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Review

Emerging Perfluorinated Chemical GenX: Environmental and Biological Fates and Risks

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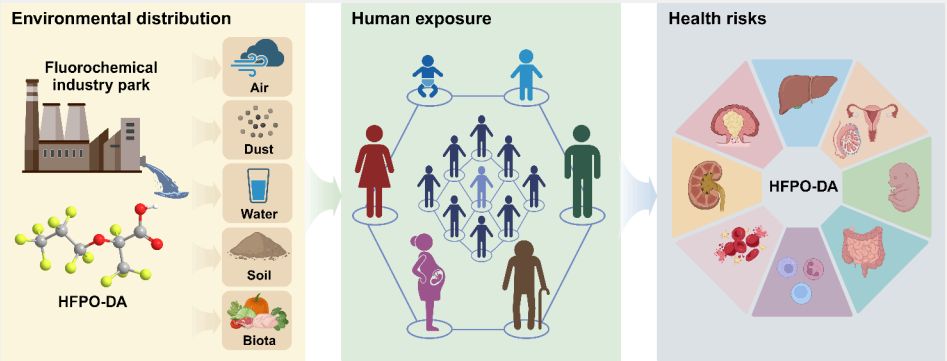

ABSTRACT: Perfluorinated chemical GenX, formally known as hexafluoropropylene oxide dimer acid (HFPO−DA), has been applied as an alternative to the forever chemical perfluorooctanoic acid (PFOA). The applications of HFPO−DA have rapidly expanded from traditional nonstick coating industries into high-tech semiconductor manufacturing. Because of such facts in conjunction with its low biodegradation rate and high potential of long-distance atmospheric transport, the presence and accumulation of HFPO−DA have been ubiquitously detected in environmental media and biological species, including animals and human beings, posing alarming and urgent needs for the risk assessment of HFPO−DA. Building on the United States Environmental Protection Agency's evaluation of HFPO−DA in 2021, this review first summarizes the interaction of HFPO−DA with the environment, elaborates on its known toxicities and potential carcinogenicity, along with their possible mechanisms, and briefly addresses its current exposure assessment and risk management strategies. These lines of evidence support that the safety of HFPO−DA necessitates further investigation and monitoring, albeit being considered as a less toxic and low persistence substitute of traditional PFOA.

KEYWORDS: *HFPO−DA, GenX, PFAS, PFOA, Risk assessment*

1. INTRODUCTION

Per- and polyfluoroalkyl substances (PFASs), including perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS), have been widely used in daily life since the 1950s, such as in nonstick coatings, food packaging materials, firefighting foam, electronic products, and medical equipment.[1] These chemicals are best known as "forever chemicals" due to their extremely high physical and chemical stability, which poses great risks to the environment and biological ecosystems, leading to worldwide restriction or prohibition of their applications.[2,3] As a result, PFAS alternatives have recently been developed. Hexafluoropropylene oxide dimer acid (HFPO−DA), an alternative to PFOA and commercially known as the GenX chemical, was first introduced in 2010 as part of Dupont's GenX processing aid technology for the production of fluoropolymers.[4] HFPO−DA belongs to a class of perfluoroether carboxyl acids (PFECAs), which are short-chain perfluorinated compounds with ether bonds and carboxyl groups.[5,6] Due to the presence of an oxygen atom in their perfluorocarbon chain, PFECAs are believed to be more likely to break down and form hydrogen bonds in water, making them more hydrophilic and degradable.[7] Since its introduction, HFPO−DA has been extensively produced and widely used due to its shorter fluorinated carbon chain, lower bioaccumulation, and reduced toxicity. For instance, annual emissions of HFPO−DA from the Chemours Fayetteville Works plant into North Carolina's Cape Fear watershed were estimated to exceed 1.22 tons in 2017−2018.[8] In China,

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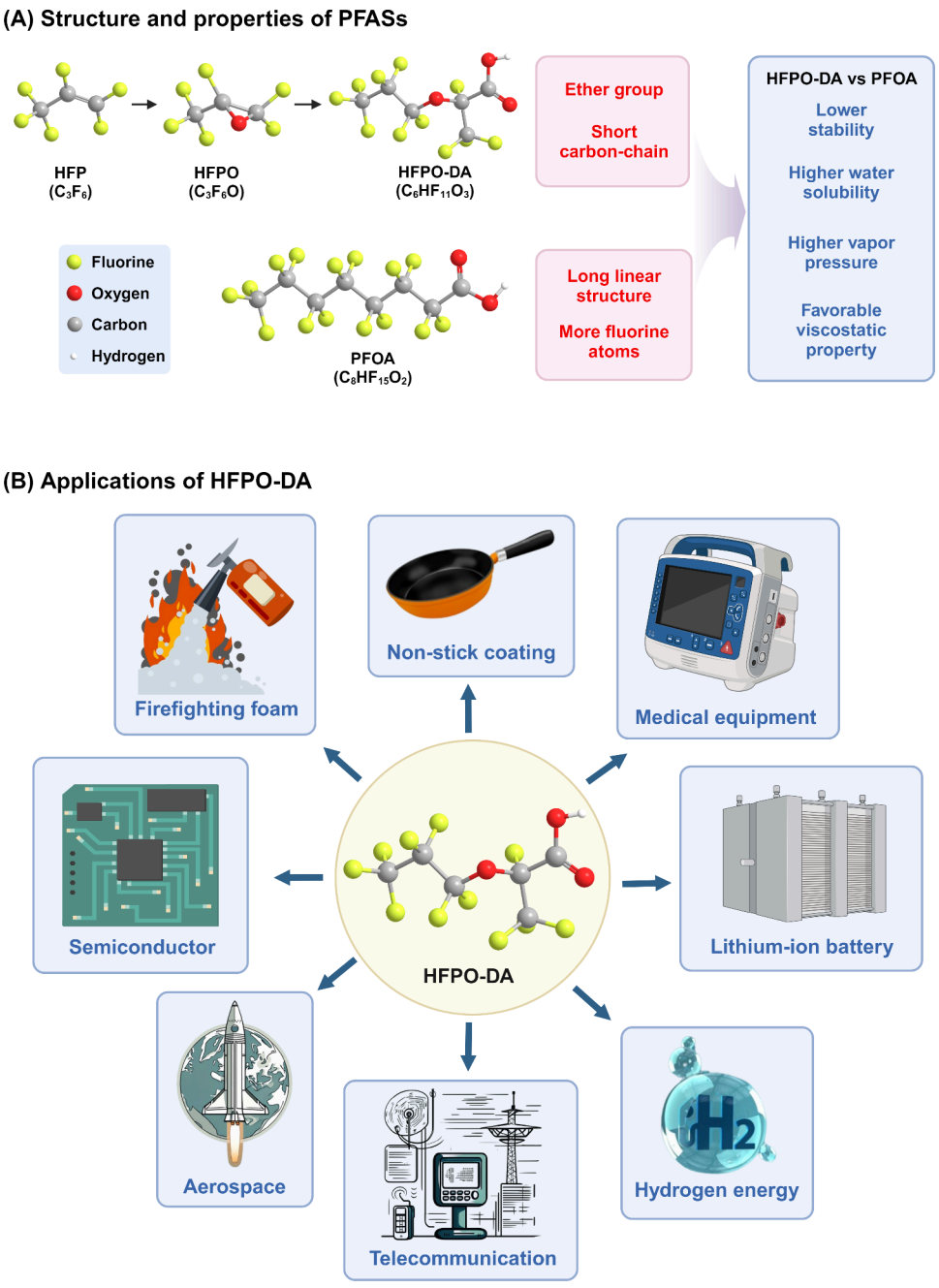

Figure 1. Structure and applications of HFPO−DA. (A) Structure and properties of PFASs. (B) Applications of HFPO−DA. Hexafluoropropylene: HFP; HFPO: hexafluoropropylene oxide; HFPO−DA: hexafluoropropylene oxide dimer acid; PFOA: perfluorooctanoic acid. This graphic was created using BioRender.

HFPO−DA emissions across five major river systems were estimated to be over 2.6 tons.[9] As a result of these industrial and anthropogenic activities, HFPO−DA has emerged as a widespread environmental contaminant and has been classified as a substance of very high concern by the European Chemicals Agency, promoting stricter monitoring and regulation of its use and disposal.[10]

The United States Environmental Protection Agency (EPA) released its final human health toxicity assessment for HFPO−DA and its ammonium salt in October 2021, and mandated the monitoring of 29 PFASs, including GenX.[4] Recent studies have revealed that human beings are exposed to HFPO−DA through food, drinking water, and indoor dust, with estimated daily intakes (EDIs) for many individuals exceeding the EPA's reference dose (RfD) of 3.0 ng/kg/day.[11] Epidemiological and experimental data have indicated potential toxic effects of HFPO−DA on the liver, reproductive and endocrine systems, kidneys, and other organ systems.[12−14] While HFPO−DA has garnered growing attention, many aspects of this compound remain poorly understood, such as environmental behaviors, possible toxic mechanisms, and risks to human health. Therefore, in this review, we aim to provide up-to-date knowledge on HFPO−DA, including its environmental fates, biological effects and their mechanisms, and potential risks to human populations.

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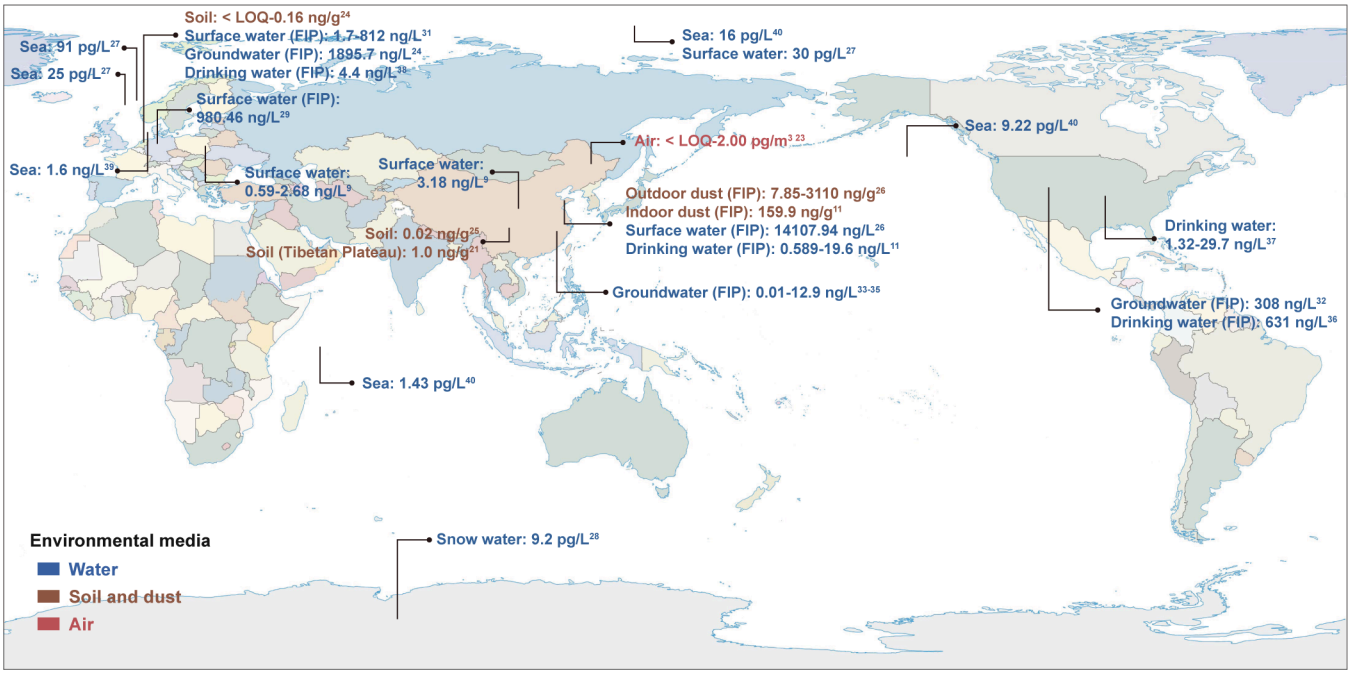

Figure 2. Global distribution and concentration of HFPO−DA in the environment. LOQ: limit of quantification. This graphic was created using BioRender.

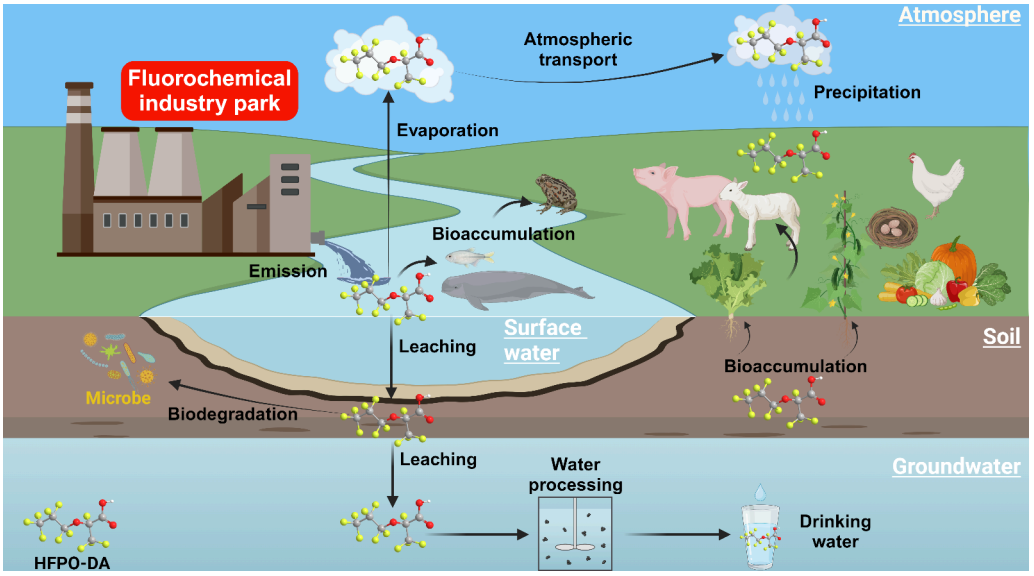

Figure 3. Environmental fates of HFPO−DA. This graphic was created using BioRender.

2. PHYSICAL AND CHEMICAL PROPERTIES OF HFPO−DA

HFPO−DA is a six-carbon PFECA synthesized through the condensation of two molecules of hexafluoropropylene oxide (HFPO), a derivative of hexafluoropropylene (HFP) (Figure 1A). HFPO−DA can further react with additional HFPO molecule(s) to form HFPO trimer acid (HFPO-TA) or longer chain fluoropolymers. Notably, HFPO−DA and its ammonium salt are the primary chemicals of GenX technology.[4] Like other PFASs, HFPO−DA exhibits high physicochemical stability and superior corrosion resistance compared to its hydrocarbon analogs due to the presence of fluorine atoms.[15−17] As an ether-PFAS, HFPO−DA also possesses favorable viscostatic properties because of the flexibility of its C−O bond. Such molecular flexibility prevents crystallization, resulting in a low glass transition temperature and keeping HFPO−DA in liquid form across a wide range of temperatures. Consequently, HFPO−DA has been commonly used as lubricants in semiconductor fluid processing, telecommunication cables, aerospace, hydrogen energy, and lithium-ion batteries in addition to its classical applications in nonstick coating, firefighting foam, and others (Figure 1B).[18,19]

Furthermore, the presence of oxygen atom and a shorter carbon-chain make HFPO−DA less stable and more degradable than other PFASs, positioning it as a promising alternative to PFOA.[7] In contrast to the eight-carbon, linear molecular structure of PFOA, HFPO−DA exhibits distinct physicochemical properties due to its shorter carbon chain and

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ether linkage (Figure 1A). For instance, HFPO−DA remains in liquid form at room temperature and has high water solubility (>751 g/L, at 20 °C), whereas PFOA is in a solid state at room temperature and has a water solubility of 3.3 g/L at 25 °C. Both compounds exhibit strong acidity (pK_a = 2.8) and are primarily present as anions in most biological fluids, except for gastric secretions.[4,20] Recent reports indicate that HFPO−DA has a vapor pressure of 101 kPa at 20 °C,[21] significantly higher than 306 Pa reported by the EPA under the same temperature.[4] This high vapor pressure facilitates the evaporation of HFPO−DA into the atmosphere and allows it to dissolve in aerosolized water droplets or adhere to suspended particles. Such a discrepancy in vapor pressure values can be partially attributed to HFPO−DA's surfactant properties and amphiphilic nature (*i.e.*, containing both hydrophilic and hydrophobic atoms). This characteristic leads to HFPO−DA accumulation at interfaces, such as water−air interface and glass surfaces, complicating its quantification in aqueous solutions.[4] In contrast, PFOA has a much lower vapor pressure of 2.3 Pa at 20 °C.[20] The differences in these physical properties underscore the distinct environmental behaviors of these two compounds (see the detailed discussion below).

3. ENVIRONMENTAL FATES OF HFPO−DA

3.1. Environmental Distributions of HFPO−DA

HFPO−DA is widely used in Europe, the United States, and China, making it frequently detected in the environment of these regions (Figure 2). Currently, fluorochemical industry parks (FIPs) are the major sources of HFPO−DA emission, with the highest concentrations detected in nearby environmental media. Surprisingly, HFPO−DA has also been discovered in both the Antarctic and Arctic poles, highlighting its capacity for long-range transport.[22] This may be attributed to the high volatility of HFPO−DA, which facilitates its atmospheric transport to remote areas (Figure 3). In the atmosphere, HFPO−DA readily and predominantly dissolves in aerosolized water droplets or attaches to suspended particles, with only a small fraction remaining in the air. Consequently, its concentration in the air is relatively low. For instance, at a ski resort in Zhangjiakou City, China, HFPO−DA levels were detected between below the limit of quantification (LOQ) to 2.00 pg/m^3.[23]

HFPO−DA in Soils and Dust. Unlike atmospheric air, HFPO−DA is frequently detected in soils and dust (Figure 2). For instance, Gebbink and van Leeuwen measured HFPO−DA and PFOA levels in soils in The Netherlands, and found that HFPO−DA levels (<LOQ to 0.16 ng/g) were much lower than those of PFOA (1.0−21 ng/g).[24] Similarly, in residential areas of 89 cities in China, the concentration of HFPO−DA (0.02 ng/g) was also lower than that of PFOA (0.35 ng/g).[25] Of note, HFPO−DA content in soils on the Tibetan Plateau increased with altitude and the highest concentration was 1.0 ng/g.[21] This trend may be due to the high volatility of HFPO−DA, which facilitates its atmospheric transport and deposition in areas distant from the original pollution sources. Strikingly, HFPO−DA is present at a relatively higher concentration in dust (Figure 2). Feng and colleagues found the concentrations of HFPO−DA in outdoor dust ranged from 7.85 ng/g to 3110 ng/g, accounting for 6.29% of the total 18 PFASs in Shandong Province, China.[26] This research group also reported a concentration of 159.9 ng/g of HFPO−DA in indoor dust samples from the same region, where it was the fourth most abundant PFAS tested.[11] These profiles suggest that HFPO−DA may accumulate more readily in dust than in soils, possibly due to its high volatility.

HFPO−DA in Water Sources. HFPO−DA has high hydrophilicity, leading to its widespread detection in water sources at varying levels across the globe (Figure 2). Its concentrations in major rivers in Europe ranged from 0.59 ng/L to 2.68 ng/L,[9] while its levels in the Delaware River of the United States were from 2.02 ng/L to 8.75 ng/L.[9] Comparable levels (up to 10.3 ng/L) of HFPO−DA were also detected in five major rivers in China.[9] Notably, HFPO−DA is present in the Arctic surface water and the Antarctic snowmelt, despite being at much lower levels of 30 pg/L and 9.2 pg/L, respectively.[27,28] As expected, the levels of HFPO−DA in surface water near FIP sources were significantly higher than those of other regions (Figure 2). For instance, in Germany, the average concentration of HFPO−DA was reported to be 980.46 ng/L in the Altes River near a FIP facility,[29] while a peak concentration of 86.08 ng/L of HFPO−DA was found in Lower Rhine rivers near FIPs, which even exceeded the levels of PFOA (7.10 ng/L).[30] In The Netherlands, HFPO−DA levels near FIPs ranged from 1.7 ng/L to 812 ng/L with an average of 130 ng/L.[31] The level of HFPO−DA was found to be 14107.94 ng/L in surface water surrounding the largest FIP facility in Shandong Province, China.[26] Furthermore, HFPO−DA can rapidly leach to groundwater from soils, leading to its accumulation in groundwater (Figure 2). The average levels of HFPO−DA in groundwater were 1895.7 ng/L in The Netherlands,[24] 308 ng/L in North Carolina of the United States,[32] and up to 12.9 ng/L in China.[33−35]

Given that HFPO−DA is present in both surface water and groundwater systems, concerns have been raised about its presence in drinking water (Figure 3). Indeed, a concentration of 631 ng/L of HFPO−DA was found in the finished drinking water from a treatment plant near the Cape Fear River in North Carolina.[36] The Kentucky Department of Environmental Protection showed the presence of HFPO−DA in 11 drinking water treatment plants with concentrations ranging from 1.32 ng/L to 29.7 ng/L.[37] In The Netherlands, the average concentration of HFPO−DA in drinking water near a FIP source was 4.4 ng/L, which was slightly higher than that of PFOA (4.1 ng/L).[38] In Shandong Province of China, a wide range of 0.589 ng/L to 19.6 ng/L of HFPO−DA was also detected in tap water of five villages near a FIP facility.[11]

Additionally, HFPO−DA is also detected in oceanic water with distinct distribution patterns, which are influenced by regions, ocean currents, and atmospheric transports (Figure 2). The seawater near German coastline contained an average level of 1.6 ng/L HFPO−DA,[39] while its level in Norway's coastal regions was 1−2 order(s) of magnitude lower. A sharp decrease in concentration was observed from the Norwegian Coastal Current (91 pg/L) to the Norwegian Atlantic Current (25 pg/L), indicating that water transported from the European coast to the Arctic may have a greater influence than water from the North Atlantic.[27] The concentrations of HFPO−DA in the Northwest Pacific and the Northeast Indian Ocean were 9.22 pg/L and 1.43 pg/L, respectively, while its level near the Arctic was 16 pg/L.[40] This may be related to the "grasshopper effect" of HFPO−DA, resulting in its migration from low-latitude to high-latitude regions and ultimately accumulation in oceanic water near the Arctic.

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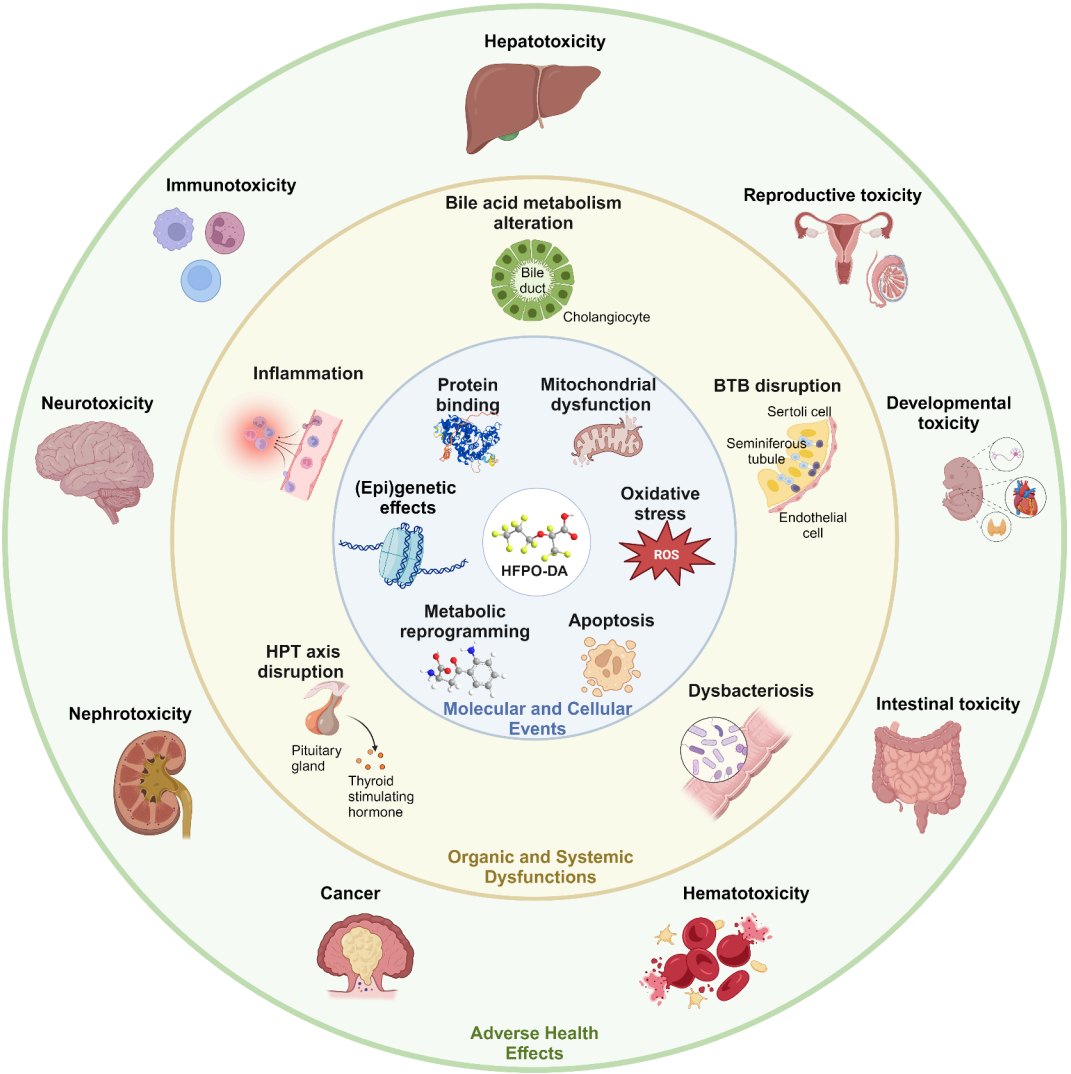

Figure 4. Toxic effects and their underlying mechanisms of HFPO−DA. BTB: blood-testis barrier; HPT: hypothalamic-pituitary-thyroid; KA: kynurenic acid. This graphic was created using BioRender.

In summary, since the environmental distributions of HFPO−DA have been studied primarily in European countries, the United States, and China, its comprehensive distribution profiles in the globe remain scarce. Further studies are needed to assess the distribution profiles of HFPO−DA in other regions, particularly in the Southern Hemisphere and developing countries, where industrialization is on the rise.

3.2. Bioaccumulation of HFPO−DA in Aquatic and Terrestrial Species

Due to its widespread presence in water bodies, HFPO−DA accumulation can be detected in many aquatic and terrestrial species (Figure 3). For example, the average concentration of HFPO congeners in fish was 0.59 ng/g, much less than 1.45 ng/g for PFOA.[41] Notably, the relative ratio of HFPO−DA in fish liver and muscle was 4.08, which was higher than a ratio of 1.78 for PFOA, indicating that both chemicals more preferentially deposit in the liver than muscle with a greater extent for HFPO−DA.[41] In amphibians, Shu and colleagues found that HFPO−DA was the most abundant PFAS among all 39 PFASs detected in *Chinese toads* of Chaohu Lake, China.[42] Specifically, the intestine (0.13 ng/g) had the highest concentration of HFPO−DA, followed by the brain (0.11 ng/g) and then the liver (0.05 ng/g). The presence of HFPO−DA in the brain implies that it could potentially pass through the blood-brain barrier (BBB) and cause damage to this specific organ.

As mentioned earlier, HFPO−DA is present in soils (despite being at relatively low levels), which partially contributes to its accumulation in various terrestrial organisms (Figure 3). Feng *et al.*[11] detected HFPO−DA in vegetables and wheats with concentrations of 20.1 ng/g and 6.70 ng/g, respectively, albeit its absence in terrestrial animal food, including meat, eggs, and sheep milk. A separate study found that HFPO−DA was the dominant contaminant in vegetables, grains, pork, and lamb in 33 cities of China, indicating a widespread dietary contamination,[43] which alarms an urgent need for assessing the potential risk of HFPO−DA. Of note, emerging evidence has suggested that HFPO−DA readily accumulates in the aerial parts of plants with concentrations often exceeding those of PFOA. For example, the levels of HFPO−DA and PFOA in lettuce leaves were 182 ng/g and 121 ng/g, respectively, and their corresponding levels were 95.0 ng/g and 82.7 ng/g in cucumbers.[44] Such phenomena may be attributed to the large proportion of HFPO−DA in the soluble fraction (55%−74%) and its high abundance in phloem and xylem saps.[45] Since the

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Table 1. Summary of the NOAELs and LOAELs of HFPO−DA[a]

Study design	Species	NOAEL (mg/kg/day)	LOAEL (mg/kg/day)	Observed toxic end points
Subacute oral toxicity	Rats	0.3	3	Hematological toxicity
Subacute oral toxicity	Mice	0.1	3	Liver toxicity; Hematological toxicity; Immunotoxicity
Subacute oral immunotoxicity	Mice	10	100	Immunotoxicity
Chronic oral toxicity	Rats	0.1	10	Hematological toxicity
Chronic oral toxicity	Mice	0.5	5	Liver toxicity
Chronic toxicity and carcinogenicity	Rats	1	50	Liver toxicity
Reproductive and developmental toxicity	Mice	F0: 0.1	F0: 0.5	Liver effects; Reproductive toxicity; Developmental toxicity
		F1: 0.5	F1: 5	
Prenatal and developmental toxicity	Rats	F0 and F1: 10	F0 and F1: 10	Developmental toxicity
Reproductive and developmental toxicity	Rats	F0 and F1: 62.5	F0 and F1: 125	Reproduction/developmental toxicity; Thyroid dysfunction
Reproductive and developmental toxicity	Mice	N/A	2	Reproductive/developmental toxicity
Reproductive and developmental toxicity	Rats	F0: 30	F0: 62.5	Thyroid dysfunction; Reproductive/developmental toxicity
		F1: 10	F1: 30	

[a]The data was originally from the 2021 EPA report.[4] N/A: not applicable.

aerial parts of vegetables are usually edible, these accumulative patterns may imply a possible high dose exposure of HFPO−DA by food. Future investigations are warranted to determine whether and how HFPO−DA can be bioaccumulated and biomagnified through the food chain and to evaluate its resultant potential health effects on animals and humans.

3.3. Degradation of HFPO−DA

HFPO−DA is typically resistant to biodegradation, leading to its persistency in environmental media and a half-life longer than 6 months.[4] The removal efficiency and defluorination ratio of HFPO−DA by native microbes under anoxic conditions were 5.45% ± 2.99% and 3.02% ± 0.62% in a 10-month period, respectively.[46] Compared with the slow natural manner, removal of HFPO−DA can be achieved more efficiently by artificial methods, such as electrochemical and photocatalytic degradation. Using a Zn-doped SnO_2−Ti_4O_7 anode, Yang *et al.*[47] showed an oxidation rate of 96.4% within 3.5 hours for HFPO−DA degradation, and also predicted that its resulting products, *i.e.*, $CF_3CF_2CF_2O^-$ and $CF_3CF_2COO^-$, were less toxic to aquatic organisms,[47] suggesting a detoxification action. Cathodic reduction was also shown as an efficient approach for HFPO−DA degradation.[48] In addition, Bao and colleagues compared two photocatalytic degradation approaches: oxidation by ultraviolet (UV)-activated persulfate (UV/PS) system and reduction by UV-activated sulfite (UV/sulfite) system.[49] They found that the latter was able to effectively degrade HFPO−DA by attacking its C−F bonds, while the UV/PS system was relatively inefficient, despite being an effective method for PFOA removal.[50] Furthermore, Zhu *et al.*[51] reported that HFPO−DA could be degraded with high adsorption and degradation efficiency by an adsorption photocatalyst method.[51] It is worthwhile to note that these efficient approaches also have drawbacks. Electrochemical oxidation is expensive due to the use of costly electrodes and poses safety concerns because of the potential generation of toxic byproducts, while photocatalysis requires high energy input and has limited light penetration capability. Thus, improved degradation approaches and technologies for the removal of HFPO−DA will be of great research interest in the future.

4. ADVERSE HEALTH EFFECTS OF HFPO−DA

Compared with PFOA, HFPO−DA exhibits faster clearance and lower acute toxicity due to its distinct chemical structure. Yet, long-term and low-dose exposure to HFPO−DA can cause toxic effects in multiple organ systems. For instance, HFPO−DA primarily accumulates in the liver, and hepatotoxicity is the most prominent and sensitive end point. Consequently, the United States EPA has established the RfD value based on the no observed adverse effect level (NOAEL) for hepatotoxicity (as detailed in section 5.2). Other reported adverse effects of HFPO−DA include reproductive and developmental toxicity, endocrine disruption, intestinal toxicity, immunotoxicity, hematological toxicity, renal toxicity, and carcinogenicity (Figure 4). Mechanistically, activation of peroxisome proliferator-activated receptor α (PPARα) is a best-known pathway for HFPO−DA induced hepatotoxicity. Multiomics technologies have identified other signaling pathways and metabolic dysregulation as potential toxic mechanisms (Figure 4).

4.1. Toxicokinetics of HFPO−DA

HFPO−DA is absorbed through gastrointestinal tract in rodents and primarily distributed to the liver, with higher concentrations observed in males than in females.[4] It can cross the placenta and milk and thus be transferred to offspring. The metabolic transformation of HFPO−DA appears very limited as its concentration remains largely unchanged after a 6-hour incubation with rat liver microsomes *in vitro*.[52] Urine is the main route of excretion, while fecal elimination of HFPO−DA is minor in rodents.[4] Of note, Gannon *et al.*[53] reported that HFPO−DA had shorter half-life time in female rats than in males: 0.2 hour *vs.* 2.8 hours for distribution phase and 67.4 hours *vs.* 72.2 hours for elimination phase. This could explain the higher accumulation of and greater vulnerability to HFPO−DA in male rats. Interestingly, such phenomena were not observed in mice.[53] The liver half-life time of HFPO−DA was 3.7 days in mice, much shorter than PFOA's 198.6 days, indicating faster clearance and lower tissue accumulation of HFPO−DA.[52] This perhaps because the binding affinity of HFPO-DA for bovine serum albumin was approximately 5-fold lower than its long linear chain counterpart PFOA. Such reduced affinity is likely due to the shorter hydrophobic tails and non-linear structure of HFPO−DA, where the CF_3-group near its carboxylic head sterically impedes protein binding.[54] While this property could contribute to lower tissue accumulation than PFOA, it also reduced protection against HFPO−DA-induced cytotoxicity in human umbilical vein endothelial cells, and unlike PFOA,

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whose stronger binding affinity to albumin helps mitigate its cytotoxicity.[54]

4.2. Toxic Effects of HFPO−DA

Systemic Toxicity. The acute toxicities of HFPO−DA are relatively low since its oral LD_{50} values were estimated to be 1730 mg/kg and 1750 mg/kg in male and female rats, respectively,[4] which were higher than that of PFOA (<1000 mg/kg in rats).[55] Common poisoning signs include wet fur, body weight changes, altered posture, lethargy, and discoloration in multiple organs such as lungs, stomach, skin, lymph nodes, liver, and esophagus. Enlarged liver and hepatocytes were noted in young male rats orally treated with a single dose of ammonium salt of HFPO−DA at 2250−5000 mg/kg.[4] Subacute and (sub)chronic studies indicated that GenX is toxic at 0.5−1000 mg/kg/day. The NOAELs and lowest observed adverse effect levels (LOAELs) of HFPO−DA by oral exposure were summarized in Table 1.[4]

Hepatotoxicity. Liver toxicity is the most-documented adverse effect of HFPO−DA, including single cell necrosis, cytoplasmic vacuolation, hepatocyte swelling and damage, and elevated levels of serum alanine aminotransferase (ALT). These effects are believed to involve various mechanisms, with activation of PPARα being essential (Figure 4).[4] Specifically, Ren and co-workers found that HFPO−DA induced increases in liver coefficient and liver injury markers were significantly attenuated in *PPARα* knockout (PPAR$\alpha^{-/-}$) mice compared to wild-type littermates.[12] Such protective effects were not observed in liver-specific *PPARγ* knockout mice (PPAR$\gamma^{\Delta Hep}$) when compared with their wild-type animals, indicating that PPARα but not PPARγ selectively mediates HFPO−DA induced hepatotoxicity.[12] In addition, HFPO−DA treatment also altered DNA methylation in $HepG_2$ cells characterized by a decrease in global methylation at low doses (100−400 μmol/L) but an increase at high doses (600−800 μmol/L), which correlated with downregulation of DNA methyltransferases with no obvious effects on DNA demethylase ten-eleven translocation dioxygenases.[56]

Bile acid metabolic dysregulation is another important mechanism underlying liver enlargement caused by HFPO−DA (Figure 4). An increase in total bile acid level in conjunction with elevation of primary bile acids and reduction of secondary bile acids were found in HFPO−DA treated mice at a dose of 2 mg/kg/day.[7] Mechanistically, dysregulation of bile acid metabolism by HFPO−DA was mediated by upregulation of nuclear receptor pregnane X receptor; by contrast, PFOA did so through the upregulation of vitamin D receptor and liver X receptor α.[57]

In addition, HFPO−DA could also impair mitochondrial functions and metabolic activity. Blake and colleagues treated pregnant CD-1 mice with HFPO−DA (2 or 10 mg/kg/day) *via* oral gavage and observed increased mitochondrial counts in hepatocytes.[58] The expression of mitochondrial metabolic genes, such as pyruvate dehydrogenase kinase 4 and carnitine palmitoyltransferase 2, was also upregulated in HFPO−DA treated dams and their fetuses.[59] Importantly, metabolomic profiling studies revealed that HFPO−DA treatment differentially altered numerous metabolites in serum and the liver of mice, among which kynurenic acid (KA) had the strongest correlation with liver injury.[60] HFPO−DA induced a metabolic shift in KA metabolic pathway to reduce KA levels in the liver, which led to inhibition of AMP-activated protein kinase (AMPK) expression and aryl hydrocarbon receptor activity, and as a result triggered endoplasmic reticulum stress and activated the nuclear factor-κB (NF-κB) signaling pathway.[60] Pretreatment with KA significantly mitigated liver injuries induced by HFPO−DA, indicating that KA reduction is a causal factor and that KA could serve as a sensitive biomarker for HFPO−DA induced hepatotoxicity.[60]

Interestingly, a recent mechanism of action (MOA)-based study revealed species specificity in HFPO−DA induced hepatotoxicity.[18] The first three key events (KEs) were identified as PPARα activation, alteration of cell growth pathways, and perturbation of cell growth/survival using *in vitro* and *in vivo* data.[18] However, the authors suggested that the HFPO−DA-mediated liver toxicity in mice is unlikely to occur in human, as only the initial KE (PPARα activation) was shared by the two species and because only a small subset of PPARα-mediated gene expression observed in human were present in rodents (*i.e.*, lipid-regulating effect).[18] The molecular mechanisms underlying such species-specific liver toxicity warrant further investigation.

Reproductive and Developmental Toxicity. HFPO−DA could affect androgen levels and damage testicular functions. Utilizing *in vivo* mouse model and mouse Leydig cell line TM4 cells *in vitro*, Peng and colleagues found that HFPO−DA disrupted the integrity of the blood-testis barrier (BTB), resulting in decreased testosterone levels and impaired spermatogenesis.[13] Mechanistic studies revealed that HFPO−DA activated p38 mitogen-activated protein kinase signaling pathway, upregulated matrix metalloproteinase-9 expression, and promoted tight junction protein occludin degradation, which together led to destruction of tight junctions and increase in BTB permeability.[13] Oral administration of HFPO−DA for 15 days downregulated the expression of testicular genes in H3K27me3-mediated polycomb pathway and changed gene expression related to DNA methylation in mice.[61] HFPO−DA also suppressed the transcriptional activity of androgen receptor (AR) and directly bound to AR as an antagonist, resulting in blockade of testicular development, acceleration of ovarian development, and eventually promotion of feminized characteristics in zebrafish larvae during sex differentiation.[62]

Likewise, HFPO−DA also impairs female reproductive functions. Lv *et al.*[63] found that gestational exposure to HFPO−DA increased weight gain of dam animals and significantly reduced birth weight and body length of neonates, leading to developmental retardation until weaning age. Placental histopathology showed that HFPO−DA exposure induced neutrophil infiltration, which correlated with altered expression levels of inflammation-related proteins in the RAS-related protein 1 (RAP1) signaling pathway, suggesting that placental inflammation and the RAP1 pathway are implicated in HFPO−DA induced reproductive dysfunctions.[63] Furthermore, using high-throughput toxicity screening assay, Blake and co-workers demonstrated that HFPO−DA treatment upregulated placental efflux transporter ATP-binding cassette subfamily G member 2 and 17β-hydroxysteroid dehydrogenase 1 (an enzyme catalyzing estradiol synthesis) expression in human placental trophoblast JEG-3 cells.[64] In *C. elegans*, HFPO−DA exposure (2−4 g/L) in nematodes delayed progeny production, which was associated with downregulation of *vitellogenin* (the precursor of egg yolk).[65]

Mounting evidence from *C. elgans* and zebrafish models supports that HFPO−DA is a developmental toxicant. *C. elegans* nematode development was delayed by HFPO−DA

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treatment (1.5−4 g/L) from the first larval stage (L1) to sexual maturation (young adult), which correlated with upregulation of detoxification enzymes and downregulation of ribosomal proteins.[65] HFPO−DA also reduced the hatching rate of zebrafish embryos, increased malformation rate and mortality, and downregulated apoptosis and autophagy genes.[66] Recent evidence showed that HFPO−DA disturbed the hypothalamic-pituitary-thyroid (HPT) axis in zebrafish embryos, leading to impairment of the central nervous system development and decrease in motor ability.[14] These effects were accompanied by dysregulation of thyroid hormone synthesis and function as well as increases in the activities of acetylcholinesterase and glutathione peroxidase, and ATP production, suggesting that thyroid hormones, energy metabolism, and redox homeostasis were impaired.[14] Consistently, Zhao *et al.*[67] also found that HFPO−DA caused thyroid hormone disruption in zebrafish embryos and altered the expression of genes related to nervous system development, such as elav like neuron-specific RNA binding protein.[67]

By contrast, the effects of HFPO−DA on cardiac development and functions are controversial. Two independent studies found that HFPO−DA decreased heart rate in zebrafish embryos and/or increased nitric oxide production.[67,68] However, an increase in heart rate induced by HFPO−DA was observed in this species.[14] HFPO−DA exposure also induced developmental toxicity in chicken embryo hearts as presented by thinned right ventricular wall thickness and elevated heart rate, which were mitigated by knockdown of *PPARα*, indicating that PPARα is involved in the developmental toxicity of HFPO−DA.[69] Using 3D cultures of human induced pluripotent stem cell (hiPSCs) line IMR90−1 cells, HFPO−DA impaired cardiac development and contractility of embryoid bodies when treated at concentrations of 25 mmol/L and above.[70] However, in different hiPSC line BiONi010-C cells, no cardiac developmental effects were found by HFPO−DA treatment. In contrast, both PFOS and PFOA disturbed cardiac development at much lower dosage (3.13 mmol/L and 6.3 mmol/L, respectively), indicating that HFPO−DA is a relatively weak developmental cardiotoxicant.[70]

Neurotoxicity. In addition to the neurodevelopmental toxicity mentioned above in zebrafish embryos, HFPO−DA also induced neurotoxicity in *in vitro* and *in vivo* models. For instance, Wu and co-workers reported that HFPO−DA treatment prior to differentiation resulted in changes in nuclear morphology, and increases in epigenomic markers (*e.g.,* H3K4me3 and H3K27me3) and mitochondrial membrane potential in differentiated human dopaminergic-like neuron line SH-SY5Y cells.[71] These alterations were accompanied by changes in neuronal markers, such as elevation in intracellular calcium levels, downregulation of tyrosine hydroxylase (the rate-limiting enzyme of catecholamine biosynthesis), and enrichment of α-synuclein in cell bodies, collectively supporting that HFPO−DA is neurotoxic.[71] RNA-sequencing results revealed sex-specific differences in brain gene expression characterized by a larger number of differentially expressed genes (DEGs) in HFPO−DA treated adult female fruit flies than males and by a higher number of DEGs under low doses and short exposure durations.[72] Pathway analysis identified immune response as the major cluster of DEGs, indicating neuroinflammation is likely involved in HFPO−DA induced neurotoxicity.[72] In addition, HFPO−DA could disrupt the integrity of BBB in adult rats by inhibiting P-glycoprotein and breast cancer resistance protein activities, two key efflux transporters, leading to increased permeability.[73] This allows toxic xenobiotics and endogenous metabolites to easily reach the brain and thus cause cognitive dysfunction and other neurological disorders. Together, these findings indicate that HFPO−DA may pose significant threats to neurological health.

Intestinal Toxicity. HFPO−DA has been shown to induce intestinal inflammation and toxicity. Wang and co-workers found that exposure to HFPO−DA resulted in oxidative damage, inflammation, and apoptosis in zebrafish intestine in a dose-dependent manner.[74] A stimulation of proinflammatory factor tumor necrosis factor-α (TNF-α) expression was found at low dose (5 μg/L) of HFPO−DA, whereas inhibition of proinflammatory factor interleukin-1β (IL-1β) expression was noted at high dose (500 μg/L).[74] HFPO−DA also reduced the diversity of intestinal microbiota and caused an imbalance in intestinal flora. The abundance of certain specific genera was negatively correlated with the expression of genes related to lipid metabolism and apoptosis, indicating that HFPO−DA disturbs intestinal microbiota niche to influence lipid metabolism and apoptosis.[74] Similarly, Xie and others found that HFPO−DA induced colonic inflammation in mice, as evidenced by increased serum TNF-α levels and upregulated mRNA levels of *TNF-α*, *p65*, *toll-like receptor 4*, and *monocyte chemoattractant protein 1* in colon tissues. Such inflammatory changes were correlated with decreased expression of mucin 2 and zonula occludens-1, indicating intestinal barrier dysfunction.[75] A recent study explored the toxicity of PFOA or HFPO−DA combined with polystyrene nanoplastics in *Crassostrea hongkongensis* oysters.[76] The authors observed that the combination of HFPO−DA with nanoplastics induced higher bioaccumulation of these particles and more severe histopathological damage to the intestines compared to nanoplastics alone or in combination with PFOA, which correlated with higher expression levels of antioxidant enzymes and a comparable upregulation of immune-related genes.[76]

Immunotoxicity. The immune system is another target of HFPO−DA. Rushing and colleagues treated C57BL/6 mice with HFPO−DA by oral gavage at doses of 1−100 mg/kg/day for 28 days and found that T cell-dependent antibody responses (TDARs) was suppressed in females as demonstrated by a 7.3% decrease in IgM production, while the number of T lymphocytes increased in males, despite no change in B lymphocyte population in both genders.[77] Yao *et al.*[68] found that HFPO−DA exposure led to increases in the number of neutrophils and the levels of proinflammatory factors, such as interferon α (IFN-α), IL-1β, TNF-α, and C3 complement in zebrafish larvae. Transcriptomic sequencing and gene ontology enrichment analyses further revealed that 30 out of 837 DEGs were immune-related genes, which were predominantly enriched in the myeloid differentiation primary response protein 88 (MyD88)-independent toll-like receptor (TLR) and the inhibitor κB kinase/NF-κB signaling pathways.[68] Protein−protein interaction network and molecular docking analyses further showed that HFPO−DA mediated immunotoxicity mainly through the TLR, RIG-I-like receptor, and NOD-like receptor signaling pathways.[68] In addition, Lee *et al.*[78] found that mice treated with HFPO−DA exhibited attenuated innate immune responses to inhalation of carbon black nanoparticles possibly by suppressions of neutrophil chemokine (CXCL-1/2) production and of neutrophil recruitment into the lungs.[78] HFPO−DA also stimulated the proliferation of lung macrophages and alveolar epithelial

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cells, which may increase susceptibility to lung diseases such as asthma, fibrosis, and cancer.[78] Thus, HFPO−DA exposure perturbs the functions and homeostasis of the immune system.

Hematological and Renal Toxicity. The EPA's 2021 report showed that HFPO−DA could induce anemia.[4] For example, male ICR mice and SD rats treated with 3−100 mg/kg/day of HFPO−DA for 28−180 days had 11%−12% decreases in hemoglobin, hematocrit, and red blood cell counts.[4] Such hematological effects were also observed in female animals, typically at higher doses (*i.e.*, 50 mg/kg/day and above). The maximum decreases in hemoglobin, red blood cell counts, and hematocrit were 24%, 28%, and 20% in females, respectively.[4]

Evidence from subchronic studies elucidated that HFPO−DA treatment at doses of 0.1−1000 mg/kg/day increased kidney coefficient in ICR mice and SD rats with a 16% maximal increase in male animals and a 23% elevation in females. These increases in kidney weight were accompanied by elevated blood urea nitrogen levels, an indication of kidney injury.[4] Consistently, results from chronic toxicity studies uncovered that the changes in biochemical damage marker and kidney weight in female rats were also accompanied by renal pathological damages, such as transitional cell hyperplasia, tubular dilation, pelvic and tubular mineralization, papillary edema, and even necrosis.[4] Thus, HFPO−DA is a hematologic and nephrotoxic agent.

Carcinogenic and Genetic Effects. The carcinogenicity of HFPO−DA remains inconclusive. Based upon evidence mainly from animal studies, the EPA classified HFPO−DA as *Suggestive Evidence of Carcinogenic Potential* for humans in 2021.[4] This was supported by the observations that liver tumor in females as well as pancreatic carcinoma and Leydig cell adenoma in males were observed in rats chronically treated with 500 mg/kg/day of HFPO−DA.[4] However, Leydig cell tumor was reported to have low relevance between humans and rodents.[79] In addition to that, this specific study observed large number of animal premature deaths, which led the EPA to conclude that the evidence was insufficient.[4]

Of note, HFPO−DA induced chromosomal aberrations in Chinese hamster ovary cells *in vitro*.[4] A recent study revealed that HFPO−DA induced both DNA fragmentation and chromatin damage in rat FRTL-5 thyroid cell line as evidenced by increases in comet formation, tail length, DNA abundance in comet tail, and micronucleus formation.[80] However, other genetic mutation results showed that HFPO−DA ammonium salt failed to induced mutations in prokaryotic *Escherichia coli* and *Salmonella typhimurium* as well as mouse lymphoma cells with and without metabolic activation.[4] *In vivo* mammalian studies also found no evidence of chromosomal mutations and aberrations, micronucleus formation, or DNA damage following oral exposure of HFPO−DA.[4]

The possible carcinogenic and genotoxic mechanisms of HFPO−DA are also incompletely understood. PPARα activation was a possible mechanism mediating HFPO−DA induced carcinogenesis in the chronic rat study referred by the EPA.[4] In cultured human bone marrow mesenchymal stem cells *in vitro*, transcriptomic and enrichment analyses showed that genes in apoptotic signaling pathways were significantly enriched by HFPO−DA treatment, many of which were related to KEGG terms like "metabolic pathways", "colorectal cancer", "bladder cancer", "Ras signaling", "HIF-1α signaling", and "AMPK pathway", implying possible links between these pathways with HFPO−DA.[81] HFPO−DA could also inhibit the expression of lysosome-associated protein transmembrane-4β and cathepsin B genes in zebrafish, which were typically known for tumor proliferation, invasion, and metastasis.[66] Taken together, more evidence is definitely required to assess the potential carcinogenicity and genotoxicity of HFPO−DA.

5. RISK ASSESSMENT OF HFPO−DA

5.1. Human Exposure and Risks of HFPO−DA

Exposure of HFPO−DA to human beings and its potential health effects have been increasingly appreciated. The dietary intake of HFPO−DA in 33 cities in China was estimated to be 2.33−3.96 ng/kg/day, which exceeds the EPA's RfD value (3.0 ng/kg/day) by 0.78−1.32 times.[43] The fifth percentile, median, and 95th percentile EDIs of HFPO−DA were 5.06 ng/kg/day, 17.9 ng/kg/day, and 87.07 ng/kg/day in residents living near a FIP facility in Shandong Province, China, respectively.[82] Over 89.8% of this population exposed to HFPO−DA levels exceeding the RfD value, with 33.4% experiencing exposure up to 10-fold higher.[82] Similarly, another study conducted in residents surrounding a large FIP plant in the same region also found that the EDI of HFPO−DA *via* food, drinking water, and indoor dust was 52.1 ng/kg/day, despite it was much lower than that of PFOA (457 ng/kg/day).[11] Nevertheless, these lines of evidence indicate that the external exposure levels of HFPO−DA in the human population are alarming, and its resulting health risks merit further investigation in the future.

Unlike daily intake levels of HFPO−DA, trace amounts of HFPO−DA have been detected in human specimens. In blood samples collected from the Anniston Community Health Survey (ACHS) I in 2005−2006 and ACHS II in 2014 in the United States, the average concentrations of HFPO−DA were 0.05 ng/mL and 0.07 ng/mL (LOQ = 0.05 ng/mL), respectively, with detection rates of 4.4% and 8.9%.[83] Kotlarz *et al.*[84] found that HFPO−DA did not exceed a LOQ of 2 ng/mL in blood of 344 residents near the Cape Fear River in North Carolina. By contrast, another study reported that HFPO−DA was detected in 48% of serum samples of residents near the Cape Fear River, with a mean concentration of 1.9 ng/mL (ranging from <LOQ 0.24 ng/mL to 5.85 ng/mL).[85] Data from the National Health and Nutrition Examination Survey (NHANES) showed that only one out of 1672 blood specimen had HFPO−DA level above the LOQ value (0.1 ng/mL).[86] Results from Chinese residents near a FIP facility showed an average of 0.028 ng/mL HFPO−DA in blood (below the LOQ of 0.05 ng/mL) with a detection rate of 8.4%.[87] Likewise, Calafat *et al.*[88] analyzed 2682 urine samples of NHANES participants in 2013−2014 and found that HFPO−DA was one of the few PFAS detected with a detection rate of 1.2% and a maximum concentration of 0.4 μg/mL (LOQ = 0.1 μg/mL). Using HPLC-tandem mass spectrometry (LOQ = 1.68 ng/mL), no HFPO−DA was detected in urine samples of residents near a large FIP in China.[11] These findings suggest that HFPO−DA levels in humans are generally low.

Nevertheless, epidemiological evidence supports a correlation between HFPO−DA exposure and reproductive dysfunctions. In a case-control cohort of infertile women with polycystic ovary syndrome (PCOS; n = 366) in China, the detection rate of HFPO−DA in plasma was 94.3%, which was higher than that of 89.9% in control individuals (n = 577).[89] Importantly, each one standard deviation increase in HFPO−

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DA level correlated with a 39% higher risk of PCOS, and multipollutant analysis models revealed that exposure to PFASs was associated with a 9% increase in the prevalence rate of PCOS, with HFPO−DA being one of the main contributors, particularly in overweight/obese women.[89] In addition, the same research group also found that the detection rate of HFPO−DA in plasma was 97.4% in unexplained recurrent spontaneous abortion patients (n = 464) and that exposure to HFPO−DA was associated with a 35% additional increase in the risk of this disease [adjusted odds ratio = 1.35 (95% CI: 1.15, 1.59)].[90]

In addition to the reproductive system, a suggestive link was also observed between HFPO−DA and glioma. HFPO−DA was detected in 31% of human brain glioma tissue samples (n = 26, LOQ = 0.05 ng/g wet weight), with a median concentration of 0.64 ng/g higher than that of PFOA (0.33 ng/g).[91] The median level of HFPO−DA in glioma tissues (n = 137) was estimated to 0.75 ng/g, which was three folds higher than that of surrounding noncancerous tissues (0.25 ng/g; n = 40).[92] However, this specific study was exclusively done in glioma patients without healthy control individuals, making it inconclusive in the causal relationship between HFPO−DA and glioma.[93]

Together, these preliminary findings in the human population highlight the potential risks of HFPO−DA exposure on the reproductive system and beyond.

5.2. Current Health Toxicity Values of HFPO−DA

Based upon these known health impacts of HFPO−DA on animals and humans, its health toxicity values have been proposed. The North Carolina Department of Health and Human Services established a value of 0.140 μg/L as the provisional drinking water health goal for HFPO−DA in 2017. This limit value was nonregulatory and nonenforceable and was based on considerations of 0.1 mg/kg/day as the NOAEL value, liver single cell necrosis of mice as the sensitive end point, and the bottle-fed infants as the most susceptible population. The total uncertainty factors (UFs) were 1000, which includes a value of ten for each factor: interspecies variability, intraspecies variability, and extrapolation from subchronic to chronic exposure duration. Thus, an RfD value of 0.0001 mg/kg/day was recommended. The provisional goal was calculated based on this RfD by considering the infant's body weights, the intake of drinking water for bottle feeding, and a 20% relative contribution from the contamination source.[94]

In 2021, the United States EPA re-evaluated the health toxicity values of HFPO−DA by using the aforementioned NOAEL of 0.1 mg/kg/day and considering liver lesions in rodents as relevant to humans. Using benchmark dose modeling and allometric scaling of the toxicologically equivalent dose, a human equivalent dose of 0.01 mg/kg/day was determined. By taking a value of 3000 as the UFs (a factor of 10 for each: intraspecies variability, database deficiencies, and extrapolation from sub-chronic to chronic duration, and a factor of 3 for interspecies differences), the established RfD was 0.003 μg/kg/day.[4] In 2022, the EPA released 10 ng/L as the final drinking water health advisory for HFPO−DA by considering lactating women as the most sensitive population and their intake of drinking water per body weight.[95] In April 2024, this value of 10 ng/L was legally set as the maximum contaminant levels for HFPO−DA by the EPA.[96]

The National Institute for Public Health and the Environment in The Netherlands set safety limit values for HFPO−DA: 118 ng/L as the water quality standard, 100 ng/g dry weight as the risk limit for general soils, 8 ng/g dry weight as the risk limit for vegetable garden soils, and 660 ng/L as the risk limit for groundwater. For drinking water, a tolerable daily intake (TDI) of 21 ng/kg/day was established based upon the NOAEL of 0.1 mg/kg/day and UFs. A provisional guidance value of 0.15 μg/L for HFPO−DA was determined by considering this TDI, the average body weights of adults, the intake of drinking water, and a 20% relative contribution from the source.[97] These administrative guidelines underscore global recognition of the potential health risks of HFPO−DA and are required to be updated as new scientific knowledge emerges.

6. CONCLUSIONS AND FUTURE DIRECTIONS

Although HFPO−DA is currently considered to have low toxicity and persistence, its increasingly wide applications as an alternative to PFOA have raised concerns about its potential environmental and health risks. Human exposure to HFPO−DA occurs through multiple routes, leading to exposure levels that exceed the current RfD in certain populations. Epidemiological and toxicological evidence has documented a plethora of adverse effects associated with HFPO−DA. However, the underlying mechanisms are complex and remain poorly understood. While health toxicity values for HFPO−DA have been established in the United States and The Netherlands, further studies are urgently needed to refine these values and understand the potential long-term health effects.

In addition, the following areas also warrant future investigation. First, the environmental behaviors of HFPO−DA. Considering its slow degradation rate and long-distance transport capability, assessment of the long-term fates of HFPO−DA in the environment is needed. New and advanced liquid chromatography−mass spectrometry approaches are required to identify the intermediate degradation products of HFPO−DA and their environmental fates. Computational, mathematical, and statistical models are helpful to elucidate the interactions of HFPO−DA with other environmental contaminants and natural chemicals. Second, assessment of health risks of HFPO−DA. Perspective and large cohort epidemiological studies are optimal to investigate the long-term health effects of HFPO−DA exposure on humans, with special focuses on the vulnerable populations, such as infants, children, pregnant women, and the elderly. Furthermore, the distribution profiles and potential bioaccumulation target organs of HFPO−DA remain to be defined. Limited evidence on HFPO−DA content has been reported in human brain tissues, while its presence and distribution in other tissues (*i.e.*, the liver and reproductive organs) remain unknown. Animals treated with tracer-labeled HFPO−DA, such as F^{18}-labeling with positron emission tomography and fluorescein-labeling with *in vivo* optical imaging, will be helpful to answer these questions. Lastly, the toxic mechanisms of HFPO−DA. Recent studies proposed to use un(semi)supervised machine learning, molecular docking models, and PBT/PMT-based (persistence, P; bioaccumulation, B; mobility, M; and toxicity, T) grouping strategies to predict the bioactivity of PFASs and inform risk assessment.[98,99] Such approaches are also applicable to studying the potential effects and to identifying molecular targets of HFPO−DA based upon structural similarity with other PFASs. Despite the identification of reproductive and

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developmental toxicities, there is a lack of multigeneration reproductive and developmental toxicity studies to understand the potential transgenerational impacts of HFPO−DA on offspring, particularly in light of its known epigenetic alterations. Future toxicological studies in conjunction with high-throughput OMICs approaches are also needed to identify specific molecular targets and signaling pathways regulating the adverse effects of HFPO−DA *per se* or its combination with both legacy and emerging PFASs, particularly when exposed at environmentally relevant concentrations.

In conclusion, the potential risks of HFPO−DA to humans and the environment warrant active monitoring and examination. Better understanding the environmental behaviors, toxic effects, and underlying mechanisms and exposure routes of HFPO−DA is essential for developing effective strategies to minimize its harmful impacts and protect human beings and the ecosystems.

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Notes

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