


Review of the zebrafish as a model to investigate per- and polyfluoroalkyl substance toxicity

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Abstract

The existence of thousands of per- and polyfluoroalkyl substances (PFAS) and evidence that some cause adverse health effects has created immense need to better understand PFAS toxicity and to move beyond one-chemical-at-a-time approaches to hazard assessment for this chemical class. The zebrafish model enables rapid assessment of large libraries of PFAS, powerful comparison of compounds in a single *in vivo* system, and evaluation across life stages and generations, and has led to significant advances in PFAS research in recent years. The focus of this review is to assess contemporary findings regarding PFAS toxicokinetics, toxicity and apical adverse health outcomes, and potential modes of action using the zebrafish model. Much of the peer-reviewed literature has focused on a small subset of PFAS structural subclasses, such as the perfluoroalkyl sulfonic acids and perfluoroalkyl carboxylic acids. However, recent data on more diverse PFAS structures are enabling prioritization of compounds of concern. Structure-activity comparisons and the utilization of modeling and 'omics technologies in zebrafish have greatly contributed to our understanding of the hazard potential for a growing number of PFAS and will surely inform our understanding and predictive capabilities for many more PFAS in the future.

Keywords: PFAS; developmental toxicity; toxicokinetics; mode of action; mechanisms

Per- and polyfluoroalkyl substances (PFAS) are a broad class of anthropogenic chemicals defined by carbon-hydrogen bonds replaced in part (polyfluorinated) or in full (perfluorinated) by carbon-fluorine bonds (Buck *et al.*, 2011). More specifically, PFAS are any fluorinated substances consisting of at least one fully fluorinated methyl (-CF₃) or methylene (-CF₂-) group (Wang *et al.*, 2021). The strength of the C-F bond imparts high chemical stability and oleophobic and hydrophobic properties that are highly beneficial for many industrial, manufacturing, and consumer product uses. Most recognizably, PFAS have been used as components in aqueous film-forming fire-fighting foams (AFFFs) and in coatings for textiles, cookware and food packaging; however, there are numerous other applications related to aerospace, electronics, the energy sector, and more (Gluge *et al.*, 2020). The C-F bond is highly persistent in the environment, resisting degradation (Buck *et al.*, 2011). Thus, PFAS are detected globally in drinking, surface, and groundwater (Kurwadkar *et al.*, 2022), food (Domingo and Nadal, 2017; Pasecnaja *et al.*, 2022), biota, and in human tissues (De Silva *et al.*, 2021; Jian *et al.*, 2018). The ubiquity of human exposure to PFAS paired with evidence that some cause adverse health effects has raised public concern internationally regarding the toxicity and continued use of this chemical class (Cousins *et al.*, 2020).

A significant amount of toxicological data is available for a select few PFAS, such as perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS). PFOA and PFOS are 2 legacy PFAS (ie, long-chain perfluoroalkyl acids that have been largely

phased out of production in many developed countries due to growing evidence of persistence and health effects) with 7- and 8-fully-fluorinated-carbon chains, respectively. They induce a range of adverse health effects, such as low birth weights, liver, kidney, and thyroid disease, and immune dysfunction, evidenced by both epidemiological and animal studies (Fenton *et al.*, 2021). Widespread public and government concern prompted major PFAS manufacturers in the United States (U.S.) to initiate phase-outs of PFOS between 2000 and 2002 and of PFOA between 2006 and 2015 (USEPA, 2022b). Such phase-outs have generally decreased the concentrations of these long-chain PFAS in human biomatrices, most often measured in blood and serum (Land *et al.*, 2018). However, declining concentrations over the past 2 decades are not universal, depending on chemical and region (Fan *et al.*, 2022), and organ-specific trends are not as well-understood. Additionally, there is evidence of steady or increasing concentrations of legacy PFAS in biota, with implications for human dietary exposure (Barbo *et al.*, 2023; Schultes *et al.*, 2020). Phase outs have also led to the adoption of alternative, often shorter-chain PFAS typically with limited toxicity data (Ateia *et al.*, 2019; Brendel *et al.*, 2018). The U.S. Environmental Protection Agency (EPA) recently announced proposed drinking water regulation for 6 PFAS (ie, PFOS, PFOA, perfluorohexane sulfonic acid [PFHxS], perfluorobutane sulfonic acid [PFBS], perfluorononanoic acid [PFNA], and GenX) (USEPA, 2023), and total PFAS concentrations in drinking water are regulated in the European Union (EU, 2022). While perfluoroalkyl carboxylic acids (PFCAs;

eg, PFOA) and sulfonic acids (PFSAs; eg, PFOS) have been the most frequently studied and have the most evidence to support such regulation, PFAS are a diverse chemical class with many emerging contaminants (Table 1). A more complete scientific understanding of PFAS toxicity will not happen without comprehensively examining a much broader representation of PFAS structural diversity.

The PFAS chemical class consists of thousands of compounds. In 2018, the Organization for Economic Co-operation and Development (OECD) identified 4730 PFAS with unique CAS numbers (OECD, 2018), and more than 12 000 PFAS structures have been identified by the U.S. EPA (USEPA, 2022a). A one-chemical-at-a-time approach to all PFAS toxicity assessment and regulation based on mammalian testing is not feasible, given the low-throughput nature of this testing and the large number of PFAS without toxicity data. *In vitro* and *in silico* models are increasingly important but are limited in biological complexity. Therefore, we need an *in vivo* model capable of assessing PFAS for potential human health hazards. The zebrafish is a widely used alternative model that can rapidly assess the toxicity of these chemicals. The zebrafish is robust and can bridge the gap between *in vitro* and higher vertebrate models; it is amenable to high-throughput developmental toxicity testing and intergenerational assessments. Embryos develop rapidly and transparently, are genetically tractable, and manifest simple assayable behaviors as early as 24 h post fertilization (hpf). Thus, zebrafish can be leveraged as a whole animal biosensor, to quickly rank PFAS hazard potential, prioritize compounds of high concern for additional testing, and characterize toxicity. Assessing a broad diversity of PFAS in a single system offers a powerful platform that eliminates the challenges of cross-species extrapolation and enables direct comparisons between compounds. This review addresses PFAS structure-activity relationships pertaining to toxicokinetics and toxicity, apical adverse health outcomes, and potential modes of action using the zebrafish model. Throughout this review, all waterborne exposure concentrations have been converted to molar units to facilitate comparisons between studies (Supplementary Table 1). To facilitate readability, chemical abbreviations are used throughout the review, and full chemical names can be found in Table 2.

Exposure and toxicokinetics

The trope that all PFAS are “forever chemicals” stems from the incredible strength of the C-F bond. Perfluoroalkyl acids, like PFOS and PFOA, are highly resistant to degradation and metabolism, and considered terminal degradation products (Buck et al., 2011). Half-lives in humans exposed to highly contaminated drinking water have recently been estimated between 1.05–3.4 years for PFOS and 1.77–3.9 years for PFOA, typically measured in serum (Li et al., 2018; Worley et al., 2017; Xu et al., 2020; Li et al., 2019). However, growing evidence shows that resistance to degradation in the environment and metabolism *in vivo* is not characteristic of all PFAS; many compounds are considered precursors that may be broken down into terminal products. A better understanding of PFAS absorption, distribution, metabolism, and excretion (ADME) is essential to understand the hazards posed by this chemical class (Tal and Vogs, 2021) and has been the focus of numerous recent studies.

Developmental zebrafish Bioconcentration

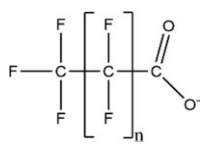
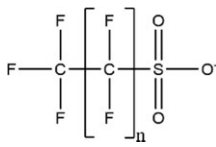
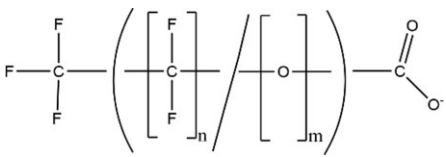
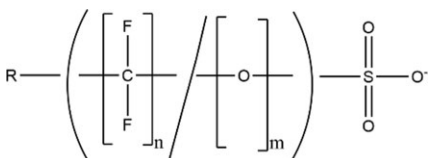
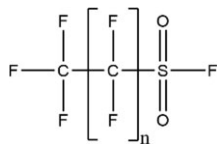
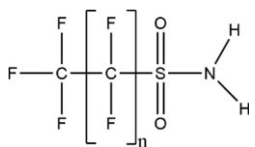
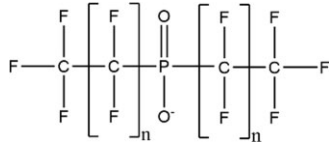
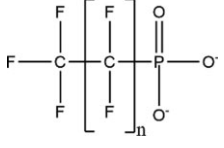
Recent studies in developmental zebrafish have related structural features to bioconcentration factor (BCF), a cumulative measure of the competing uptake and elimination rates that encompasses biotransformation of the parent compound (Amot and Gobas, 2006). Developmental zebrafish exposure studies addressed in this and following sections typically occur aqueously from 1 to 5 days post fertilization (dpf), a window that encompasses key events such as neurogenesis, cardiovascular, hepatic (3 dpf) and gill development (Villeneuve et al., 2014). The dynamic nature of this life stage is beneficial for interrogation of chemical perturbation and is important to consider when interpreting developmental exposure data.

Among PFAS with similar chain length, the highest bioconcentration potential is ranked as follows: compounds with sulfonamide head groups (Han et al., 2021; Rericha et al., 2022a) > sulfonic acids > fluorotelomer sulfonic acids (Menger et al., 2020; Rericha et al., 2022a) > carboxylic acids (Gaballah et al., 2020; Han et al., 2021; Menger et al., 2020; Rericha et al., 2022a; Vogs et al., 2019) and emerging fluoroethers, such as GenX (Gaballah et al., 2020; Satbhai et al., 2022). For instance, embryonic exposure to a mixture of 9 PFAS (2–3.8 μM of each) yielded BCF values for PFHxS, 6:2 FTSA, and PFPeA of 100, 35, and 0.9, respectively (Menger et al., 2020). For PFAS with the same head group, bioconcentration increased with increasing fluorinated chain length (Gaballah et al., 2020; Han et al., 2021; Menger et al., 2020). For those with carboxylic acid groups, BCF values of 0.9, 3.0, 18, 100, and 610 were observed for the 4-fluorinated-carbon compound (PFPeA) through the 8-fluorinated-carbon (PFNA) (Menger et al., 2020). Similarly, sulfonic acid PFAS exhibited BCF values of 230 and 2600 for the 6- and 8-fluorinated-carbon compounds PFHxS and PFOS, respectively, following approximately 0.8 μM exposures (Vogs et al., 2019). GenX was consistently reported to have a low BCF relative to other PFAS and was rapidly eliminated (Gaballah et al., 2020; Satbhai et al., 2022).

Interestingly, an inverse relationship between BCF and exposure concentration across a variety of PFAS has recurred across studies (Gaballah et al., 2020; Han et al., 2021; Satbhai et al., 2022; Vogs et al., 2019). For example, PFOS exhibited a BCF of 1348 at 1.8 μM , but a BCF of 684 at 3.1 μM (Gaballah et al., 2020). This trend may be attributed to the saturation of substrate binding sites that facilitate uptake. Considering other properties, no trends have been identified between PFAS BCF and L-FABP (liver-fatty acid binding protein) binding affinity, with only a weak positive association to $\text{Log } K_{ow}$. Instead, retention time in an HPLC C18 analytical column, which approximated hydrophobicity, positively correlated with BCF (Han et al., 2021). While exposure paradigms varied somewhat between studies, BCF trends related to carboxylic acid or sulfonic acid head group and fluorinated chain length largely agree.

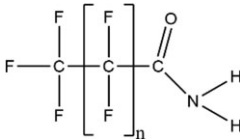
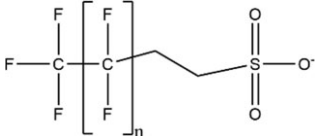
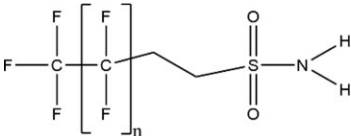
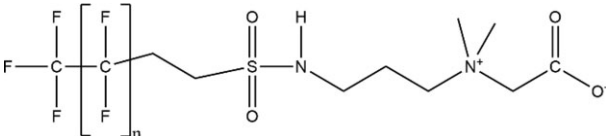
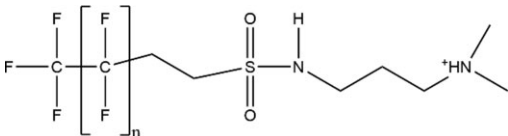
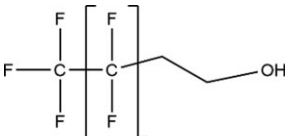
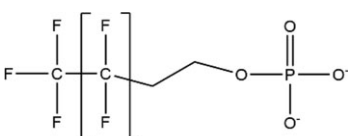
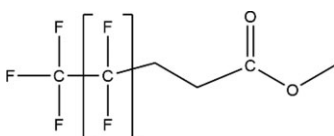
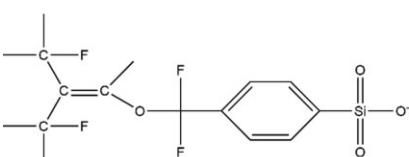
In addition to PFCAs and PFSAs, lesser-studied emerging alternatives, such as perfluoropolyether carboxylic acids (PFECAs) also bioconcentrate. Internal PFECA concentrations increased with an increasing number of OCF_2 moieties. PFOSDoDA, with 5 OCF_2 moieties and a total of 6 fluorinated carbons, bioconcentrated more readily than homologs with 4 or 3 ether moieties and more than PFOA after exposure to nominal concentrations of 90, 106, 128, and 97 μM , respectively (Wang et al., 2020). Han et al. also reported another PFECA, PFO3TDA, as particularly bioaccumulative (Han et al., 2021). Relatively high BCF values for PFECAs suggest some polyether alternatives may not be

Table 1. PFAS subclasses and structures

Subclass	Representative Structure	Compounds Discussed in This Review
Perfluoroalkyl Carboxylic Acids (PFCAs)		PFBA ($n = 2$; CAS: 375-22-4) PFPeA ($n = 3$; CAS: 2706-90-3) PFHxA ($n = 4$; CAS: 307-24-4) PFHpA ($n = 5$; CAS: 375-85-9) PFOA ($n = 6$; CAS: 335-67-1) PFNA ($n = 7$; CAS: 375-95-1) PFDA ($n = 8$; CAS: 335-76-2) PFUnDA ($n = 9$; CAS: 2058-94-8) PFDoA ($n = 10$; CAS: 307-55-1) PFHxS ($n = 5$; CAS: 355-46-4) PFOS ($n = 7$; CAS: 1763-23-1) Cl-PFOS ^a (CAS: not available)
Perfluoroalkyl Sulfonic Acids (PFSA)		GenX ^a (CAS: 13252-13-6) PFMOBA ($n = 5, m = 1$; CAS: 863090-89-5) PFDMMOBA ^a (CAS: 801212-59-9) PFO2HpA ($n = 3, m = 2$; CAS: 151772-58-6) ADONA ^a (CAS: 958445-44-8) PFO3TDA ($n = 8, m = 3$; CAS: 330562-41-9) PFO3DA ($n = 5, m = 3$; CAS: 151772-59-7) PFO3OA ($n = 3, m = 3$; CAS: 39492-89-2) PFO4DA ($n = 4, m = 4$; CAS: 39492-90-5) PFO5DoDA ($n = 5, m = 5$; CAS: 39492-91-6) 6:2 Cl-PFESA ($n = 8, m = 1, R = Cl$; CAS: 73606-19-6) 8:2 Cl-PFESA ($n = 10, m = 1, R = Cl$; CAS: 83329-89-9) PFESA 1 ^a (Nafion Byproduct 1; CAS: 29311-67-9)
Per/poly-fluoroethers Carboxylic Acids (PFECAs)		GenX ^a (CAS: 13252-13-6) PFMOBA ($n = 5, m = 1$; CAS: 863090-89-5) PFDMMOBA ^a (CAS: 801212-59-9) PFO2HpA ($n = 3, m = 2$; CAS: 151772-58-6) ADONA ^a (CAS: 958445-44-8) PFO3TDA ($n = 8, m = 3$; CAS: 330562-41-9) PFO3DA ($n = 5, m = 3$; CAS: 151772-59-7) PFO3OA ($n = 3, m = 3$; CAS: 39492-89-2) PFO4DA ($n = 4, m = 4$; CAS: 39492-90-5) PFO5DoDA ($n = 5, m = 5$; CAS: 39492-91-6) 6:2 Cl-PFESA ($n = 8, m = 1, R = Cl$; CAS: 73606-19-6) 8:2 Cl-PFESA ($n = 10, m = 1, R = Cl$; CAS: 83329-89-9) PFESA 1 ^a (Nafion Byproduct 1; CAS: 29311-67-9)
Per/poly-fluoroether Sulfonic Acids (PFESAs)		GenX ^a (CAS: 13252-13-6) PFMOBA ($n = 5, m = 1$; CAS: 863090-89-5) PFDMMOBA ^a (CAS: 801212-59-9) PFO2HpA ($n = 3, m = 2$; CAS: 151772-58-6) ADONA ^a (CAS: 958445-44-8) PFO3TDA ($n = 8, m = 3$; CAS: 330562-41-9) PFO3DA ($n = 5, m = 3$; CAS: 151772-59-7) PFO3OA ($n = 3, m = 3$; CAS: 39492-89-2) PFO4DA ($n = 4, m = 4$; CAS: 39492-90-5) PFO5DoDA ($n = 5, m = 5$; CAS: 39492-91-6) 6:2 Cl-PFESA ($n = 8, m = 1, R = Cl$; CAS: 73606-19-6) 8:2 Cl-PFESA ($n = 10, m = 1, R = Cl$; CAS: 83329-89-9) PFESA 1 ^a (Nafion Byproduct 1; CAS: 29311-67-9)
Perfluoroalkyl Sulfonyl Fluoride		PFOS-F ($n = 7$; CAS: 307-35-7)
Sulfonamides (FASAs)		FBSA ($n = 3$; CAS: 30334-69-1) FOSA ($n = 7$; CAS: 754-91-6)
Perfluoroalkyl Phosphinic Acids (PFPIAs)		8:8 PFPiA ($n = 7$; CAS: 500776-69-2)
Perfluoroalkyl Phosphonic Acids		-

(continued)

Table 1. (continued)

Subclass	Representative Structure	Compounds Discussed in This Review
Perfluoroalkyl Carboxamides		-
Fluorotelomer Sulfonic Acids (FTSAs)		4:2 FTSA (n = 3; CAS: 757124-72-4) 6:2 FTSA (n = 5; CAS: 27619-97-2)
Fluorotelomer Sulfonamide (FTSAm)		6:2 FTSAm (n = 5; CAS: not provided)
Fluorotelomer Sulfonamide Alkylbetaine (FTAB)		6:2 FTAB (n = 5; CAS: 34455-29-3)
Fluorotelomer Sulfonamide Alkylamine (FTAA)		6:2 FTAA (n = 5; CAS: 34455-22-6)
Fluorotelomer Alcohol (FTOHs)		6:2 FTOH (n = 5; CAS: 647-42-7)
Phosphate Esters		-
Carboxyesters		-
Other		OBS (CAS: 70829-87-7)

Specified structures and CAS numbers are representative. Additional CAS numbers for a given PFAS (eg, for a salt form of the compound) may have been used in the referenced studies.

^a Not represented by representative structure due to additional R group or branching.

Table 2. Chemical abbreviations

4:2 FTSA—4:2 fluorotelomer sulfonic acid
6:2 Cl-PFESA—perfluoro(2-((6-chlorohexyl)oxy)ethanesulfonic acid)
6:2 FTAA—fluorotelomer sulfonamide alkylamine
6:2 FTAB—fluorotelomer sulfonamide alkylbetaine
6:2 FTSA—6:2 fluorotelomer sulfonic acid
6:2 FTSAm—6:2 fluorotelomer sulfonamide
8:2 Cl-PFESA—2-[[8-Chloro-1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8-hexadeca-fluorooctyl)oxy]-1,1,2,2-tetrafluoroethanesulfonic acid
8:8 PFPiA—8:8 perfluoroalkyl phosphinic acid
ADONA—4,8-dioxo-3H-perfluorononanoate
Cl-PFOS—1-chloro-perfluorooctane sulfonic acid
F-53B—perfluoro(2-((6-chlorohexyl)oxy)ethanesulfonic acid)
FBSA—perfluorobutane sulfonamide
FOSA—perfluorooctane sulfonamide
GenX—perfluoro-2-methyl-3-oxahexanoic acid
OBS—perfluorous nonenoxbenzenesulfonate
PFBS—perfluorobutane sulfonic acid
PFDA—perfluorodecanoic acid
PFDMMOBA—4-(heptafluoroisopropoxy)hexafluorobutanoic acid
PFDoA—perfluorododecanoic acid
PFESA 1—Perfluoro-3,6-dioxo-4-methyl-7-octene-1-sulfonic acid
PFHxA—perfluorohexanoic acid
PFHxS—perfluorohexane sulfonic acid
PFMOBA—perfluoro (4-methoxy butanoic) acid
PFNA—perfluorononanoic acid
PFO2HpA—perfluoro-3,6-dioxahexanoic acid
PFO3DA—perfluoro-3,6,9-trioxadecanoic acid
PFO3OA—perfluoro (3,5,7-trioxadecanoic) acid
PFO3TDA—perfluoro-3,6,9-trioxatridecanoic acid
PFO4DA—perfluoro (3,5,7,9-tetraoxadecanoic) acid
PFO5DoDA—perfluoro (3,5,7,9,11-pentaoxadodecanoic) acid
PFOA—perfluorooctanoic acid
PFOS—perfluorooctane sulfonic acid
PFOS-F—perfluorooctanesulfonyl fluoride
PFPeA—perfluoropentanoic acid

less bioaccumulative than legacy PFAS. Functional head group type, chain length, and ether moieties clearly potentiate bioconcentration.

Uptake, elimination, and metabolism

Similar to BCF trends, PFSAs (eg, PFHxS and PFOS) induced higher uptake rates than PFCAs (eg, PFBA and PFOA), up to approximately 10 times higher (Vogs et al., 2019). Following exposures to PFOS, PFHxS, and PFOA, but not the shorter-chain PFBA, uptake was biphasic with slower initial uptake that increased after hatching (48 hpf) from the chorion, a protective acellular envelope. Some studies remove the zebrafish chorion prior to exposure, whereas others leave it intact. Vogs et al. concluded that a 2-compartment toxicokinetic model was the best fit, accounting for the chorion as a significant barrier that led to slower initial perfluoroalkyl acid uptake, particularly for higher concentrations. Uptake rates were more dependent on carbon chain length and functional head group than were elimination rates (Vogs et al., 2019). Comparing toxicokinetics of PFOS and its alternative OBS after approximately 0.02 μM exposures from 72 to 120 hpf, uptake rate constants were similar whereas the elimination rate constant was lower for PFOS, leading to a higher PFOS BCF value (Zou et al., 2021). Metabolism also plays a key role in bioconcentration. Studies largely support a lack of larval metabolism of perfluoroalkyl acids, like PFOS and PFOA (Vogs et al., 2019), but rapid interdigitation of *in vivo* metabolism of a 74 PFAS library has demonstrated metabolism of other diverse PFAS structures (Han et al., 2021). Hydrolysis is a major metabolism pathway particularly for PFAS with carboxamide, sulfonamide, and carboxyester functional groups. Perfluoroalkyl carboxamides were the most rapidly metabolized into terminal PFCAs. Fluorotelomer alcohols

(FTOHs) with 1–2 hydrocarbons were predominately metabolized via β -oxidation and Phase II reactions, whereas those with more than 2 hydrocarbons were mostly metabolized via taurine conjugation (Han et al., 2021). Metabolism of numerous structurally diverse PFAS into a variety of transformation products highlights the need for additional studies of this nature and magnitude, along with reverse toxicokinetic models to apply findings to human risk assessment (Tal and Vogs, 2021).

Adult zebrafish

Adult zebrafish are more amenable to the measurement of ADME endpoints, such as tissue distribution, given their larger size and fully developed organ systems. As in developing zebrafish, PFAS uptake rates in adults increased with chain length and were greater for PFSAs than PFCAs (Wen et al., 2019), whereas elimination rate constants were less affected by these structural features (Wen et al., 2017). Uptake rates for PFCAs and PFSAs were highest in the liver and blood, followed by gill, ovary, then brain and muscle, with similar findings for tissue concentrations and BCF values (Wen et al., 2017). Longer-chain compounds accumulated to a greater extent in the blood, and shorter-chains in the liver (Wen et al., 2019). Short-chain PFCAs and PFSAs (mixture of 5 at 0.027–0.047 μM each) reached a steady state in approximately 5 days, whereas co-exposure with long-chains (mixture with 6 at 0.016–0.024 μM each), delayed the short-chain steady state to 14–21 days, which suggests that long-chain PFAS compete for transporters and binding sites (Wen et al., 2017).

Other lesser studied PFAS, 6:2 FTAB and 6:2 FTAA, exhibited differences in ADME in adult zebrafish exposed to 65% and 35% mixtures (0.057/0.034 μM and 0.57/0.34 μM , respectively) for 180 days (Shi et al., 2019). 6:2 FTAB was undetectable in adult tissue and offspring. 6:2 FTAB was rapidly metabolized into 6:2 FTAA, which accumulated in a sex-dependent manner and composed approximately 92% of all quantified PFAS. Metabolic products of 6:2 FTAA, 6:2 FTSAm, and 6:2 FTSA, contributed to 2.8–8.5% of quantified PFAS. Concentration-dependent maternal transfer of chemical to embryos occurred following 6:2 FTAA exposure, and concentrations in F1 embryos of parent compound and metabolites combined were as high as 25% that in F0 adult tissues (Shi et al., 2019). Earlier studies reported maternal transfer rates of 10% following PFOS exposure (Sharpe et al., 2010). Recent adult zebrafish studies were not sufficiently numerous to establish trends in BCF, but did provide evidence of tissue-specificity, metabolism, and maternal transfer.

Toxicity: adverse apical outcomes

Developmental zebrafish

PFOS has been the most often studied PFAS in zebrafish developmental studies, with extensive evidence that it elicits adverse apical outcomes. PFOS increased mortality in zebrafish by 5 dpf after developmental exposure as low as 14 μM (Martinez et al., 2019) and caused 100% mortality by 5.5 dpf (9.3 μM) (Shi et al., 2008). When considering all affected morphological features, a median benchmark dose lower confidence limit (BMDL) was 4.7 μM at 5 dpf (Martinez et al., 2019). Spinal deformities, altered head-trunk angles, and body axis curvature were among the most pronounced malformations and have been observed from PFOS exposures as low as 1 μM (Shi et al., 2008), 1.9 μM (Martinez et al., 2019), and 20 μM (Lee et al., 2021). Other common morphological effects of developmental exposure include decreased body length (Jantzen et al., 2016a; Martinez et al., 2019; Wu et al., 2022) or relative liver size (Wang et al., 2022), reduction in swim bladder

inflation (Martinez et al., 2019; Shi et al., 2008), yolk sac edema (Lee et al., 2021; Martinez et al., 2019; Shi et al., 2008), pericardial edema (Lee et al., 2021), reduced eye-snout distance (Martinez et al., 2019), epiboly deformities (Shi et al., 2008), and altered heart rate (Shi et al., 2008). While some noted normal hatch rates after developmental exposure to PFOS up to 186 μM (Martinez et al., 2019), others noted dose-dependent lower hatch rates and hatch delay relative to controls (Shi et al., 2008). One study reported no behavior changes associated with 32 μM PFOS exposures from 1 to 5 or 1 to 15 dpf (Sant et al., 2021), whereas others demonstrated an array of photomotor behavior effects. Within the range of 0.02–20 μM , PFOS caused hyperactive locomotor activity at 5 dpf in the light, and also at 14 dpf, with mixed hyperactivity and hypoactivity in the dark and a decreased incidence and duration of swimming bursts (Jantzen et al., 2016a; Lee et al., 2021, 2022; Wu et al., 2022) and 0.5–5 nM (Haimbaugh et al., 2022) induced hypoactive behavior in the F0 generation that manifested as hyperactivity in the F1 generation (Haimbaugh et al., 2022). The current literature provides ample evidence of PFOS-induced morphological and behavioral effects in developing zebrafish.

C-F chain length

Assessment of PFOS and PFOA homologs showed that increased PFAS toxicity was correlated with increasing C-F chain length, up to 8 fluorinated carbons. Gaballah et al. reported no effects of PFBS exposure, but abnormal body axis phenotypes and failed swim bladder inflation were associated with exposures to PFOS, PFHpS, PFHxS, and PFPeS (descending from 8 to 5 fluorinated carbons), with respective EC_{50} values of 28.2, 168.1, 227.9, and 48.8 μM . Inclusion of the high EC_{50} for PFPeS in the analysis resulted in no linear correlation between EC_{50} and chain length for the PFSAs; otherwise, a clear trend in toxicity based on C-F chain length was evident. Menger et al. (2020) observed a similar trend as PFHxS caused malformation and mortality in exposures as low as 12 μM , and PFOS as low as 4.3 μM . In a PFCA homologous series consisting of compounds with 3- to 12-fluorinated-carbon chains, only PFOA and PFNA (7 and 8 fluorinated carbons) induced malformations and mortality at as low as 16.4 and 74.8 μM , respectively. Besides PFDoA (11 fluorinated carbons), all PFCA homologs caused abnormal larval behavior (Rericha et al., 2021), though PFDoA (0.4–10 μM) has otherwise been associated with delayed decrease in swimming activity and slower swimming speeds (Guo et al., 2018). PFOS and PFHxS (8 and 6 fluorinated carbons) both reduced swimming distance in the dark and induced a greater startle response (Menger et al., 2020) and increased swimming distance regardless of the light phase (Gaballah et al., 2020). Studies generally agree on the presence of abnormal morphology and behavior following exposure to the majority of assessed PFCAs and PFSAs, with C-F chain length influencing effects (Gaballah et al., 2020; Liu et al., 2022; Menger et al., 2020; Wasel et al., 2021).

Functional groups

PFAS with sulfonic acid functional head groups elicited greater toxicity than those with carboxylic acid head groups in developing zebrafish. While all PFSAs evaluated by Gaballah et al. elicited morphological effects, PFNA was the only PFCA that caused malformations and lethality. PFPeA, PFHxA, PFHpA, and PFOA did not induce morphological effects, though exposures to most caused altered larval behavior (Gaballah et al., 2020; Menger et al., 2020). PFOA and PFNA consistently altered locomotor activity in exposures as low as 0.2 μM (Jantzen et al., 2016a; Menger et al.,

2020) and 0.06 μM (Yu et al., 2022). The concentration that caused mortality in 50% of the exposed zebrafish (LC_{50}) for PFOA was 725 μM , and it caused malformations (ie, bent tail, yolk sac, and pericardial edema) at exposure concentrations as low as 48 μM (Gaballah et al., 2020; Menger et al., 2020; Pecquet et al., 2020); however PFSAs induced effects at lower concentrations. Differences in toxicity values between studies are likely related to slight variations in exposure paradigms, including whether exposure solutions were buffered to neutralize pH which can greatly influence observed toxicity (Wasel et al., 2021). Overall, while PFCAs have the potential to induce morphological effects, they often do so at higher concentrations relative to PFSAs.

Lesser-studied PFAS subclasses, including fluoroethers, phosphinic acids, and those with fluorotelomer chemistries (Table 1), have been the focus of several recent studies. Emerging fluoroethers ADONA and PFESA 1 did not induce toxicity when tested between 4.4 and 80.0 μM (Gaballah et al., 2020). After early developmental exposure for 24 h to a variety of perfluoropolyether carboxylic acids, PFO3TDA elicited the lowest LC_{50} of 38 μM , followed by PFO3DA (202 μM), PFOA (232 μM), PFDMMOBA (248 μM), GenX (383 μM), PFO2HpA (441 μM), and PFMOBA (499 μM) (Gebreab et al., 2020). PFOA and PFECAs both reduced interocular distance and increased listing incidence or impaired righting behavior. These findings suggest that some PFECAs may not be safer alternatives to PFOA (Gebreab et al., 2020). LC_{50} was inversely correlated with alkyl chain length, but there was no correlation with substitution of CF_2 by ether groups in PFECAs (Gebreab et al., 2020). The uninflated swim bladder is a frequently identified malformation following PFOA and PFECA exposures, as well as yolk sac edema for PFOA and PFO4DA or spinal deformity for PFO3OA and PFO5DoDA. EC_{50} rankings were PFO5DoDA < PFO4DA < PFOA < PFO3OA, with EC_{50} values for uninflated swim bladders of 52, 310, 606, and 3932 μM , respectively. Toxicity increased with an increase in backbone OCF_2 moieties (Wang et al., 2020). When tested between 0.01 and 5.79 μM , 8:8 PFPiA, a phosphinic acid, did not affect larval survival, hatch rate, or cause malformations; however, 0.34–5.79 μM exposures reduced swimming activity at 120 hpf during the dark photoperiod (Kim et al., 2020). 6:2 FTSA did not cause malformations but altered behavior at 180 μM (Menger et al., 2020). Beyond PFCAs and PFSAs, it is difficult to determine toxicity trends among other PFAS subclasses based on the available smaller-scale studies, ie, those that evaluated 1–8 compounds. A shift towards assessments of larger PFAS libraries is necessary.

Toward rapid testing of all PFAS

PFAS toxicity research is driven by the thousands of PFAS on the global market and the urgent need to understand which structures are hazardous and why (OECD, 2018). Because testing in zebrafish is an efficient approach to examine large numbers of structurally diverse PFAS, 2 immediate goals emerged: (1) to establish basic structure-bioactivity relationships for as many PFAS as can be tested in zebrafish, and (2) prioritize thousands of PFAS down to just those structures for which bioactivity merits closer scrutiny in rodent testing. After all, it is doubtful that a PFAS that is developmentally inactive in zebrafish would be found to present a hazard liability in higher vertebrates. The current limiting factor for such high-throughput testing is the number of analytically validated PFAS available for procurement.

The largest PFAS zebrafish developmental toxicity assessment to date was of 139 structures. It identified 49 (35%) as bioactive in at least one morphology or behavior endpoint (Truong et al., 2022). Among the 139 PFAS, 31 induced aberrant morphology, 11 altered

embryonic behavior at 24 hpf, and 25 altered larval behavior at 120 hpf. PFDA was the most potent, with a modeled benchmark dose to elicit 10% of any morphological effect (BMD₁₀) of 0.22 μM . Among commonly studied PFAS identified as bioactive by Truong *et al.*, potency rankings were PFDA > FOSA > PFOS > PFHpS > PFHxS > PFOS-F. Even with a 139 PFAS dataset, the authors concluded it was insufficient to establish broadly applicable structure-activity relationships. Analyses revealed limited correlation between bioactivity and physiochemical properties, such as LogP, mass, and number of fluorinated carbons. However, there was an association between bioactivity and specific chemotypes, including sulfonyl, sulfenamide, sulfonamide, and alkyl hydroxide chemotypes (Truong *et al.*, 2022). In another recent study, high prevalence of altered larval behavior (36%) following developmental exposure to a diverse set of 58 PFAS at a single concentration of approximately 0.1 μM was associated with PFCAs, PFSAs, and PFAS with fluorotelomer carboxylic acids, ether/polyether carboxylic acids, phosphinic acids, phosphate esters, and phosphonic acids (Rericha *et al.*, 2021). Clearly, diverse PFAS spanning structural subclasses exhibit bioactivity.

Notably, multiple studies identified FOSA, which contains a sulfonamide head group and an 8-fluorinated-carbon chain, as particularly bioactive. It was the sole compound to alter morphology, and embryonic and larval behavior in the 139 PFAS study (Truong *et al.*, 2022). FOSA was also the most potent developmental toxicant in 2 other high-content screening studies that evaluated 38 (Dasgupta *et al.*, 2020) and 74 PFAS (Han *et al.*, 2021). Following earlier exposures beginning at 0.75 hpf, FOSA induced significant delays to epiboly (25 μM), and 0.75–24 hpf exposures (0.78 μM) decreased liver area and increased yolk sac neutral lipids by 128 hpf (Dasgupta *et al.*, 2020). A shorter sulfonamide PFAS homolog—perfluorobutane sulfonamide (FBSA)—was identified as the most toxic in a 4-fluorinated-carbon homologous series tested from 1 to 100 μM . While PFPeA, PFBS, and 4:2 FTSA induced abnormal larval behavior, FBSA was the only one to elicit abnormal morphology (Rericha *et al.*, 2022a). FOSA and other sulfonamide PFAS have been shown to bioconcentrate to a greater extent than other PFAS (Han *et al.*, 2021; Rericha *et al.*, 2022a), which may explain their higher toxicity. Some have argued that toxicokinetics explains the majority of variability in toxicity between PFAS, and that once within the organism, nonunique toxic mechanisms are operant, specifically for perfluoroalkyl acids (Vogs *et al.*, 2019). However, Han *et al.* suggested that FOSA may have a unique mode of action as it is more toxic than other compounds with similar or even slightly higher BCF values. Ultimately, apical organism-level endpoints cannot discern whether PFAS induce toxicity via similar or different modes of action. Such inquiries require 'omics and mechanistic investigation.

Adult zebrafish

Adult zebrafish studies enable the interrogation of organ-level, sex-specific, intergenerational effects, and considerably more complex behavior endpoints. PFOS, F-53B, and OBS 1 μM exposures for 28 days caused vacuolation in the liver and an increased liver somatic index induced by F-53B (Huang *et al.*, 2022a). Following a developmental aqueous exposure (2 nM) to PFOA that was immediately followed by dietary exposure to 8 picomolar PFOA, fish were significantly smaller in weight and length, with no effect on survival (Jantzen *et al.*, 2017). Similarly, exposures to 6:2 FTAB and 6:2 FTAA mixtures (0.057/0.034 μM or 0.57/0.34 μM , respectively) for 180 days decreased body weight and K-factor, a concentration-dependent effect in both sexes. It also decreased the average number of eggs produced and increased the

incidence of malformations (uninflated swim bladder, pericardial and yolk sac edemas, and bent spines) and mortality in the developing F1 generation (Shi *et al.*, 2019). Altered behavior following PFAS exposure is also frequently observed; a study of sublethal exposure from 3 to 120 hpf to 2.0 μM PFOS, PFOA, and PFNA found no change in adult body length or weight but various altered adult behaviors. PFNA elicited broad effects on behavior in male fish, including reduced distance traveled, increased thigmotaxis, and increased aggressive behavior, and they spent more time in light arenas in a light/dark assay than controls. Conversely, PFOS-exposed males showed reduced aggression and PFOA-exposed females spent less time in the lighted arenas relative to controls (Jantzen *et al.*, 2016b). A dietary exposure to PFHxA, extending from the juvenile into the subadult life stage, found no effect on body weight and length but altered behavior in the F0 and subsequent generations (Rericha *et al.*, 2022b). Altered behavior across generations was observed following developmental exposures to PFOS and PFOA, and was associated with altered gene expression patterns that were exacerbated in successive generations (Haimbaugh *et al.*, 2022). Aqueous exposures to 0.27–0.83 μM PFBS for 1 week altered embryo development, specifically nutrient loading. Spawning immediately after exposure yielded embryo concentrations of 99–253 pg/embryo, with PFAS concentration dropping below detection in embryos after the 4th or 5th spawning, all of which averaged 1.71 days apart. PFBS loading in embryos led to decreased survival, though there was no difference in the number of embryos produced by the F0 (Annunziato *et al.*, 2022). In adult zebrafish, exposure to a variety of PFAS, primarily PFCAs and PFSAs, largely did not induce significant organism-level malformations in F0 populations but did affect fecundity, behavior, and development of the next generation.

Toxicity: modes of action and mechanisms

Developmental zebrafish

Recent strides towards elucidating the mechanisms of PFAS toxicity in zebrafish have predominately employed transcriptomics (Table 3). 'Omics endpoints are more sensitive and specific than macroscopic morphological effects and provide insight into the modes of action by which PFAS induce toxicity (Annunziato *et al.*, 2019; Gebreab *et al.*, 2020; Martinez *et al.*, 2019; Tu *et al.*, 2019). While the field is making progress towards understanding the mechanistic pathways leading to adverse effects of PFAS exposures by employing 'omics technologies, *in silico* molecular docking studies, and systems biology approaches, more work is needed to determine molecular initiating events that drive toxicity (Figure 1).

Metabolic pathways and energetics

Key events related to metabolic pathways and altered energetics are frequently identified as characteristic of PFAS toxicity. PFOS and F-53B exposures as low as 0.46 and 0.024 μM , respectively, increased oxidative energy expenditure of 2 dpf embryos and reduced feed intake, whereas PFOS and OBS exposures as low as 4.6 and 0.06 μM reduced embryo oxygen consumption rate (Tu *et al.*, 2019). The liver and pancreas have been identified as target organs of toxicity. Hepatic toxicity in the form of steatosis, vacuolation, increased total cholesterol and triglyceride levels, and decreased levels of low-density lipoprotein at 7 dpf were induced by 1 μM exposures to PFOS, Cl-PFOS, 6:2 Cl-PFESA (F-53B), or 8:2 Cl-PFESA (Yi *et al.*, 2019). While Sant *et al.* found no change in cholesterol, triglycerides, glucose, or unsaturated fatty acid concentrations at 4 dpf following PFOS exposure at 16 or 32 μM , they did

Table 3. Summary of disrupted systems and pathways based on transcriptomic studies

Summarized Major Systems and Functions	Representative Disrupted Pathways	Chemical	References		
Nervous system development, neurological function	Neurodevelopment, neuron projection regeneration, notochord development, dopamine biosynthesis, dopamine neuron function	PFOS	(Dong et al., 2021; Huang et al., 2021; Lee et al., 2021)		
		PFOS	(Wu et al., 2022)		
		PFOA	(Yu et al., 2021)		
		OBS	(Huang et al., 2021)		
		PFDoA	(Guo et al., 2018)		
Sensory system development	Visual perception	FBSA	(Rericha et al., 2022a)		
		PFOS	(Dong et al., 2021)		
		–			
Lipid synthesis, transport, and metabolism	Lipid metabolism, fatty acid synthesis, β -oxidation, cholesterol transport, peroxisome proliferator-activated receptor (PPAR) signaling, energy expenditure	PFOS	(Cheng et al., 2016; Christou et al., 2020; Dong et al., 2021; Haimbaugh et al., 2022; Martinez et al., 2019; Mylroie et al., 2021; Tu et al., 2019; Wang et al., 2022; Yi et al., 2019; Zhu et al., 2021) (F2 generation)		
		–			
		Cl-PFOS	(Yi et al., 2019)		
		PFBS	(Gong et al., 2022; Sant et al., 2019)		
		F-53B	(Wang et al., 2022; Yi et al., 2019)		
		8:2 Cl-PFESA	(Yi et al., 2019)		
		PFNA	(Gong et al., 2022)		
		FBSA	(Rericha et al., 2022a)		
		–			
		OBS	(Wang et al., 2022)		
		FOSA	(Dasgupta et al., 2020)		
		PFOS	(Diaz et al., 2021; Guo et al., 2019; Huang et al., 2022b; Lee et al., 2021; Martinez et al., 2019)		
		Immune response	Chemokine activity, proteases implicated to antigen presentation, inflammatory response	PFOA	(Diaz et al., 2021)
PFOS	(Liu et al., 2022)				
PFOA	(Liu et al., 2022)				
PKCO signaling in T lymphocytes, B cell receptor signaling, role of NEAT in regulation of the immune response	FOSA		(Liu et al., 2022)		
	–				
	Immune cell function and trafficking		PFOA	(Haimbaugh et al., 2022) (F2 generation)	
	–				
Cellular signaling	Hepatotoxicity	F-53B	(Huang et al., 2022b)		
		OBS	(Huang et al., 2022b)		
		PFBS	(Tang et al., 2020) (F1 eggs)		
		6:2 FTAB	(Shi et al., 2018)		
		FBSA	(Rericha et al., 2022a)		
		PFOS	(Lee et al., 2021)		
Liver development	Hepatotoxicity	PFOS	(Lee et al., 2021)		
		FOSA	(Dasgupta et al., 2020)		
Oxidative stress	Nrf2 signaling	PFOS	(Lee et al., 2021; Sant et al., 2018)		
		F-53B	(Wu et al., 2019)		
Endocrine system	Steroid synthesis	PFOS	(Haimbaugh et al., 2022) (F2 generation)		
		–			
		PFBS	(Sant et al., 2019)		
	Estrogen receptor signaling	PFOS	(Haimbaugh et al., 2022) (F1 generation)		
		–			
		PFOA	(Wang et al., 2020)		
		PFO5DoDA	(Wang et al., 2020)		
Cardiac development and function	Cardiac hypertrophy signaling	PFO4DA	(Wang et al., 2020)		
		PFO3OA	(Wang et al., 2020)		
		PFOS	(Liu et al., 2022)		
		PFOA	(Liu et al., 2022)		
		FOSA	(Liu et al., 2022)		
	Cardiac muscle contraction	Wnt/ β -catenin pathway	PFBS	(Gong et al., 2022)	
			F-53B	(Shi et al., 2017b)	
			–		
			Erythrocyte-related	2-(Perfluorohexyl) ethanoic acid	(Shi et al., 2017a)
			–		

see an increase in saturated fatty acids. PFOS exposure up to 15 dpf caused aberrant pancreatic islet morphologies and lipid accumulation (Sant et al., 2021). PFBS exposure at 16 and 32 μ M also decreased pancreas length and increased the incidence of severely hypomorphic and fragmented islets at 4 dpf (Sant et al., 2019). Lipid dysregulation, as well as altered liver and pancreas morphology, are strong evidence of altered energetics following PFAS exposures.

Transcriptomic studies have further revealed enrichment of biological pathways involved in lipid metabolism and energetics following exposure to PFBS (Sant et al., 2019), PFOS (Christou et al., 2020; Haimbaugh et al., 2022; Zhu et al., 2021), chlorinated PFOS homologs (Yi et al., 2019), FOSA (Dasgupta et al., 2020), and FBSA (Rericha et al., 2022a) (Table 3). PFOS, Cl-PFOS, and 6:2 Cl-PFESA (F-53B) (1 μ M), all of which have 8 fluorinated carbons, had comparable disruptive potency on lipid metabolism responsive genes,

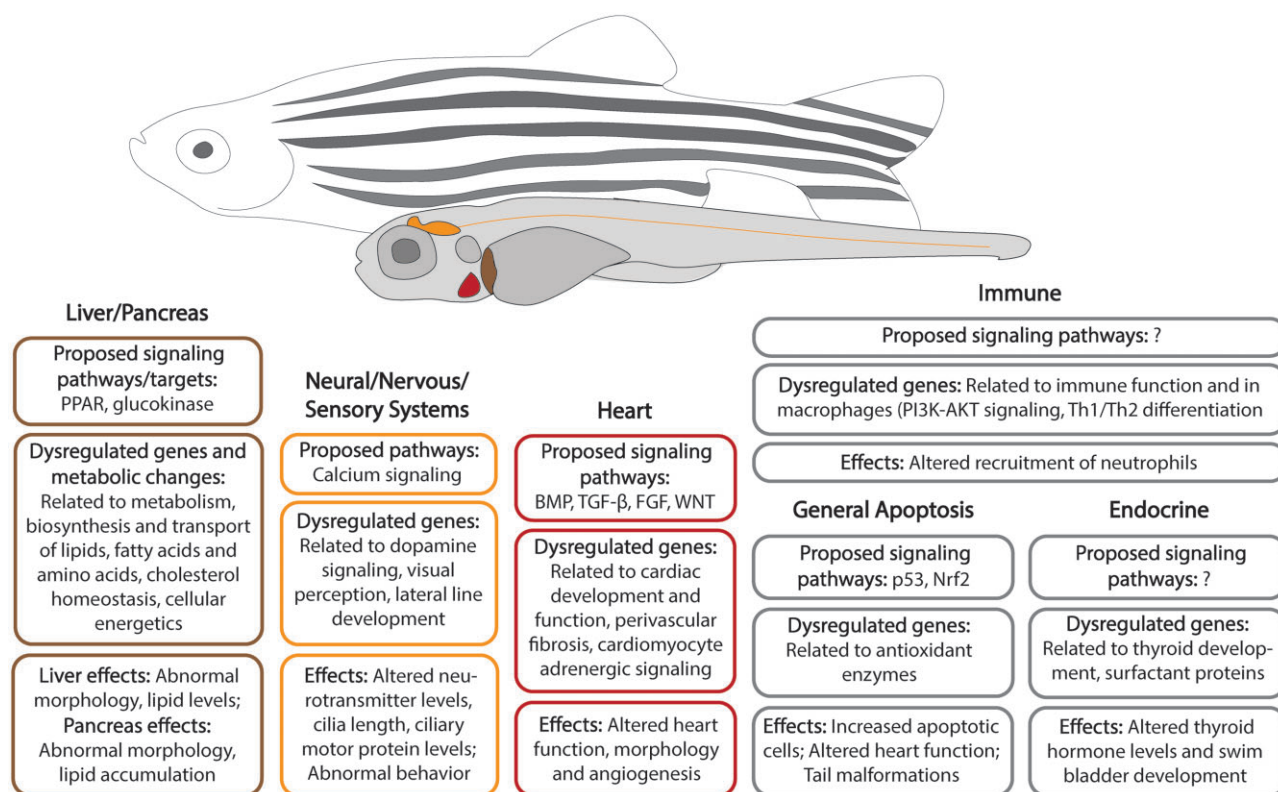


Figure 1. Representative organs and systems, biological pathways, and endpoints affected by PFAS exposures in zebrafish.

increasing expression of genes involved with fatty acid β -oxidation (Supplementary Table 2). 8:2 Cl-PFESA (1 μ M) exhibited the opposite trend, and both suppressed genes involved in fatty acid export (Yi et al., 2019). At 72 hpf, following exposure to 1 μ M PFOS, pathways involving biosynthesis of fatty acids and cholesterol homeostasis and regulation were among the most significantly enriched biological pathways (Dong et al., 2021). Following exposure to PFOS at similar concentrations (0.046–4.6 μ M), and also following F-53B (0.026–2.6 μ M), or OBS (0.064–6.4 μ M), transcript abundance at 96 hpf related to central regulation of energy expenditure, glucose metabolism, and lipid metabolism was decreased (Tu et al., 2019). OBS uniquely induced dose-dependent decrease in GK protein abundance, and PFOS reduced NPY protein abundance (Tu et al., 2019). Zhu et al. (2021) proposed that PFOS-induced metabolic reprogramming and increased fatty acid uptake may contribute to tumor cell proliferation. Several studies point to peroxisome proliferator-activated receptors (PPARs) as a modulator of PFAS toxicity due to their prevalent role in lipid metabolism. Developmental PFOS exposure led to decreased PPAR gene expression at 96 hpf (*pparaa* and *pparg* at 32 μ M, and *pparg* at 16 μ M) (Sant et al., 2021). PFNA and PFBS (20 μ M) exposures also enriched PPAR signaling at 120 hpf (Gong et al., 2022). In another study PFBS exposure did not alter expression of PPAR genes at 96 hpf but affected numerous PPAR targets (Sant et al., 2019). Similarly, no PPAR genes were differentially expressed after FBSA exposure, but PPARA was predicted to regulate 36 differentially expressed genes (DEGs) (Rericha et al., 2022a).

Metabolic profiling has also identified PFAS targets involved in lipid metabolism and energetics. The metabolic effects of GenX (303 μ M) and PFO3TDA (17.8 μ M) exposures were similar to PFOA (120.8 μ M) in that after exposure from 72 to 96 hpf they were related to carbohydrate, lipid and amino acid metabolism, and associated cellular energetics. Reduced molar ratio of

branched-chain to aromatic amino acids relative to controls was a metabolic indication of liver damage, also significantly correlated with LC₅₀. Furthermore, PFO3TDA led to decreased trimethylamine N-oxide, a proposed biomarker of metabolic syndrome. PFOA and PFO3TDA decreased carnitine, which is essential for fatty acid transport and related to β -oxidation, and decreased cholesterol, and PFO3TDA exposure decreased fatty acid levels. Metabolic changes also suggested mitochondrial dysfunction and possibly PPAR-mediated effects (Gebreab et al., 2020). PFOS exposures at 2 μ M from 48 to 120 hpf were not associated with altered lipid metabolism despite increased yolk sac area at 120 hpf; PFOS-affected pathways were related to amino acid, purine, carbon, and 2-oxocarboxylic acid metabolism (Ortiz-Villanueva et al., 2018). An integrated biomolecule network analysis based on transcriptomic, proteomic, and metabolomic data from developmental PFOS exposures (0.1–20 μ M) identified clusters of affected pathways related to proteasome-linked actin-binding protein and amino acid metabolisms, lipid metabolism and cell proliferation and apoptosis, and phospholipid-translocating ATPase activity mechanisms (Lee et al., 2021).

Two molecular docking studies provided insight into potential biological targets. Molecular docking revealed that 8:2 Cl-PFESA exhibited higher PPAR antagonism than PFOS and other chlorinated homologs (binding energy -7.6 kcal/mol for PPAR α and -8.8 kcal/mol for PPAR β) (Yi et al., 2019). The order of binding affinities to glucokinase (zGK) for PFOS and several alternatives were: F-53B (-7.8 kcal/mol) > PFOS > OBS. (Tu et al., 2019).

Apoptosis and oxidative stress

Apoptosis is a recurring endpoint in zebrafish PFAS studies that is often linked to oxidative stress. In a recent study of the PFOS alternative 6:2 FTAB, developmental exposure to 70.1 μ M significantly increased apoptotic cells detected in the tail area at 96 hpf

(Shi et al., 2018). An earlier study reported an increase in apoptotic cells, especially around the heart and tail after 10 μM PFOS exposure, and suggested that apoptosis may help explain observations of altered heart rates and tail malformations (Shi et al., 2008). Similar findings of increased apoptotic cells in the heart region at 72 hpf and decreased heart rate at 48 hpf following 2–30 hpf PFOA exposures as low as 0.06 μM were noted (Yu et al., 2022). The abundance of *p53* and *bax* transcripts at 96 hpf was increased in a dose-dependent manner, up to 5.3-fold after exposure to 10 μM PFOS (Shi et al., 2008). Heightened expression of *p53* (1.5-fold) and *bax* (1.54-fold), *apaf1* (1.33-fold), and *mdm2* (1.48-fold), and increased activity of at least 138% for caspase-3, -8, and -9 was reported following exposure to 70.1 μM 6:2 FTAB. Shi et al. suggested that PFAS-induced cell apoptosis may occur via activation of *p53* and may then be caspase-dependent, ultimately leading to tail malformations as a result of the cell death, though they also noted that apoptosis may be induced by reactive oxygen species (ROS) (Du et al., 2017; Shi et al., 2018).

Exposure to 6:2 FTAB (Shi et al., 2018), PFOS (Du et al., 2017; Wu et al., 2022), and F-53B (Wu et al., 2019) increased ROS levels in larvae. At 96 hpf, early exposure to 35 and 70 μM 6:2 FTAB increased malondialdehyde (MDA) levels, another indicator of potential oxidative damage, along with catalase (CAT) enzyme activity by 3.0-fold (70 μM); glutathione peroxidase (GPx) activity decreased (Shi et al., 2018). PFOS exposure (3.0 μM) increased MDA, SOD, and Gpx, and decreased CAT activity in one study (Du et al., 2017) but had only a significant decrease in MDA levels in another study 24 h after a 72–120 hpf exposure (10 μM) (Zou et al., 2021). At 120 hpf, all antioxidant enzyme activities decreased after 0.35 μM F-53B exposure, except for GPx. Along with changed activity levels, gene expression of antioxidant enzymes (Supplementary Table 2) was reduced and expression of *nrf2* was heightened. After western blot and molecular docking analysis, the authors proposed F-53B inhibits PI3K, an upstream Nrf2 activator, leading to decreased protein expression of Nrf2 through the PI3K/Akt pathway (Wu et al., 2019). PFOS and OBS (10 μM) exposures also lead to decreased Nrf2 expression after a 24 h depuration period (Zou et al., 2021). Metabolomic evidence of oxidative stress induced by PFOA (121 μM), GenX (303 μM), and PFO3TDA (17.8 μM) exposures included increased taurine levels and decreased glutathione levels, both associated with heightened response to oxidative stress and with the Nrf2 pathway (Gebreab et al., 2020). Disruption of embryogenesis by PFOS may be in part modulated through the Nrf family of genes and their capacity to induce antioxidant response. The incidence of pericardial edema, reduced growth, and apoptosis was ameliorated in mutant *nrf2a*^{fn318-/-} zebrafish exposed to PFOS, though mutants exhibited increased yolk utilization. There is evidence of other compensatory antioxidant responses despite Nrf2a deficiency such as dose-dependent increased expression of *pparg* and its targets *apoa1a* and *fabp1b1* in mutants. *In silico* evidence of Nrf family-PPAR-response elements crosstalk led the authors to suggest that PPAR γ may play an important role; ultimately, there may be multiple mediators of oxidative stress response to PFOS (Sant et al., 2018).

Cardiac system

Altered heart rate and incidence of pericardial edema have been observed following exposures to several PFAS, along with altered erythrocyte numbers and disrupted angiogenesis. PFAS exposures hindered cardiac function, leading to decreased heart rate and lower stroke volumes and output (Liu et al., 2022) and histological heart malformations (PFNA, PFBS) (Gong et al., 2022). Exposure to 37.2 μM PFOS or its alternative OBS (31.9 μM)

shortened intersegmental vessel length and enhanced dorsal aorta vessel formation in *Tg(fli1: eGFP)* zebrafish, a vascular reporter line. OBS exposure also decreased formation of dorsal longitudinal anastomotic vessels, whereas PFOS decreased posterior cardinal vein formation (Huang et al., 2021). FOSA exposure between 0.002 and 0.2 μM led to dose-dependent elongation of the heart in the cardiomyocyte reporter line *Tg(myl7: eGFP)*, ie, increased sinus venosus and bulbus arteriosus lengths, expanded pericardial sac area, and caused irregular heart beat and cardiac output at 72 hpf (Chen et al., 2022). At this same timepoint, developmental exposure to 11–32 μM 2-(Perfluorohexyl)ethanoic acid (CAS: 53826-12-3) and 2.6–21.0 μM F-53B decreased numbers of erythrocytes and decreased heart rate. 2-(Perfluorohexyl)ethanoic acid exposure altered expression of erythrocyte-related genes, and F-53B affected the Wnt/ β -catenin pathway, critical for cardiac development (Table 3) (Shi et al., 2017a,b).

In fact, numerous studies employed transcriptomics to identify altered gene expression and biological pathways related to cardiac development and function, and specifically related to WNT signaling. Single-cell RNA sequencing following 0.06 μM PFOA exposure revealed that the highest number of DEGs were found in heart cells. Affected biological functions included gas transport, muscle cell differentiation, actin cytoskeleton, adrenergic signaling in cardiomyocytes, lysosome, autophagy, and apoptosis (Yu et al., 2022). PFOS, PFOA, and FOSA exposures all altered biological pathways related to cardiac hypertrophy signaling at 120 hpf within the range of 0.002–0.02 μM , and further analysis revealed miR-16-5p as an activated upstream regulator among all the chemicals (Liu et al., 2022). At 120 hpf, PFOS exposure to 1 μM decreased expression of genes related to muscle fiber regeneration and hematopoietic processes (Dong et al., 2021). By 120 hpf, exposure to 0.002 and 0.02 μM FOSA caused dysregulation of genes with predicted functions related to dysfunction of the heart, fibrosis left ventricular dysfunction, and perivascular fibrosis. BMP, TGF- β , and FGF signaling pathways mediated affected processes. WNT signaling was activated through the WNT/ β -catenin, WNT/ Ca^{2+} , and WNT/Rho pathways. Following 0.02 μM exposure, there was also significant enrichment of the aryl hydrocarbon receptor (AHR) pathway, an important regulator of xenobiotic metabolism and more (Chen et al., 2022). Though WNT and AHR signaling pathways have been proposed, additional investigation is needed to determine the mechanisms of cardiac toxicity.

Neural/nervous/sensory systems

Dysregulation of the brain and nervous system development, and probable motor function, are commonly observed following PFAS exposures, identified using transcriptomics (Annunziato et al., 2019; Dong et al., 2021; Huang et al., 2021; Kim et al., 2020; Rericha et al., 2022a; Wu et al., 2022) and metabolomics (Gebreab et al., 2020). PFOA exposure (2.4 μM) promoted the proliferation of BrdU/HuC/D positive cells in the preoptic area of the hypothalamus at 48 hpf and disrupted the expression of genes essential to dopamine neuron development and function. Concentrations as low as 0.24 μM altered gene expression that suggests perturbation of dopamine neurotransmission signaling and homeostasis (Wu et al., 2022; Yu et al., 2021). PFOS (0.6–6 μM) similarly affected gene expression related to dopamine synthesis, corroborated by measured increases in protein levels and brain dopamine content (Wu et al., 2022). KEGG pathway analysis of DEGs after 3.83 μM PFOS exposure revealed enrichment of extracellular matrix-receptor interaction, cardiac muscle contraction, and calcium signaling (Christou et al., 2020). Christou et al. investigated effects on

calcium signaling related to ryanodine receptors (RyR) through co-exposures with caffeine or dantrolene. Dantrolene decreased swimming speed in controls but had no effect upon co-exposure with PFOS; perhaps PFOS has a higher affinity to RyR binding sites than dantrolene. PFOS altered endogenous neurotransmitter levels after exposure at 10 and 20 μM , and altered several genes associated with seizures (Lee et al., 2022). Across multiple studies, *bdnf* expression was decreased by exposure to PFOS and OBS (Huang et al., 2021), PFHxA, PFHxS, and 6:2 FTOH (Annunziato et al., 2019). 8:8 PFPiA exposure decreased mRNA expression of neuronal genes *elavl3* and *tuba1* (Kim et al., 2020) and a similar effect was observed after PFHxA exposure, along with decreased protein levels (Guo et al., 2021). Integrated multi-omics analyses following 0.1–20 μM PFOS exposure identified network clusters of pathways related to Ca^{2+} binding-related, producing resource substances of neurotransmission, and Ca^{2+} -regulated muscle contraction (Lee et al., 2021). Behavior effects following PFDaA (10 μM) exposure coincided with decreased ACh content, AChE activity, increased dopamine levels, and altered gene expression related to nervous system development (Guo et al., 2018).

Dysregulation of genes related to the sensory and motor systems has also been reported. Visual perception was among the most highly enriched biological processes at 72 hpf following 1 μM PFOS exposure (Dong et al., 2021). The lateral line development gene *ap1s1* was induced in a dose-dependent manner following PFHxA exposure at 2.0 (3.63-fold) and 20.0 (13.2-fold) μM , and was induced by 20 μM 6:2 FTOH exposure, whereas 0.2 μM PFHxS decreased expression (Annunziato et al., 2019). Related to muscle development, *tgfb1a* expression increased after PFHxA and 6:2 FTOH exposures as low as 0.2 μM . Length of cilia, an important organelle for signal transduction, in the retinal neuroepithelium was reduced by PFOS (37.2 μM) and OBS (47.9 μM) exposures. Expression of genes related to cilia in the dynein arm family was increased, whereas that of genes in the kinesin and tubulin families was decreased. Abundance of ciliary motor proteins was decreased, and molecular docking revealed potential for PFAS-protein interaction; for instance, PFOS exhibited a binding affinity of -9.4kcal/mol towards DYNC1H1 (Huang et al., 2021). Overall, PFAS exposures altered development and function of the brain and sensory systems through several key events. Integrated multi-omics analyses identified widespread dysregulation of calcium signaling, though the driving mechanisms remain uncertain.

Endocrine system

Investigation of PFAS as endocrine disrupting compounds has predominately revealed effects related to the thyroid system. PFOA and alternative PFECAs lowered T3 and T4 levels at 5 dpf in a dose-dependent manner, at as low as 22.5 μM following PFO5DoDA developmental exposure. PFO5DoDA, PFO3OA, and PFOA exposures also increased abundance of transcripts related to TH synthesis (*dio2*) and regulation (*crh*) at 5 dpf. These PFAS and PFO4DA increased expression of genes involved in TH metabolism (*ugt1ab*, *st1*, and *st5*) and downregulated *st4*, whereas only PFOA increased *tsh β* and *ttr* transcript abundance. Increased expression of metabolizing genes likely increased TH clearance, leading to the decreased TH levels. T3 and T4 supplementation partially rescued PFOA- and PFECA-induced swim bladder malformations and yolk sac edemas. Decreased expression of outer mesothelial markers *anxa5* and *hprt1l* at 5 dpf following PFOA (1207.5 μM) and PFO5DoDA (22.5–90.1 μM) exposures suggested swim bladder malformation was linked to mesothelium development (Wang et al., 2020). After 4.5–6 dpf exposures to PFOA

(11 μM) and PFBA (640 μM), larvae exhibited decreasing swim bladder surface area, the same effect observed after T3 exposure, also increasing expression of *tpo* and 2 genes encoding surfactant proteins, *sp-a* and *sp-c* at 6 dpf. PFOA also heightened expression of *tshB* and *trA*, whereas PFBA increased *ttf-1*. Following 4.5–28 dpf exposures, PFOA and PFBA increased percentage of fish without anterior swim bladders, but heightened expression of *sp-a* and *sp-c* only persisted in PFOA-exposed groups. The shorter chain PFBA (3 fluorinated carbons) had similar phenotype to PFOA (7 fluorinated carbons), but at 28 times higher concentrations (Godfrey et al., 2017). 8:8 PFPiA (5.79 μM) also increased expression of genes related to thyroid hormones, including *crhb*, *dio3a*, and *tshr* (Kim et al., 2020). PFOS exposure as low as 0.2 μM increased expression of thyroid development marker genes *hhx* and *pax8*, and also reduced expression of sex hormone regulating genes *cyp19a* and *cyp19b*, all at 96 hpf (Shi et al., 2008).

Immune system

While immunotoxicity is a frequently noted effect of PFAS exposure, only a couple of recent studies have investigated effects in developmental zebrafish. RNA-seq studies have identified immune-related biological pathways associated with PFAS-induced gene expression changes (Haimbaugh et al., 2022; Liu et al., 2022; Rericha et al., 2022a) (Supplementary Table 2). Additionally, PFOS (200 nM) exposure exacerbated chemically induced inflammation (pooled larvae analysis), specifically expression of *il17a*, *tnfa*, and *il1b* at 120 hpf, and increased neutrophil recruitment to the intestine (Diaz et al., 2021). Exposure to 1.2 or 12 μM PFOA decreased the number of neutrophils at inflicted wound sites at 48 hpf by 1.4- and 1.8-fold, respectively (Pecquet et al., 2020). Exposure to 6:2 FTAB at 8.8 μM led to dose-dependent decreased expression of *ccl1* at 96 hpf, whereas 17.5 μM increased expression of *il1 β* , *il8*, and *tnfa*, and 70 μM heightened *cxcl-c1c* and *ifn* expression at 96 hpf (Shi et al., 2018). Single-cell RNA sequencing after 0.06 μM PFOA exposure showed that macrophages were among the most affected cell populations, exhibiting altered fatty acid metabolism, PI3K-AKT signaling, and Th1 and Th2 differentiation (Yu et al., 2022). Limited data on key events related to immunotoxicity warrant additional studies in zebrafish.

Adult and intergenerational

Studies conducted in adult zebrafish corroborate affected systems and biological pathways identified in developmental studies and provide insight into intergenerational and sex-specific effects. Adult exposures caused lipid dysregulation, oxidative stress, and immunotoxicity. Chronic exposure to 0.5 μM PFOS for 5 months caused liver steatosis and altered serum lipid profiles, with more frequent and severe effects observed in males. Male zebrafish exhibited lower hepatosomatic index, reduced ATP concentration and altered expression of nuclear receptors and genes related to fatty acid oxidation in the liver (Cheng et al., 2016). PFOS, F-53B, and OBS (1 μM for 21 days) exposures affected lipid metabolism-related pathways, specifically pertaining to PPAR signaling (Wang et al., 2022). Expression of genes related to lipid transport and synthesis, and fatty acid β -oxidation was decreased, including *ppary* after PFOS and F-53B. Molecular docking demonstrated similar binding affinities to *zfpPARY* of -8.5 and -9.1 , respectively (Wang et al., 2022). PFOS, F-53B, and OBS exposures also repressed expression of pro-inflammatory cytokines, increased renal interstitium in the head kidney, reduced expression of immune-related genes in the liver and altered expression in the intestine, shortened average intestine villus height, and caused dysbiosis of intestinal microbial

communities (Huang et al., 2022b; Wang et al., 2022). Morphological and transcriptomic evidence of immunotoxicity in the spleen was observed after exposure to 0.12–1.2 μM PFOA (Zhong et al., 2020). Molecular docking revealed the 3 PFAS bound to zNF- κB , with the highest affinity of -7.4 kcal/mol for F-53B, which likely mediated immune-related responses (Huang et al., 2022b). In adults developmentally exposed to PFNA, PFOA, or PFOS (0.02–2 μM), alterations in *slco2b1*, *slco1d1*, *tgfb1a*, and *bdnf* expression were chemical- and sex-dependent, and several persisted from the juvenile life stage into adulthood (Jantzen et al., 2016b).

Adult exposures also provided insight into intergenerational effects. Exposures to 0.033 or 0.33 μM PFBS disrupted maternal transcript transfer to F1 eggs, resulting in differentially enriched pathways related to immune function, along with adheren junctions, protein dimerization, and DNA packaging processes (Tang et al., 2020). Nrf2a was again suggested as a key pathway involved in effects on energy balance. F0 PFBS exposures to 0.27–0.83 μM disrupted lipid homeostasis and altered nutrient profiles and fatty acid composition in F1 embryos, particularly in *nrf2a*^{fh318/fh318} mutants (Annunziato et al., 2022). Following a 2 nM PFOA developmental aqueous exposure combined with a 4 month 8 pM dietary exposure up to 6 months old, F0 adults exhibited decreased expression of organic anion transporters *slco2b1*, *slco4a1*, *slco3a1*, increased *slco1d1*, and decreased *tgfb1a*. F1 offspring exhibited significant developmental delay and an increase in *ap1s1* expression, related to protein cargo sorting and vesicular trafficking (Jantzen et al., 2017). Evidence of disrupted biological processes intergenerationally warrants particular scrutiny when assessing the hazard potential of these compounds.

Conclusions and future directions

A significant amount of data on PFAS ADME and toxicity in zebrafish exists for what is still a relatively small number of PFAS, including the PFCAs, PFSAs, and PFECAs. Trends generally show greater bioconcentration and toxicity for longer chain compounds and those with sulfonamide and sulfonic acid functional groups. While some studies have tested PFAS from lesser-studied subclasses and larger libraries, evaluation of many more PFAS is needed to sufficiently interrogate structure-activity relationships, differential ADME, and modes of action. Knowledge of PFAS modes of action is growing, and the recent zebrafish literature indicates affected biological pathways and measured key events related to lipid metabolism and energetics, oxidative stress, the nervous system, as well as the cardiac, endocrine, and immune systems. Studies have highlighted a few mediating receptors, such as Nrf2a, PPAR, and AHR. Additional pharmacokinetic modeling (Khazaei and Ng, 2018), modeling to inform development of quantitative Adverse Outcome Pathways (Warner et al., 2022), functional, 'omics and integrated multi-omics analyses are needed. Large-scale chemical assessment using the throughput advantages of zebrafish will be essential to evaluate more PFAS in a vertebrate system. While PFAS mixture toxicity was outside the scope of this review, mixture studies are essential to determine PFAS hazard potential, as exposures often occur as complex environmental mixtures. Comparison of PFAS sensitivity among species, aquatic and otherwise, was beyond the present scope but is an important consideration for translational and risk assessment purposes that others have begun to address (Savoca and Pace, 2021; Wasel et al., 2021).

This review has identified several important considerations for future PFAS testing in zebrafish. GenX, a PFECA, has been

shown to degrade in dimethyl sulfoxide, a commonly used solvent for toxicity testing (Gaballah et al., 2020). Compatibility between chemicals and solvents to ensure stock solution integrity is essential. Similarly, shifts in pH alone can cause toxicity for developing embryos and confound observed PFAS toxicity and comparison between studies if exposure solutions are not buffered. Also essential is the need for analytically characterized PFAS standards so that investigators know that the label compound is responsible for the observed bioactivity rather than an industrial contaminant or a degradation product. The chorion status of embryos can also significantly influence PFAS toxicity. While simple automated removal of the chorion en masse has been available to the zebrafish community for over a decade, it is still not a common practice. Laboratories that do not practice the technique should be cognizant of its potential for false negative estimations of PFAS bioactivity. As the scientific community and international regulatory agencies work to determine how PFAS should be regulated, we must clearly establish a broader foundation of data for structurally diverse PFAS so that what we learn from hundreds of PFAS structures can be leveraged to predict the hazard potential of thousands more.

Supplementary data

Supplementary data are available at Toxicological Sciences online.

Declaration of conflicting interests

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