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Interagency Coordinating Committee on the Validation of Alternative Methods

New Approach Methodologies for Characterizing Potential Human and Environmental Health Effects of PFAS



A Report of the ICCVAM Per- and Polyfluoroalkyl Substances Workgroup

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Abbreviations and Acronyms

AFFF	Aqueous film-forming foams
EPA	United States Environmental Protection Agency
ICCVAM	Interagency Coordinating Committee for the Validation of Alternative Methods
NAM	New approach methodology
OECD	Organisation for Economic Co-operation and Development
PBTK	Physiologically based toxicokinetics
PFAS	Per- and polyfluoroalkyl substances
PFDA	Perfluorodecanoate
PFHxS	Perfluorohexane sulfonate
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid
PPAR	Peroxisome proliferator-activated receptor
QSAR	Quantitative structure–activity relationship
TK	Toxicokinetics
TSCA	Toxic Substances Control Act

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Disclaimer

This report has been developed as a resource for U.S. federal agencies and stakeholders seeking to establish confidence in new approaches that replace, reduce, or refine the use of animals in testing. The principles described in this report were developed with input from staff from 18 federal agencies, multiple interagency workgroups, and the Scientific Advisory Committee on Alternative Toxicological Methods. As such, this report does not necessarily reflect the opinions or policy of any agency or workgroup. It does not create rights for any person or party and should not be taken as a commitment by any federal agency. This report does not establish any legally enforceable responsibilities. Instead, this report describes the Interagency Coordinating Committee on the Validation of Alternative Methods' (ICCVAM) current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word "should" means that something is suggested or recommended but not required.

Executive Summary

The Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM) coordinates information and evaluates use of new approach methodologies (NAMs) across the U.S. government. To address the need for more efficient understanding and characterization of per- and polyfluoroalkyl substances (PFAS) toxicity, ICCVAM established a PFAS workgroup. The workgroup compiled this document, which explores the current use of NAMs to evaluate potential adverse effects associated with PFAS and discusses challenges and data gaps. It is intended to provide background information for future collaborative efforts.

PFAS are a diverse set of fluorinated chemicals, the unique properties of which have led to their broad use in commerce. While these properties make PFAS extremely effective in many applications, they might also lead to unintentional effects on human and environmental health. Exact definitions of PFAS differ and the number of chemicals considered to be PFAS varies widely under those various definitions. However, it is broadly agreed that there is a need for better characterization of the human and environmental health effects of PFAS, and that addressing this need will require characterization of many thousands of chemicals.

Due to the inherent impracticality of testing thousands of chemicals using animal methods, NAMs have been proposed as promising screening tools to inform on potential human and environmental hazards of PFAS. The term “NAM” encompasses any technology, methodology, or combination thereof that can provide toxicological information useful for chemical screening and hazard assessment without reliance on traditional approaches using vertebrate animals. NAMs can include in silico, in chemico, and in vitro test methods, as well as non-testing approaches such as data reviews to support testing waivers.

Examples of NAMs that have been used to evaluate the effects of PFAS on human and environmental health include in vitro and in silico models used to predict PFAS hazard for biological systems and to understand the underlying mechanisms of PFAS toxicity. Studies using in vitro NAMs have suggested that PFAS can affect the immune system and cause developmental toxicity, including deleterious effects on the developing nervous system. Other studies have shown that PFAS can interact with metabolic and endocrine pathways. In silico NAMs demonstrate potential utility in predicting properties of PFAS relevant to human and environmental health, including toxicokinetics, biological targets, and bioaccumulation.

In addition to challenges that have been identified generally for qualification and validation of NAMs, some challenges specific to PFAS need to be considered to effectively implement NAMs for human health and environmental hazard assessment of those substances.

- As PFAS as a group do not have a common mechanism of action, it will be important to develop a battery of NAMs with broad biological coverage to evaluate the scope of PFAS hazard potential.
- Diversity of PFAS presents a challenge in implementing predictive computational approaches such as read-across. Recent studies suggest that structural consideration alone may not be sufficient for performing read-across without mechanistic information or additional data such as toxicokinetic and physicochemical characteristics.
- The chemical properties that make PFAS useful in manufacturing also make them challenging to evaluate using NAMs in a laboratory setting. Thus, consideration of assays for PFAS testing

should take into account issues such as cellular bioavailability and partition coefficients of test substances.

- The large number of PFAS and complexity of physicochemical properties remains a challenge for acquiring reliable analytical chemistry information. The National Institute of Standards and Technology is establishing a PFAS database to address this critical need.
- Assessment of PFAS toxicity is complicated by the fact that mixtures of PFAS that commonly occur in environmental media also include other environmental pollutants. Future work should focus on strategies for testing PFAS mixtures, considering factors such as PFAS exposure and differences in toxicity effects across the PFAS chemical space.

The ICCVAM PFAS Workgroup considers that NAMs encompassing a range of in vitro and computational approaches will be important sources of data for initial characterization and prioritization of PFAS with regards to considerations for assessing human or ecological health. Initial efforts should focus on understanding the diversity of PFAS chemical properties, assessing effects of PFAS co-exposures, comparing NAMs results to traditional testing outcomes and validating the assays for regulatory use, and relating NAM results to human exposure to increase confidence in NAMs for PFAS hazard assessment. Integrative tools such as adverse outcome pathways may be useful to link molecular initiating events associated with PFAS to specific toxicity outcomes. Future studies evaluating susceptible and vulnerable subpopulations will also be critical for a comprehensive assessment of PFAS toxicity potential.

New Approach Methodologies for Characterizing Potential Health and Environmental Effects of PFAS

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a diverse set of fluorinated chemicals. Their use in commerce is widespread due to their unique properties such as thermal and chemical stability, water and stain resistance, and electrical insulating properties. Commercial and industrial applications include use as water- and oil-repellents, metal finishing agents, processing aids in the manufacture of plastics, and firefighting foams. The definition of PFAS varies, but the grouping is generally considered to include thousands of substances.

The properties of PFAS that make them useful in commerce have also raised concerns about their effects on human and environmental health. PFAS have been found to be present and highly persistent in the environment, humans, and biota. However, only a few PFAS are in the Toxic Substances Control Act (TSCA) inventory (EPA, 2021b), and even fewer have been extensively studied, resulting in limited information being available on human health and environmental hazards for the majority of PFAS. These concerns have been highlighted within the National Science and Technology Council PFAS Report (NSTC, 2024) and by activities proposed by the U.S. Environmental Protection Agency (EPA) to combat PFAS contamination (EPA, 2025; NSTC, 2023).

New approach methodologies (NAMs), including high-throughput in vitro assays and in silico models, have been proposed as promising tools to screen PFAS for potential hazard (EPA, 2021b). NAMs activities across the U.S. government are coordinated by the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM). ICCVAM, a congressionally mandated group of federal agency representatives, collects information on and evaluates use of NAMs via targeted workgroups (ICCVAM Authorization Act of 2000, 2000). ICCVAM established its PFAS workgroup in 2023 to provide expertise on the application of NAMs to evaluate PFAS. The workgroup compiled this document, which explores the current use of NAMs to evaluate potential toxicities associated with PFAS and discusses challenges and data gaps. It is intended to provide background information for future efforts, including a workshop.

2. Existing Definitions of PFAS

The term “PFAS” has been broadly used in research and regulatory contexts. Although most organizations generally agree on whether a specific chemical might be considered a PFAS, the chemical features that define a PFAS may vary across organizations and contexts (**Table 1**). In 2021, the Organisation for Economic Co-operation and Development (OECD) adopted a broad definition of PFAS that specifies that such compounds should contain at least one fully fluorinated methyl or methylene carbon atom. This definition produced a list of approximately 40,000 substances (OECD, 2021; Wang et al., 2021). However, a 2024 query of PubChem’s Classification Browser returned over 6 million substances that met the OECD PFAS definition. A more specific definition is utilized by the EPA Office of Pollution Prevention and Toxics, which in 2023 proposed a significant new use rule that included a definition specifying that PFAS contain at least one of three specific structures (EPA, 2023b) (**Table 1**). This definition is intended to capture chemicals that could be manufactured in the future, regardless of

whether a given PFAS is currently present as a contaminant in the environment. All currently existing definitions range from broad to narrow, with updates made on an as-needed basis according to improved understanding of the science (NSTC, 2023).

To address the need for additional toxicological data on PFAS, in October 2021 Congress directed EPA (15 U.S.C. § 8962, 2021) to develop a process for prioritizing which PFAS or "class of PFAS" should be subject to additional testing under TSCA Section 4 (EPA, 2021b). To facilitate this prioritization, several publications have proposed applying read-across, which is a data-gap filling approach used to predict the properties of a target substance by leveraging data from similar source substances, to develop a categorization approach and tiered-testing strategies for hazard assessment (Patlewicz et al., 2024; Patlewicz et al., 2022; Richard et al., 2023). Characteristics that may be considered in read-across approaches include similarities in structure, physicochemical properties, toxicokinetic (TK)/absorption distribution metabolism and elimination profiles, molecular targets, target organs or systems, performance or activity measured by NAMs, or the likelihood of co-exposure with other chemicals (Mansouri et al., 2024; Patlewicz & Shah, 2023). Ideally, application of these approaches should include a quantification of uncertainty across chemicals within groupings to consider in the interpretation of toxicological information (Patlewicz et al., 2024). Toxicity predictions for PFAS by structure-based groupings may also be improved by considering additional information such as TK data (Patlewicz et al., 2024). As has been demonstrated in previous ICCVAM workgroup assessments of agency needs, an understanding of current research and regulatory needs will be foundational for developing PFAS grouping strategies (Ceger et al., 2022; Chang et al., 2022; Choksi et al., 2019; NSTC, 2024; Petersen et al., 2022; Strickland et al., 2018; Strickland et al., 2019).

Table 1: Chemical Structure-based Definitions of the PFAS Class*

Source	Definition
National PFAS Testing Strategy - 2024 TSCA PFAS Workshop (Patlewicz, 2024)	TSCA Section 8(a)(7) Data Collection Rule (see below) plus TSCA-plausible simulated/computationally derived PFAS degradation products.
European Chemicals universal PFAS restriction proposal (ECHA, 2026)	Any substance that contains at least one fully fluorinated methyl (CF_3-) or methylene (-CF_2-) carbon atom, without any H/Cl/Br/I atoms attached to it. A substance that only contains the following structural elements is excluded from the scope of the restriction: CF_3-X or X-CF_2-X', where X = -OR or -NRR' and X' = methyl (-CH_3), methylene (-CH_2-), an aromatic group, a carbonyl group (-C(O)-), -OR'', -SR'' or –NR''R'''; and where R/R'/R''/R''' is a hydrogen (-H), methyl (-CH_3), methylene (-CH_2-), an aromatic group or a carbonyl group (-C(O)-).

Source	Definition
TSCA Section 8(a)(7) data collection rule (EPA, 2023c)	Any chemical substance or mixture containing a chemical substance that contains at least one of the following three substructures: • R-(CF_2)-CF(R')R", where both the CF_2 and CF moieties are saturated carbons. • R-CF_2OCF_2-R', where R and R' can either be F, O, or saturated carbons. • $CF_3C(CF_3)$R'R", where R' and R" can either be F or saturated carbons.
TSCA New Chemicals Program PFAS framework (EPA, 2023a)	A chemical substance that contains at least one of these three structures: • R-(CF_2)-CF(R')R", where both the CF_2 and CF moieties are saturated carbons. • R-CF_2OCF_2-R', where R and R' can either be F, O, or saturated carbons. • $CF_3C(CF_3)$R'R'', where R' and R" can either be F or saturated carbons. Note: EPA considers both the substance itself and potential metabolites or degradants when reviewing a PFAS under TSCA Section 5.
Drinking Water Contaminant Candidate List 5 (EPA, 2022)	A chemical that is not perfluorooctanoic acid (PFOA) or perfluorooctane sulfonic acid (PFOS), which are already in the regulatory process, and contains at least one of these three structures: • R-CF_2-CF(R')(R''), where both the CF_2 and CF moieties are saturated carbons and none of the R groups is hydrogen. • R-CF_2OCF_2-R', where both the CF_2 and moieties are saturated carbons and none of the R groups is hydrogen. • $CF_3C(CF_3)$RR', where all the carbons are saturated and none of the R groups is hydrogen.
National PFAS Testing Strategy (EPA, 2021b)	A structure that contains the unit R-CF_2-CF(R')(R''), where R, R', and R'' are not hydrogen and the carbon-carbon bond is saturated (branching, heteroatoms, and cyclic structures are included).
OECD PFAS terminology report (OECD, 2021)	A fluorinated substance that contains at least one fully fluorinated methyl or methylene carbon atom (without any H/Cl/Br/I atom attached to it); with a few noted exceptions, any chemical with at least a perfluorinated methyl group (–CF_3) or a perfluorinated methylene group (–CF_2–) is a PFAS.
FY21 National Defense Authorization Act Section 332 (William M.	Man-made substances that are either single molecules in which all of the carbon atoms are fully fluorinated carbon atoms or mixtures of molecules

Source	Definition
(Mac) Thornberry National Defense Authorization Act for Fiscal Year 2021, 2021)	having fully fluorinated carbon atoms, partially fluorinated carbon atoms, and nonfluorinated carbon atoms.
Buck et al. 2011 (Buck et al., 2011)	Aliphatic substances containing one or more C atoms on which all the H substituents present in the nonfluorinated analogs from which they are notionally derived have been replaced by F atoms in such a manner that they contain the perfluoroalkyl moiety C_nF_{2n+1}–.

* Modified from the Report to Congress (NSTC, 2023)

3. State of the Science for Evaluating PFAS with NAMs

The term "NAM" refers to any technology, methodology, or combination thereof that can provide toxicological information useful for chemical screening and hazard assessment without reliance on traditional protocols based on vertebrate animals (EPA, 2021a; Fragki et al., 2023; Yao et al., 2026). NAMs can include in silico, in chemico, and in vitro methods, as well as non-testing approaches such as data reviews to support testing waivers. Considering the limited availability of toxicity data for PFAS (Shirke et al., 2026), the small number of PFAS and mixtures containing them that have been adequately studied, and the uncertainty surrounding the results from in vivo studies, NAMs have been identified as a promising tool for efficiently evaluating a large number of PFAS for toxicological potential (EPA, 2021b). Several NAMs have already been used to evaluate the effects of PFAS on human and environmental health. For example, predictions derived from in silico NAMs indicate that a subset of PFAS can be considered persistent, bioaccumulative, and/or toxic, summarized below in Section 3.1.2. In silico NAMs and in Appendix 1, Section 1.2. While the summaries provided in this document are not exhaustive, they highlight the fact that studies such as these using NAMs have been helpful in filling data gaps. A clearer understanding of how NAMs can be employed to this end and which NAMs should be used for testing will help advance their predictive capability for assessing PFAS toxicity.

3.1. NAMs for Evaluating PFAS Effects on Human Health

Over the past few years, several in vitro and in silico models have been utilized to predict PFAS hazard for different biological systems and to understand the underlying mechanisms. Examples that demonstrate the utility of NAMs for assessing PFAS hazard and dose-response are discussed below, with additional case examples provided in Appendix 1.

3.1.1. In Vitro NAMs

NAMs have been used to evaluate the effects of a subset of PFAS on the immune, endocrine, hepatic, renal, neurological, reproductive and developmental systems. For example, in vitro NAMs have been used to study potential links between PFAS and immunosuppressive activity:

- Houck et al. (2023) examined 147 PFAS to assess potential for immunosuppressive and other bioactivities using an in vitro platform including 12 different primary cell and coculture assays

encompassing 148 endpoints that evaluated multiple pathobiological states. The study found that 21% of the tested substances were considered bioactive in at least one assay. A subset of PFAS, including PFOA, demonstrated some similarities to reference immunosuppressant response profiles.

- Similarly, mixtures of six short- and/or long-chain PFAS were reported to reduce activation of multiple T cell types in vitro (T lymphocytes and basophils) in a human cell model (Maddalon et al., 2023).

Other NAMs have shown that PFAS can disrupt lipid accumulation and gene expression in human hepatocytes (Marques et al., 2022). Studies with NAMs have also demonstrated that PFAS can interact with metabolic enzymes, mitochondrial proteins, and oxidative stress pathways (Fang et al., 2010; Permadi et al., 1992). Studies conducted using cell-based models have identified perturbations in steroid hormone synthesis, thyroid receptor signaling, and/or hormone receptor agonism/antagonism after PFAS exposure (Chaparro-Ortega et al., 2018; Coperchini et al., 2021; De Toni et al., 2022; Hallberg et al., 2019; Huang et al., 2022; Paul Friedman et al., 2016). Two studies that used in vitro NAMs and zebrafish models to screen over 100 PFAS for developmental and neurodevelopmental toxicity reported activity in approximately 30% of the tested substances (Britton et al., 2024; Carstens et al., 2023).

A trend between chain length and toxicity potential has yet to be clearly determined across NAMs studies. While some studies report no associations, others have reported trends such as longer-chain PFAS being more bioactive in NAMs assays or polyfluoroalkyl alcohols with shorter chain lengths being more potent than longer-chain PFAS (Carstens et al., 2023; Cooper et al., 2025; Judson et al., 2024; Marques et al., 2022; Patlewicz et al., 2026; Patlewicz et al., 2024). In studies examining developmental effects, the presence of a long-chain PFAS or sulfonamide moiety was reported to be associated with increased development toxicity potential (Britton et al., 2024; Carstens et al., 2023; Gaballah et al., 2020; Han et al., 2021). Additional work is needed to determine whether these discrepancies may be explained by differences in the types of samples.

In aggregate, the studies summarized in this section have provided valuable information that will inform PFAS hazard.

3.1.2. In Silico NAMs

In silico NAMs include tools such as quantitative structure–activity relationship (QSAR) models, quantitative structure–property relationship models, read-across, machine-learning approaches, molecular dynamics, and TK modeling. These have been extensively applied to evaluate PFAS.

- Sosnowska et al. (2023) assessed 38 QSAR models to predict physicochemical, toxicological, and ecotoxicological properties of PFAS. The models were evaluated for compliance with OECD principles of good practice for developing QSAR models (JRC, 2020). Out of 38 models, 22 models were considered to be scientifically valid and reproducible such that they could be used for predicting properties of new PFAS.
- Generalized read-across approaches have been applied to predict toxicity by structure-based category and to study PFAS mixture toxicity (Gallagher et al., 2023; Patlewicz et al., 2024).
- Machine-learning approaches have been used to predict physicochemical parameters, TK parameters, toxicity, and bioactivity. For example, Dawson et al. (2023) developed a

machine-learning model to predict TK half-lives for 11 PFAS in multiple species, which predicted that 56% of the evaluated substances have half-lives longer than two months.

- Molecular docking, a technique to identify interactions between proteins and ligands, has been used to identify PFAS binding sites. Ng and Hungerbuehler (2015) used molecular docking to study perfluorooctane sulfonate binding to human serum albumin. Studies such as this may be informative for predicting PFAS biological targets or their amenability to in vitro test systems.
- TK modeling and physiologically based TK (PBTK) modeling have been widely used to predict exposure, toxicity, and bioaccumulation potential (Chou & Lin, 2019; Fenton et al., 2021; Kabadi et al., 2020; Limbu et al., 2024; Sweeney, 2022; Worley et al., 2017). A review by Deepika et al. (2022) described a framework for risk assessment of PFAS that integrated PBTK modeling with animal and epidemiological data to estimate reference doses, which were compared with tolerable daily intake values of different regulatory agencies. Furthermore, Chou et al. (2023) developed generic PBTK models for PFAS based on oral, inhalation, and dermal exposure routes, which were used to predict internal concentrations of the PFAS in humans across exposure routes.

In summary, these studies demonstrate that in silico models can provide informative toxicological information to evaluate PFAS toxicity potential in human health.

3.2. NAMs for Evaluating PFAS Effects on the Environment

Ecotoxicology studies have been undertaken to understand the effects of chemicals and other stressors on organisms and their habitats. The ever-increasing number of different PFAS and their potential mixtures combined with the large diversity of ecological species, life history strategy, and routes of exposure significantly complicate the development and use of NAMs to understand environmental health as compared to human health evaluations.

Many NAM-based ecotoxicological studies to date focus on small model organisms such as fish embryos and invertebrates. Studies using fish embryos can predict potential effects of PFAS during early stages of development as well as hazards to adult organisms. For example, studies using zebrafish (*Danio rerio*) embryos have shown how exposure to certain PFAS, including aliphatic sulfonic acid PFAS such as perfluorooctane sulfonate and PFHxS, can lead to a variety of apical adverse outcomes such as spinal deformities, edemas, altered heart rate, and behavioral effects (Britton et al., 2024; Gaballah et al., 2020; Han et al., 2021). Zebrafish embryos have also been used to evaluate correlations between specific PFAS structural features and metabolic pathway effects (Han et al., 2021).

Omics-based approaches have been utilized in ecotoxicology as part of a tiered approach to evaluate PFAS hazard using NAMs (Flynn et al., 2025; Rericha et al., 2024; Villeneuve et al., 2025b). For instance, Villeneuve et al. (2025a) generated transcriptional points-of-departure by evaluating whole-body transcriptomes of *Daphnia magna* exposed to 18 PFAS previously demonstrated to have bioactivity in a human nuclear receptor bioactivity assay (Houck et al., 2021). The transcriptional points-of-departure were then compared to points-of-departure derived from human cell-based assays to identify variations between species. While there were some differences noted between transcriptional points-of-departure and human cell line-derived points-of-departure, this high-throughput transcriptomics approach showed potential for application to assess ecotoxicological hazard.

Additional case examples highlighting the utility of NAMs to evaluate PFAS effects on the environment are discussed in Appendix 1.

4. Challenges and Data Gaps

The examples discussed above demonstrate the potential value of NAMs for accelerating PFAS chemical screening and hazard assessment. However, as with traditional animal studies of PFAS, challenges exist in the translation of NAMs data on these substances to human health and environmental hazard assessment.

One inherent challenge to implementing NAMs to study PFAS toxicity is that, at present, PFAS as a group do not have a common mechanism of action. Multiple molecular targets, receptors, and pathways are perturbed by PFAS to different degrees, even across structural subsets of the larger PFAS family, likely contributing to varied associated health effects. Therefore, it will be important to identify a battery of NAMs with broad biological coverage to evaluate the scope of PFAS hazard potential. Moreover, while some PFAS-associated mechanisms have been well-established (e.g., liver and lipid metabolism), others are less understood (e.g., thyroid effects). NAMs modeling these mechanistic or biological domains should be prioritized for future screening and hazard assessment.

Read-across is an important tool for predicting toxicity, particularly for data-poor chemicals. However, application of read-across to predict the toxicity of data-poor PFAS is challenged by factors such as route of exposure, variability in hazard, mechanisms of action across structurally similar PFAS, and variability in toxicokinetic properties within a shared structure-based grouping. Moreover, few PFAS spanning PFAS structural categories (e.g., the OECD PFAS structural classifications (OECD, 2021)) have any in vivo data, presenting challenges in providing sufficient anchors for comparison for facilitating read-across. A recent study reporting variability in point-of-departure values from traditional in vivo toxicity data suggested that structural consideration alone may not be sufficient for performing read-across without additional data such as toxicokinetic or mechanistic-related information (Patlewicz et al., 2024). Efforts are ongoing to use NAMs data to refine PFAS categories to support read-across.

The same inherent chemical properties that make PFAS useful in manufacturing also make them challenging to evaluate using in vitro NAMs in a laboratory setting. PFAS structural features and physicochemical properties such as longer perfluorinated carbon chains and high octanol:water partition coefficients may reduce cellular bioavailability in in vitro test systems due to extensive binding to serum proteins or plastic (Carstens et al., 2023; Leung et al., 2023; Smeltz et al., 2023). Moreover, established in vitro test systems are limited in their ability to replicate the complex interactions between PFAS and cellular components such as transporter proteins, serum albumin, and organic anion transporters, any of which could impact both the TK profiles of certain PFAS and their activity in vivo (Vujic et al., 2024). Recent efforts have provided experimental human TK data for more than 100 PFAS spanning different structural categories, expanding our knowledge of in vitro TK (Crizer et al., 2024; Kreutz et al., 2023; Smeltz et al., 2023). In future work, it will be important to consider TK properties of PFAS in the interpretation of in vitro data, especially in cases where inactivity is observed, and evaluate the effectiveness of using this information to build PBTK models for PFAS as described above.

Chemical feature descriptors of PFAS, which describe the configuration of molecules, are important for understanding and predicting exposure and toxicological potential. Several analytical chemistry

challenges remain in evaluating PFAS chemical properties, including lack of reference standards and lack of methods validated both within and among laboratories. In an effort to address the lack of mass spectrometry reference standards for PFAS, a large set of PFAS stock solutions was evaluated for amenability to high-throughput screening (Smeltz et al., 2023). This study flagged 57 out of 205 PFAS for detection issues, likely explained by properties such as low boiling point, high vapor pressure, or confounding effects of the use of dimethyl sulfoxide as a solvent. Efforts are ongoing to address these issues, and the publication by the National Institute of Standards and Technology of a PFAS mass spectrometry database is a welcome development (Ragland & Place, 2023). Nonetheless, the large number of PFAS and complexity of physicochemical properties remains a challenge for acquiring reliable analytical chemistry information, which ultimately will be critical for information on exposure and hazard potential.

The translatability of PFAS NAMs data into a traditional framework of testing and hazard assessment will depend on the availability of data for model qualification and validation. Qualification and validation establish confidence in a NAM by exploring the translatability of its data with reference chemicals and assessing its repeatability and reproducibility across laboratories (Pappalardo et al., 2022). In the absence of sufficient qualifying data and analysis methods for human and ecological translation, toxicological information from NAM-based models may not be readily applicable to risk assessment. While there have been efforts to modify and repurpose existing in vitro and in silico models for PFAS toxicity prediction (Pappalardo et al., 2022), model qualification and validation will remain a challenge in the absence of adequate and relevant data, particularly for in silico approaches such as QSAR or PBTK models.

Finally, assessment of PFAS toxicity is complicated by the fact that mixtures of PFAS commonly occur in environmental media that also include other environmental pollutants such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and dioxins. While NAMs could be useful for hazard assessment of mixtures containing PFAS, the very large number of PFAS and the exponentially larger number of potential mixture combinations with other compounds present a challenge for understanding and expanding the utility of NAMs for evaluating PFAS toxicity. For example, it would be impossible to attribute specific effects to a single chemical or class of PFAS without testing individual components of an environmental mixture. Recent studies suggest the effects of PFAS mixtures can be additive (Addicks et al., 2023; Ríos-Bonilla et al., 2024), although more work is needed to understand the mathematical relationship (e.g., additive, synergism, etc.) underlying a PFAS mixtures effect. With the high likelihood of exposure to PFAS occurring via exposure to a mixture and the potential for components of that mixture to act to increase toxicity of the PFAS, future work should focus on prioritization strategies for testing PFAS mixtures, which should consider factors such as PFAS exposure and differences in toxicity effects across the PFAS chemical space.

Because of the challenges described above, experts generally concur that PFAS should not be considered to comprise a single grouping for risk assessment purposes. It may be beneficial to apply some simplifying assumptions, and smaller and more specific grouping strategies could prove useful (Anderson et al., 2022). These principles are being applied to efforts to prioritize the evaluation and selection of NAMs and support human health and the environment (Patlewicz et al., 2024; Patlewicz et al., 2022; Williams et al., 2022).

5. Conclusion: Regulatory and Future Directions

PFAS are a large and variously defined group of chemicals with diverse physicochemical properties, structures, and hazards. Their diversity and characteristics make them uniquely challenging for toxicity testing, and thus only a few have been well-studied, indicating that the development of a standardized taxonomy for PFAS may be beneficial to the conduct and prioritization of human-relevant studies. Conducting the studies needed to adequately characterize the toxicity of even a fraction of existing PFAS in laboratory animals would take too long to satisfy the requirements of state and federal agencies and the sense of urgency from the public. The financial cost of traditional models would also be a barrier. Thus, although some animal studies may have to be conducted, in vitro and computational approaches will be a very important source of data in the near future for initial characterization and prioritization of PFAS with regards to safety considerations for human or ecological health (Mumtaz et al., 2021). Given some of the understood limitations on individual NAMs approaches, it is critical for the scientific community to engage in developing and validating human-relevant integrative/combinatorial NAMs and subsequently evaluating the strengths and limitations of those models for PFAS testing. It is likely that new in vitro and computational approaches will need to be developed to help address the challenge related to the diversity of species that need to be tested as well as the diversity of PFAS.

Recent efforts by national and international regulatory agencies to apply NAMs to characterize PFAS toxicity have facilitated the screening of a broad range of PFAS compounds, identifying potential hazards that might have been missed using traditional methods. Advancements such as high-throughput transcriptomic approaches (Gutsfeld et al., 2024; Rericha et al., 2024), the EPA Transcriptomic Assessment Product for human health assessment that reduces animal use for estimating chronic toxicity values (EPA, 2024a, 2024b), and availability of high-throughput screening data in EPA's CompTox Chemicals Dashboard (EPA, 2026) will enhance our ability to evaluate PFAS toxicity potential and provide insight into their complex cellular interactions. Future work should focus on understanding the diversity of PFAS chemical properties, assessing effects of PFAS co-exposures, comparing NAMs results to traditional testing outcomes and validating the assays for regulatory use, and relating NAM results to human exposure. Each of these activities could be expected to increase confidence in NAMs for PFAS toxicological assessment. Integrative tools such as adverse outcome pathways may be useful to link molecular initiating events associated with PFAS to specific toxicity outcomes. Future studies evaluating susceptible and vulnerable subpopulations will also be critical for a comprehensive assessment of PFAS toxicity potential.

Overall, NAMs have the potential to contribute to the development of cost- and time-efficient and high-quality testing and safety assessment approaches to support regulatory processes and development of relevant, fit-for-purpose strategies for PFAS.

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Appendix

Exposure to per- and polyfluoroalkyl substances (PFAS) has been associated with effects on multiple organs, such as the liver and kidneys, due to altered lipid metabolism and active renal tubular reabsorption, respectively (Fenton et al., 2021; Starnes et al., 2022). Beyond these organs, PFAS exposure has also been linked to immune suppression (Fenton et al., 2021) and neurological effects including impaired memory and neurodevelopment, as some PFAS may cross the blood-brain barrier (Starnes et al., 2022). Further, the effects of PFAS may vary significantly across different ecosystems because of differences in local concentration, the specific chemical structures present, and the extent of occurrence (Kannankeril Khalid & Sauvé, 2026)**.** This Appendix describes examples of new approach methodologies (NAMs) that assess effects on targets or biological pathways associated with various human health endpoints and environmental effects. **This is not intended to be an exhaustive, systematic review.** Rather, it provides a snapshot of selected work that is ongoing in the NAMs space, and highlights opportunities for further development and application of NAMs for research and health assessment of PFAS. The Appendix highlights key results of research database (PubMed and other scientific databases) searches with search terms "NAM" or “new approach methodologies” AND "PFAS" conducted up to August 2024.

1. NAMs for Evaluating PFAS Effects on Human Health

1.1. In Vitro NAMs

1.1.1. Immunotoxicity

- The National Toxicology Program concluded in 2016 that both perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) are “presumed to be an immune hazard to humans” based on the suppression of antibody response in animal studies and a moderate level of evidence from epidemiology studies (NTP, 2016).
- A similar conclusion was reached in March 2023 in an evidence synthesis by the U.S. Environmental Protection Agency (EPA) (EPA, 2025). Specifically, EPA stated that exposure to the compounds is “likely to cause adverse immune effects, specifically immunosuppression, in humans under relevant exposure circumstances.”
- A systematic evidence map assessing chronic inflammatory and immunosuppressive effects of PFAS identified numerous mechanistic linkages, including increased cytokine production, decreased B cell activation, and altered levels of T cell subtypes and immunoglobulins (Zhang et al., 2023).
- Several studies have noted alterations in T cell function and cytokine signaling following exposure to certain PFAS at concentrations comparable to human exposure levels. Using human immune cells exposed to PFAS in vitro, Corsini et al. (2011) and Corsini et al. (2012) demonstrated that several PFAS affected immune cell activation and reduced pro- and anti-inflammatory cytokine production. These studies identified a role for the peroxisome proliferator-activated receptor (PPAR) in PFOA-induced inhibition of cytokine secretion in human cells. They also found that perfluorinated compounds can inhibit in vitro cytokine production and activation of NF-kB transcription factors via a PPAR-independent pathway.

1.1.2. Liver Effects, Lipid Metabolism, and Endocrine Disruption

A broad range of toxicology studies (e.g., epidemiological, animal studies, NAM-based) supports an association between PFAS exposure and endocrine disruption and liver disease. Using NAMs, certain PFAS have been reported to mimic endogenous fatty acids, disrupting endocrine metabolism and transport (Convertino et al., 2018; Vanden Heuvel et al., 2006). Houck et al. (2021) evaluated 142 PFAS using a NAMs screening platform evaluating transcription factor activities and found activity associated with known PFAS targets, including estrogen receptor-α, PPARα, and PPARγ. Moreover, using human blood samples and nuclear magnetic resonance spectroscopy to quantitate lipoprotein fractions, it was observed that PFAS that fractionate with low-to-intermediate density lipoproteins mimic the molecular action of human fatty acids (Haug et al., 2023). PFAS may affect a variety of signaling pathways relevant to liver health, including PPARs (liver receptors), the constitutive androstane receptor, and the Vitamin D receptor. PFAS administration has been also associated with increases in biomarkers of oxidative stress in the liver and other tissues (Fang et al., 2010; Kamendulis et al., 2014; Permadi et al., 1992), effects that are often associated with observation of apoptotic or necrotic changes. Increased production of reactive oxygen species was also noted in HepG2 cells (immortalized human liver cells) treated with PFOS and PFOA, although perfluorobutanesulfonic acid and perfluorohexanoic acid did not induce reactive oxygen species under the study conditions (Eriksen et al., 2010). Another study noted increased reactive oxygen species production in HepG2 cells after treatment with perfluorohexane sulfonate (PFHxS), PFOS, PFOA, perfluorononanoic acid (PFNA), perfluorodecanoate (PFDA), and perfluoroundecanoic acid, but not perfluorododecanoic acid, at doses as low as 2 µM (Wielsøe et al., 2015). PFAS-associated increases in reactive oxygen species production may be due to disruption of mitochondrial membrane potential due to interference with mitochondrial respiration, as has been demonstrated in vivo and in vitro.

A NAMs study of 30 PFAS chemicals used human hepatocytes, high-throughput transcriptomics, deep learning of cell morphology, and liver enzyme leakage assays to predict human liver injury, identifying links between transcriptomic pathway potencies and liver injury and filling mechanistic knowledge gaps for PFAS using NAMs (Mauge-Lewis et al., 2025). Continued improvement in understanding of PFAS-induced hepatotoxicity will support development of predictive NAMs able to model PFAS-induced liver disease traits, which will in turn facilitate development of greener chemistries, hazard identification, and human and ecological health assessments.

1.1.3. Renal Toxicity

Kidney disease, including renal dysfunction, nephritis, chronic kidney disease, and cancer, have been linked to PFAS exposure (ATSDR, 2021; Fenton et al., 2021). A study documenting chronic kidney disease deaths in fluorochemical workers prompted concerns that PFAS exposure may be associated with kidney disease (Steenland & Woskie, 2012). A re-evaluation of PFOA by the International Agency for Research on Cancer noted limited evidence for PFOA association with renal cell carcinoma, citing strong mechanistic evidence including epigenetic alterations, immunosuppression, oxidative stress, receptor-mediated effects, cell proliferation or death, and cellular nutrient supply (Zahm et al., 2024). Stueckle et al. (2023) acutely exposed a renal epithelial cell line (RPTEC-OAT1) to five aqueous film-forming foams (AFFFs) and seven PFAS chemicals to characterize hazards of these chemicals related to several key characteristics of carcinogens (Stueckle et al., 2022). The study found that acute AFFF exposure at the several hundred-ppm level induced cytotoxicity, while PFOS and PFOA exhibited cytotoxicity at 74 and 200 ppm, respectively, concentrations lower than all other tested shorter-chain PFAS. On an estimated equal-mass basis based on AFFF total fluorine levels, AFFF showed greater cytotoxicity than PFOS and

PFOA. PFOS and PFHxS induced mitochondrial depolarization potential with little oxygen radical generation. Follow-up RNA sequencing with pathway analysis found that AFFF exposure altered cancer-, kidney-, and liver-disease signaling pathways, alterations that were observed to be accompanied by underlying changes in cell organization, lipid metabolism, cell cycle, and survival (Stueckle et al., 2023). Studies using rodent and human renal tubule epithelial cells with overexpressed organic anion, cation, and protein transporters have examined the role of renal transport mechanisms in PFAS toxicity (Niu et al., 2023; Ryu et al., 2024). More studies are needed to understand the molecular interaction of PFAS with kidney transporters across the diverse PFAS chemical space.

1.1.4. Reproductive and Developmental Toxicity

The effects of PFAS on male and female reproductive systems have been extensively studied using in vitro models (Ding et al., 2020; Shi et al., 2024). As reviewed in Shi et al. (2024), human sperm treated with PFOS in vitro had decreased ability to penetrate synthetic mucus. Similarly, sperm treated with PFOA showed decreased penetration ability and acrosome response, likely due to chemical effects on luteinizing hormone-mediated calcium signaling (Yuan et al., 2020). Using cultured porcine granulosa and theca cells, Chaparro-Ortega et al. (2018) demonstrated that PFOA and PFOS altered steroidogenic secretion, which inhibited steroid hormone production. Qiu et al. (2021) evaluated effects of PFOS on testosterone biosynthesis using a mouse primary Leydig cell model. They found that PFOS reduced sperm count and testosterone levels, and inhibited the interaction between transcription factor CREB and cofactor CRTC2. A study by Hallberg et al. (2019) reported that PFNA exposure decreased overall survival of bovine oocytes and reduced developmental competence of surviving oocytes.

While the effects of PFAS exposure on developmental outcomes are unclear, several PFAS are known to cross the placental barrier, and epidemiological findings suggest potential associations between fetal growth restrictions and immune function (Blake & Fenton, 2020; Rappazzo et al., 2017). Using a zebrafish model to evaluate developmental toxicity, associations have been identified between certain PFAS structural properties and developmental toxicity endpoints such as impaired swim bladder inflation and photomotor response (Gaballah et al., 2020; Truong et al., 2022; Ulhaq et al., 2013; Zheng et al., 2012). Key structural properties correlated with these effects appeared to include longer carbon chain length and presence of a sulfonic functional group. The same model exhibited correlations between increasing carbon chain length and induction of a hyperactivity endpoint (Gaballah et al., 2020). Additional studies of developmental toxicity using NAMs will clarify developmental toxicity potential of PFAS.

1.1.5. Thyroid Effects

While studies have reported the association of PFAS exposure with both reduced and increased thyroid hormone levels as well as changes in thyroid size, enlarged thyroid gland, and thyroid cancer, some findings are inconsistent between animals and humans (Coperchini et al., 2020; van Gerwen et al., 2024). NAMs have expanded the scope of evaluating effects of PFAS on the thyroid. De Toni et al. (2022) suggested that legacy and new-generation PFAS, such as PFOA, PFOS, and the new-generation PFAS C6O4, can differentially influence thyroid stimulating hormone-dependent signaling pathways through direct interaction with the thyroid stimulating hormone receptor. Gene expression studies by Zhang et al. (2021) revealed that PFOA, GenX, and 4,8-dioxa-3H-perfluorononanoic acid altered gene expression in both rat and human-derived thyroid cells, with effects seeming to be both PFAS- and cell type-dependent. Conti et al. (2020) suggested that disruption of iodide homeostasis in thyroid cells may be a potential mechanism for thyroid health effects of PFOS. In a NAMs study of 136 PFAS tested in nine

in vitro thyroid assays, 85 PFAS demonstrated bioactivity in at least one assay, with a few PFAS that bound to human thyroid hormone protein transthyretin showing potency effects similar to those of known inhibitors (Degitz et al., 2024).

1.2. In Silico NAMs

The utility of in silico approaches to evaluate effects and underlying mechanisms of PFAS has drastically expanded over the past several years due to continuous advancements in the field of predictive modeling and computational toxicology.

- A study by Sosnowska et al. (2023) validated 22 out of 38 quantitative structure–activity relationship (QSAR) models for predicting biological activity of PFAS.
- Generalized read-across approaches were used to predict toxicity effects of perfluoroalkyl acids having carbon chains containing seven to nine carbon atoms and high binding affinity to human serum albumin, based on 10 structural analogs for each (Gallagher et al., 2023). Patlewicz et al. (2024) used read-across approaches in a study of over 15,000 PFAS, which were categorized into structure-based classifications and evaluated for trends related to in vivo toxicity outcomes (EPA, 2024). The authors found variability in in vivo point-of-departure values by category, and concluded that the structural considerations may not be sufficient for read-across without additional input data such as toxicokinetic information.
- Unsupervised deep learning models have been applied to visualize the chemical space of PFAS by clustering similar structures and linking related classes in a “PFAS Atlas” (Su et al., 2024). Abrahamsson et al. (2022) used a machine-learning model to predict the concentration ratio between umbilical cord blood and maternal blood, predicting 3623 PFAS compounds to be preferably partitioned into umbilical cord blood. In 2019, Cheng and Ng (2019) built machine-learning-based QSAR models to predict the bioactivity for 3486 PFAS, using as training data bioactivity information on 1012 PFAS in 26 bioassays. By applying transfer learning, Feinstein et al. (2021) established a machine-learning model to predict LD50s of PFAS. The model was trained with a data set of oral rat LD50s for 519 PFAS-like compounds, specifically fluorinated compounds containing two or more C-F bonds.
- Molecular docking, molecular dynamics, and free energy binding calculations have been applied to examine potential interactions between PFAS and the Vitamin D receptor, critical for immune and endocrine function, as well as oxidative stress targets (Azhagiya Singam et al., 2023; Rajak & Ganguly, 2023).
- Fit-for-purpose toxicokinetic and physiologically based toxicokinetic (PBTK) approaches have also been developed to predict exposure and evaluate specific toxicological endpoints. These models are particularly important because extrapolating data from animal studies is complex considering that PFAS half-lives vary significantly between species (Fenton et al., 2021). Loccisano et al. (2011) developed a PBTK model for PFOA and PFOS in monkeys and humans based on multicompartmental models previously developed by Andersen et al. (2006) and Tan et al. (2008). This model has been extended to pregnancy and lactation life stages for humans (Loccisano et al., 2013). To improve on the Loccisano et al. (2011) model, which overestimated concentrations of PFOA and PFOS in tissues of people from the Tarragona region in Spain (Domingo et al., 2012a; Domingo et al., 2012b), Fàbrega et al. (2014) developed a human PBTK model for PFOA and PFOS with tissue-to-plasma partition coefficients based on detected concentration levels from human tissues. Worley et al. (2017) developed a new PBTK model for

PFOA, with renal excretion and reabsorption of PFOA included based on in vitro data. Both the Worley et al. (2017) and Loccisano et al. (2011) models provided acceptable predictions of PFOA concentrations in plasma. More recently, Chou and Lin (2019) developed a PBTK model of PFOS for rat, monkey, and human with Bayesian statistical analysis using Markov chain Monte Carlo simulation. The model has characterized the uncertainties and interspecies variabilities for chemical-specific parameters. Kim et al. (2018) and Kim et al. (2019) developed PBTK models of PFHxS, PFNA, and PFDA in rats and extrapolated these models to human with allometric scaling equations for chemical-specific parameters. Furthermore, Sweeney (2022) further validated the PFHxS PBTK model for humans using serum and urine concentration data from biomonitoring studies, including data from the U.S. Centers for Disease Control's National Health and Nutrition Examination Survey.

2. NAMs for Evaluating PFAS Effects on Environment

Several groups have undertaken the challenge of developing models to examine the effects of PFAS on the environment. This is a complex effort due to the need to consider diverse ecological contexts and routes of exposure.

- Villeneuve et al. (2023) evaluated the use of in vitro screening to identify and predict in vivo estrogenic effects of five PFAS compounds using adult fathead minnows (Pimephales promelas). Their work showed that, when differences in bioconcentration were taken into account, in vitro screening was predictive of in vivo estrogenic effects.
- Tanabe et al. (2024) explored the effects of PFAS mixtures on embryonic and larval sheepshead minnows (Cyprinodon variegatus), suggesting that antagonistic interactions between PFOA and PFOS may reduce mixture toxicity in larvae through reduced oxidative stress.
- It is also important to understand the effects of PFAS to invertebrates due to their key roles in ecosystems. Kadlec et al. (2024) examined the effects of 17 PFAS on three different freshwater invertebrate species. Their results suggested that mechanisms of PFAS toxicity may differ depending on species and chemical classes.
- Villeneuve et al. (2026a) and Villeneuve et al. (2026b) used a transcriptional point-of-departure approach to explore PFAS effects in both Daphnia magna and Pimephales promelas, highlighting differences between species.
- Albers and colleagues linked lipidomics to morphology and behavior to better understand how PFAS-induced changes at the molecular level, particularly on lipids, could be used to explain adverse apical effects (Albers et al., 2024).

3. Mixtures

NAMs can be used to evaluate potential toxicity associated with PFAS mixtures.

- A critical review of a diverse literature set by Kim et al. (2024) suggested involvement of epigenetic mechanisms in toxicity induced by PFAS mixtures. Data reviewed included human epidemiological studies; studies of in vitro models using neuroblasts, preadipocytes, and hepatocytes; and animal studies that used both mammals and aquatic model organisms.

- A set of broadly used and accepted cytotoxicity assays was used to study cytotoxicity effects of individual and binary PFAS mixtures. The results showed that exposure to PFOA, PFOS, PFDA, PFNA, and PFHxS individually and in binary combinations caused concentration-dependent cytotoxicity to HepG2 cells (Ojo et al., 2021).
- A human liver cell line (HepG2) was employed to study cytotoxicity (cell viability) and genotoxicity (DNA damage) of selected PFAS including PFNA, PFOS, PFDA, PFOA, and PFHxS (Kim et al., 2024). The interaction toxicities of these PFAS in binary mixtures were determined using the additive index approach. The results revealed that exposure to these substances singly and in binary mixtures induced a concentration-dependent decrease in cell viability. This group also used the alkaline comet assay to evaluate the potential of PFAS and their mixtures to induce DNA damage. The results revealed that exposure to single and binary mixtures caused a moderate increase in cellular DNA damage, but no dose–response relationship was established.
- Ojo and colleagues found that, overall, PFAS caused a concentration-dependent decrease in cell viability but only a modest increase in cellular DNA damage (Ojo et al., 2020, 2021).
- To better understand the diversity of molecular targets of both legacy and new-generation PFAS, Robarts et al. (2023) conducted a comparative toxicogenomics analysis of the gene expression changes in the livers of mice exposed to 11 PFAS. A comparison of activation of PPAR, a common target of many PFAS, by the 11 substances showed that most of the alternative PFAS modulated molecular targets that overlapped with legacy PFAS.
- To address the sheer number of potential PFAS mixtures and the lack of basic toxicity data for a majority of them, machine-learning models have been developed and are undergoing refinement and validation. These new NAM techniques will be helpful in advancing the toxicity assessment methods of mixtures of PFAS and other environmental pollutants (Limbu et al., 2024).

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