of fish was selected as the exposure route of concern, which resulted in a maximum permissible concentration (MPC) of 0.65 ng/L PFOS in freshwater.

Using the RPF approach, EU Commission (2021) proposed a new EQS_{biota} of 0.077 µg/kg ww PFOA-equivalents in fish fillet and 22.3 µg/kg ww PFOA-equivalents for whole fish. Rudel et al. (2022) attempted to derive conversion factors for fillet concentrations to whole fish concentrations for selected PFAS compounds and apply RPFs relative to PFOS. However, both RPF approaches with PFOA- and PFOS-equivalents are based on toxicity endpoints in species (e.g., rodent models) that may differ from actual receptor organisms

(e.g., humans). In addition, EQS_{biota} values are intended to protect higher trophic levels and humans rather than aquatic species directly exposed to PFAS. This conclusion is consistent with the literature showing little consensus in the scientific community about how to assess ecological impacts from PFAS mixtures (ITRC 2022). A RIVM approach to evaluating PFAS mixtures for ecological protection separate from EU Commission guidelines (2018, 2021) was not found for the purposes of this evaluation.

2.5 Mixtures with Short- and Ultrashort-Chain PFAS

Some approaches to mixtures, including proposed RPF methods, make no distinction between long-chain PFAS (≥ 8 carbons in chain for perfluoroalkyl carboxylates and ≥ 7 carbons in chain for perfluoroalkyl sulfonates), short-chain PFAS (4-7 carbons in chain for perfluoroalkyl carboxylates and 4-6 carbons in chain for perfluoroalkyl sulfonates), or ultrashort-chain PFAS (≤ 3 carbons in chain). Although proponents of grouping short- and ultra-short chain PFAS with longer chain length PFAS generally agree that the shorter compounds tend to be less potent and are less bioaccumulative than their long-chain counterparts, these methods may still group all PFAS together albeit with a smaller RPF (RIVM 2018, Bil et al. 2021, EU Commission 2021). Furthermore, some regulatory agencies, such as the European Parliament (2020), have established exposure limits for the sum of all PFAS, regardless of chain length. EU Directive 2020/2184 established a drinking water limit of “PFAS Total” to 500 ppt effective January 12, 2024, provided a suitable monitoring method has been established by then. This same EU directive will also limit the sum of 20 specific compounds, including short-chain compounds with as low as four carbons, to 100 ppt in drinking water. The World Health Organization (WHO 2022) has also proposed a draft provisional guidance value (pGV) of 500 ppt for total PFAS, citing the likelihood of co-occurrence and the generally high persistence among PFAS compounds as well as the expectation that current technology can achieve removal from drinking water to such levels. However, conventional adsorption treatment technologies, such as activated carbon and ion exchange resins, generally do not remove the more hydrophilic shorter-chain PFAS as effectively as longer-chain compounds, making removal of short-chain PFAS from water challenging (Gagliano et al. 2020, Li et al. 2020). Furthermore, WHO (2022) acknowledged that this pGV is not based on similarities in toxicity within the greater PFAS class. In addition, the European Food Safety Authority (EFSA) had been asked to derive a tolerable intake for a group of 27 PFAS with different functional groups, chain lengths, physicochemical properties, and toxicokinetics, however, they settled on a tolerable weekly intake value for only four compounds (EFSA 2020). The remaining 23 PFAS were excluded either because of lower relative occurrence or short half-lives.

Despite the numerous attempts to regulate all PFAS as a single group regardless of chain length, much of the scientific literature does not support including short- and ultrashort-chain compounds in a group with long-chain PFAS. Colnot and Dekant (2022) advocated for EFSA to adopt multiple groups, namely perfluoroalkyl carboxylates and perfluoroalkyl sulfonates, to which each would be subject to an RPF with distinct index compounds. However, Colnot and Dekant (2022) warned that neither of these EFSA groups should include short-chain PFAS due to their low potency *in vivo* and rapid elimination. In

addition, *in vitro* data have also demonstrated differences in receptor activation between short-chain and long-chain PFAS; thus, PFAS with differing chain lengths should not be grouped together (Goodrum et al. 2021).

The reasons for not grouping short-chain PFAS with long-chain PFAS are even more relevant for ultrashort-chain PFAS. Ultrashort-chain compounds, such as trifluoromethanesulfonic acid (TFMS, one carbon), trifluoroacetic acid (TFA, two carbons), and perfluoropropanoic acid (PFPrA, three carbons), are highly soluble and mobile in water, and have low bioaccumulation potential in aquatic organisms. Conversely, long-chain PFAS, such as perfluorooctanoic acid (PFOA), are more hydrophobic and tend to accumulate in sediments and aquatic organisms (Gagliano et al. 2020, Li et al. 2020). Repeated dose toxicity studies of ultrashort-chain PFAS also indicate that they are significantly less toxic than long-chain PFOA. For example, NOAEL values in rats ranged from 20 mg/kg-day for PFPrA based on absence of systemic effects (Hita Laboratory 2002a, b) to 1,000 mg/kg-day for TFMS based on liver toxicity (ECHA 2022a), whereas the NOAEL for PFOA has been reported as 0.29 mg/kg-day based on liver toxicity (ATSDR 2021). The elimination potential in humans is also much greater for ultrashort-chain compounds with half-lives in the range of 25-81 hours (ECHA 2022b, 3M 2008a, b, 2009, 2019), whereas the half-life for PFOA is approximately four years (ATSDR 2021).

3 Non-Toxicity Based Approaches

Other mixtures approaches have been proposed which are not based on toxicity. One such approach involves grouping all PFAS together and is intended to simplify management of what is perceived as an otherwise complex mixture with an elusive understanding of interactions. Such methods to manage PFAS as a single group are precautionary, with an assumption of sufficiently high hazard among all compounds and are based on common features such as environmental persistency or measurable total organofluorine content.

3.1 Persistency

Cousins et al. (2020) proposed grouping strategies for PFAS. The least conservative approach proposed by Cousins et al. would group only PFAS with well documented and similar toxicological effects, modes and mechanisms of action, and elimination kinetics. The most conservative approach groups all PFAS together for phasing them out under the assumption that all PFAS have high persistence, the so-called “P-sufficient” approach. In contrast, Anderson et al. (2022) reported that most experts agreed that persistence alone is not sufficient for grouping PFAS when assessing human health risk. Only two of the 11 panel members in Anderson et al. (2022) advocated for the P-sufficient approach for regulating PFAS in drinking water; the remaining panel members noted that grouping all PFAS as “persistent” was not practical nor appropriate. Some PFAS are mineralizable and not persistent; a more suitable approach for grouping and assessing PFAS mixtures should consider the toxicological effects and potential for exposure levels of concern.

3.2 Total Organofluorine Measurements

Some researchers and organizations have proposed characterizing and managing PFAS mixtures according to measurements of total organofluorine content rather than by each constituent PFAS (Ankley et al. 2021). However, the sensitivity, selectivity, and applicability to different sample matrices among such methods vary considerably, and the sensitivity is generally too low for most environmental samples (McDonough 2019, Johnson et al. 2021). In addition, these measurements may be considered a rough estimate of total PFAS as they do not identify specific compounds, and samples with compounds containing greater proportions of fluorine may introduce bias in mixtures with varying fluorine compositions (Koch et al. 2020). Some methods only measure total fluorine at the detector and thus rely on preparation methods for selectivity to distinguish PFAS from other fluorine-containing compounds, such as pharmaceuticals and inorganic fluorine compounds (De Silva et al. 2021). For example, methods which employ detection using combustion ion chromatography (CIC) do not differentiate between organic fluorine and fluoride nor do they offer structural details about the detected compounds. Thus, methods including extractable organic fluorine (EOF) and adsorbable organic fluorine (AOF) assays, which use CIC, gain sensitivity from the sample preparation approach rather than the detection method (McDonough et al. 2019, Koch et al. 2020). Although it is generally not used for aqueous samples, particle-induced gamma ray emission (PIGE), which also is based on total fluorine at the detector, quantifies elemental fluorine in the surface of a material, but can only measure to a certain penetration depth (McDonough et al. 2019, Koch et al. 2020). The total oxidizable precursor (TOP) assay, which is the most selective among total organofluorine methods, only identifies compounds that can be oxidized to form target perfluoroalkyl acid compounds, and short-chain compounds may not be retained by traditional liquid chromatography columns; thus, this method can lead to false negative results (McDonough et al. 2019, Koch et al. 2020). Anderson et al. (2022) similarly reported that there is a lack of consensus among experts as to the suitability of total organofluorine methods, even when used in a screening level assessment for *risk*; however, total organofluorine assays may be considered acceptable for screening *exposures* only.

4 Combination of Approaches to Mixtures

Much of the scientific literature has concluded that the complexity of PFAS mixtures and their health effects requires combinations of approaches that are selected for specific purposes. Rosato et al. (2022) advocated that epidemiological studies methodologically evaluate the effect of combined exposures to multiple PFAS using multiple approaches and consider the strengths and limitations of each method with respect to the research question. Anderson et al. (2022) concluded that PFAS should be regarded in situation-dependent and case-by-case subgroups when assessing human health risk; such subgroups should be based on similar physicochemical properties, carbon chain length, and functional groups.

5 Conclusions

There is a general lack of consensus in the scientific literature about how to characterize and manage mixtures of PFAS. Dose additivity or summing has been proposed based on conservative assumptions of dose additivity or persistency, but there is a wide range of interactions among PFAS according to dose, species, and compounds. A hazard index approach might be useful, but only if limited to screening applications. RPF approaches have several limitations to their widespread applications, especially for ecological protection standards. Furthermore, mixtures approaches should consider the types of PFAS to be grouped together as short- and ultrashort-chain compounds have significantly different toxicological profiles than long-chain PFAS. Thus, there is seldom a single suitable approach to handling PFAS mixtures; mixtures of PFAS are complex and their complexities require situation-dependent approaches. The considerable uncertainties associated with the various proposed methods to grouping PFAS leads to the conclusion that the compounds are most appropriately handled on an individual basis rather than as a group.

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