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Evaluation of Developmental Toxicity, Developmental Neurotoxicity, and Tissue Dose in Zebrafish Exposed to GenX and Other PFAS

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Background: Per- and polyfluoroalkyl substances (PFAS) are a diverse class of industrial chemicals with widespread environmental occurrence. Exposure to long-chain PFAS is associated with developmental toxicity, prompting their replacement with short-chain and fluoroether compounds. There is growing public concern over the safety of replacement PFAS.

Objective: We aimed to group PFAS based on shared toxicity phenotypes.

Methods: Zebrafish were developmentally exposed to 4,8-dioxa-3H-perfluorononanoate (ADONA), perfluoro-2-propoxypropanoic acid (GenX Free Acid), perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid (PFESA1), perfluorohexanesulfonic acid (PFHxS), perfluorohexanoic acid (PFHxA), perfluoro-*n*-octanoic acid (PFOA), perfluorooctanesulfonic acid (PFOS), or 0.4% dimethyl sulfoxide (DMSO) daily from 0–5 d post fertilization (dpf). At 6 dpf, developmental toxicity and developmental neurotoxicity assays were performed, and targeted analytical chemistry was used to measure media and tissue doses. To test whether aliphatic sulfonic acid PFAS cause the same toxicity phenotypes, perfluorobutanesulfonic acid (PFBS; 4-carbon), perfluoropentanesulfonic acid (PFPeS; 5-carbon), PFHxS (6-carbon), perfluoroheptanesulfonic acid (PFHpS; 7-carbon), and PFOS (8-carbon) were evaluated.

Results: PFHxS or PFOS exposure caused failed swim bladder inflation, abnormal ventroflexion of the tail, and hyperactivity at nonteratogenic concentrations. Exposure to PFHxA resulted in a unique hyperactivity signature. ADONA, PFESA1, or PFOA exposure resulted in detectable levels of parent compound in larval tissue but yielded negative toxicity results. GenX was unstable in DMSO, but stable and negative for toxicity when diluted in deionized water. Exposure to PFPeS, PFHxS, PFHpS, or PFOS resulted in a shared toxicity phenotype characterized by body axis and swim bladder defects and hyperactivity.

Conclusions: All emerging fluoroether PFAS tested were negative for evaluated outcomes. Two unique toxicity signatures were identified arising from structurally dissimilar PFAS. Among sulfonic acid aliphatic PFAS, chemical potencies were correlated with increasing carbon chain length for developmental neurotoxicity, but not developmental toxicity. This study identified relationships between chemical structures and *in vivo* phenotypes that may arise from shared mechanisms of PFAS toxicity. These data suggest that developmental neurotoxicity is an important end point to consider for this class of widely occurring environmental chemicals. https://doi.org/10.1289/EHP5843

Introduction

Per- and polyfluoroalkyl substances (PFAS) are a structurally diverse class of industrial chemicals that contain aliphatic chains with all or some of the carbons bonded to fluorines (-C_nF_{2n}-) and carboxylic acid or sulfonic acid terminal moieties (OECD 2018). There are 4,370 unique PFAS structures (OECD 2018) with 602 compounds currently in commercial use in the United States (U.S. EPA 2019). PFAS have flame-retardant, water-resistant, and surfactant-like properties (Banks et al. 1994; Kissa 2001). This class of compounds is therefore widely used as protectants in paper and packaging products, water- and grease-repellent textiles, nonstick cookware coatings, and firefighting foams (Lindstrom et al. 2011). PFAS are extremely stable due to the carbon–fluorine bond strength (Banks et al. 1994; Kissa 2001). Based on their structurally inherent thermal and chemical stability, PFAS persist in the environment where they are generally resistant to biodegradation, photooxidation, direct photolysis, and hydrolysis (Schultz et al. 2003). As a result, they are widely detected in the environment (Dauchy et al. 2019; Pan et al. 2018), wildlife (Cui et al. 2018; Escoruela et al. 2018; Route et al. 2014), drinking water (Guelfo and Adamson 2018; Guelfo et al. 2018), and humans (Daly et al. 2018; Hurley et al. 2018; Jain 2018).

Since the voluntary phaseout of perfluoro-*n*-octanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) in the early 2000s, time trends of National Health and Nutrition Examination Survey (NHANES) PFOS and PFOA serum levels are generally indicative of reduced human exposures (Jain 2018). Despite reductions, exposures are still widespread, with PFAS detectable in 95% of NHANES subjects (2013–2014) (CDC 2019) and in pregnant women, maternal serum levels for PFOS (35.3 ng/mL) and PFOA (5.6 ng/mL) have been reported (Fei et al. 2007). Of additional concern, an examination of these compounds in U.S. children 3–11 years of age, most of whom were born after PFOS and PFOA were phased out of use, revealed detectable levels of 14 PFAS, including PFOS and PFOA, in more than 60% of study subjects (Ye et al. 2018). A longitudinal study in Finnish children and adolescents showed that although serum levels of PFOS,

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PFOA, perfluorohexanesulfonic acid (PFHxS), and perfluorohexanoic acid (PFHxA) decreased over the study period, calculated body burdens generally remained constant and, in some cases, increased (Koponen et al. 2018). In humans, PFAS exposure has been associated with reduced birth weight (Apelberg et al. 2007; Fei et al. 2007), although weak associations with low birth weight or conflicting data have also been reported (Manzano-Salgado et al. 2017; Shoaff et al. 2018; Whitworth et al. 2012). In animal studies, early life stage exposure to PFOS or PFOA have been linked to developmental toxicity in chickens and mice (Jiang et al. 2012; Tucker et al. 2015), immunotoxicity in mice (reviewed by DeWitt et al. 2009), and developmental (Huang et al. 2010; Padilla et al. 2012; Truong et al. 2014) and reproductive toxicity in zebrafish (Jantzen et al. 2017).

To address toxicity concerns, longer alkyl chain PFAS like PFOS and PFOA have been replaced with shorter alkyl chain compounds such as perfluorobutanesulfonic acid (PFBS) or large fluoroether PFAS such as perfluoro-2-propoxypropanoic acid (GenX) and 4,8-dioxa-3H-perfluorononanoate (ADONA). Alternative chemistries that retain the long-chain character, such as ADONA, were engineered with ether linkages and sites of hydrogenation in efforts to reduce biological half-lives (Fromme et al. 2017). Replacement PFAS are therefore increasingly detected in the environment, including in surface water (De Silva et al. 2011; McCord et al. 2018; Pan et al. 2018; Strynar et al. 2015; Wang et al. 2016) and drinking water (Kaboré et al. 2018; McCord et al. 2018). Environmental screening efforts have also identified relevant exposures to PFAS by-products, such as sulfonated fluorovinyl ethers (i.e., PFESA compounds), that are not strictly chemicals of commerce (McCord et al. 2018; Strynar et al. 2015). Growing concern over the safety of GenX and other replacement PFAS has unsurprisingly led to a greater demand for toxicity data (Blum et al. 2015; Borg et al. 2017; Scheringer et al. 2014). However, traditional mammalian toxicity assays can be costly and time consuming, and it is challenging to test multiple chemicals and concentrations of chemicals in parallel. Because PFAS exposures have been historically linked to complex toxicity outcomes involving whole organisms (e.g., developmental toxicity) or specific organ systems (e.g., immunotoxicity), the use of a rapid *in vivo* animal screening system is justified.

The zebrafish is a widely used *in vivo* model for toxicity testing (Hamm et al. 2019; Padilla et al. 2012). Development is rapid, with organogenesis complete by 3 d post fertilization (3 dpf). The zebrafish genome contains orthologs for ~70% of human genes (Howe et al. 2013) and ~86% of the genes that are known human drug targets (Gunnarsson et al. 2008). Zebrafish developmental toxicity testing can be completed in a matter of days by directly exposing the developing organism to xenobiotics. Post-hatch, automated locomotor behavior tests can be used to assess swimming behavior in response to a variety of stimuli as a functional neurodevelopmental outcome. One major limitation of the zebrafish model for toxicity testing relates to chemical dosimetry. Zebrafish embryos are exposed to xenobiotics via immersion. In most studies, nominal waterborne concentrations are generally reported when making determinations on compound toxicity (i.e., positive or negative for toxicity). However, based on physicochemical properties like LogP and differences in exposure parameters (e.g., static vs. semi-static exposures), both of which can affect the uptake, distribution, metabolism, and elimination of test chemicals, the internal tissue dose does not generally reflect nominal exposure media concentrations (Brox et al. 2014, 2016; Kirla et al. 2016; Souder and Gorelick 2017).

The developmental toxicity and developmental neurotoxicity of a subset of PFAS, such as PFOS and PFOA, have been previously evaluated in zebrafish (Hagenaars et al. 2011; Huang et al. 2010; Jantzen et al. 2016; Khezri et al. 2017; Spulber et al. 2014; Ulhaq et al. 2013a, 2013b). PFOS exposure results in failed swim bladder inflation, abnormal ventroflexion of the tail (Hagenaars et al. 2011; Huang et al. 2010; Jantzen et al. 2016; Ulhaq et al. 2013a), and hyperactivity (Hurley et al. 2018; Khezri et al. 2017; Spulber et al. 2014), whereas results for PFOA exposures are quite mixed for both developmental toxicity and behavior (Hagenaars et al. 2011; Huang et al. 2010; Jantzen et al. 2016; Khezri et al. 2017; Padilla et al. 2012; Truong et al. 2014; Ulhaq et al. 2013a, 2013b). However, because replacement PFAS such as GenX and ADONA are detected in the environment yet lack adequate data on their potential toxicity, the goal of this study was to assess the developmental toxicity, developmental neurotoxicity, and tissue doses of multiple aliphatic PFAS (e.g., PFOS, PFOA, PFHxS, and PFHxA), several emerging replacement PFAS (e.g., GenX and ADONA), and a polymer production by-product [e.g., perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid (PFESA1)] in parallel, using zebrafish as a test organism. In addition, the potential of sulfonic acid PFAS with varying alkyl chain lengths to elicit similar toxicity phenotypes was assessed.

Methods

Zebrafish Husbandry

All procedures involving zebrafish were approved by the U.S. Environmental Protection Agency (EPA) National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee and carried out in accordance with the relevant guidelines and regulations. Embryos were obtained from a mixed wild-type (WT) adult zebrafish line (*Danio rerio*) that was generated and maintained as previously described (Phelps et al. 2017). Briefly, to maintain genetic diversity, a minimum of one WT line (AB and/or Tupfel long fin WT strains) was added one time per year. Zebrafish adults were housed in 6-L tanks at an approximate density of 8 fish/L. Adults were fed Gemma Micro 300 (Skretting) once daily and shell free E-Z Egg (Brine Shrimp Direct) twice daily Mondays through Fridays. Both food sources were fed once daily on weekends. U.S. EPA WT zebrafish were maintained on a 14 h:10 h light cycle at 28.5°C and bred every 2–3 weeks. For embryo collection, 60–100 adults were placed in 10- or 20-L angled static breeding tanks overnight. The following morning, adults were transferred to new angled bottom tanks containing fish facility water, and embryos were collected 30–40 min later.

Chemical Preparation

ADONA [Chemical Abstracts Service Registry No. (CASRN): 958445-44-8; Catalog No. NaDONA] was purchased from Wellington Laboratories (Table 1). GenX Free Acid (CASRN: 13252-13-6; Catalog No. 2121-3-13), PFHxA (CASRN: 307-24-4; Catalog No. 2121-3-39), PFHxS (CASRN: 3871-99-6; Catalog No. 6164-3-X4), PFOA (CASRN: 335-67-1; Catalog No. 2121-3-18), PFOS (CASRN: 1763-23-1; Catalog No. 6164-3-08), perfluorobutanesulfonic acid (PFBS; CASRN: 375-73-5; Catalog No. 6164-3-09), and perfluoroheptanesulfonic acid (PFHpS; CASRN: 375-92-8; Catalog No. 6164-3-2S) were purchased from Synquest. Perfluoropentanesulfonic acid (PFPeS; CASRN 2706-91-4; Catalog No. 6164-3-2U) was synthesized for the study by Synquest Laboratories and chlorpyrifos (CASRN: 2921-88-2; Catalog No. 45395) was purchased from Sigma-Aldrich. PFESA1 (CASRN: 29311-67-9) was obtained from Chemours (Table 1). Stock solutions (20 mM or 25 mM) were prepared either by mixing liquid chemical or dissolving neat chemical into molecular-grade dimethyl sulfoxide (DMSO) (>99.9%) or deionized (DI) water, and aliquots were stored at −80°C. For each experiment, 250× working solutions were prepared by thawing single-use stock solution aliquots and performing

Table 1. Test chemicals.

Chemical	Name	CASRN	MW (g/mol)	LogP[a] (OPERA[b])	Company, catalog no.
4,8-Dioxa-3H-perfluorononanoate	ADONA	958445-44-8	400.05	3.96	Wellington Laboratories, NaNODA
Perfluoro-2-propoxypropanoic acid	GenX Free Acid	13252-13-6	330.05	3.21	Synquest, 2121-3-13
Perfluorobutanesulfonic acid	PFBS	375-73-5	300.1	3.10	Synquest, 6164-3-09
Perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid	PFESA1	29311-67-9	444.12	6.02	Obtained from Chemours
Perfluoroheptanesulfonic acid	PFHpS	375-92-8	450.12	2.83	Synquest, 6164-3-2S
Perfluorohexanoic acid	PFHxA	307-24-4	314.05	2.78	Synquest, 2121-3-39
Perfluorohexanesulfonic acid	PFHxS	3871-99-6	438.21	3.87	Synquest, 6164-3-X4
Perfluoro-*n*-octanoic acid	PFOA	335-67-1	414.07	3.79	Synquest, 2121-3-18
Perfluorooctanesulfonic acid	PFOS	1763-23-1	500.13	2.77	Synquest, 6164-3-08
Perfluoropentanesulfonic acid	PFPeS	2706-91-4	350.11	3.18	Synquest, 6164-3-2U

Note: CASRN, Chemical Abstracts Service Registration Number; MW, molecular weight.
[a]Partition coefficient.
[b]OPEn structure-activity/property Relationship App (OPERA) (https://comptox.epa.gov/dashboard).

semi- or quarter-log serial dilutions in DMSO or DI water in a 96-well polycarbonate microtiter plate. Stock plates containing 250× working solutions were sealed (Biorad; Catalog No. MSB1001) and stored at room temperature in the dark and used for the duration of each study (maximum storage time of 5 weeks).

Study Design

In Study 1 (Figure 1), the developmental toxicity and developmental neurotoxicity and the media and internal tissue doses of ADONA, GenX Free Acid, PFESA1, PFHxA, PFHxS, PFOA, and PFOS were determined, using DMSO as a vehicle. All chemicals except PFESA1 were tested in parallel and shared the same DMSO control samples for all three assays. PFESA1 was obtained subsequently from Chemours and therefore had unique, experiment-specific control data. In Study 1, GenX Free Acid diluted in DMSO was determined to be unstable, resulting in a null data set that was therefore excluded. In Study 2 (Figure 1), zebrafish were exposed to GenX Free Acid diluted in DI water and evaluated in the developmental toxicity (DevTox) and developmental neurotoxicity (DNT) assays. Measured media and tissue doses were also obtained. Last, in a sulfonic acid PFAS follow-up study (Study 3) (Figure 1), the ability of PFBS (4-carbon), PFPeS (5-carbon), PFHxS (6-carbon), PFHpS (7-carbon), or PFOS (8-carbon) exposure to cause developmental toxicity or developmental neurotoxicity was assessed. All chemicals tested in Study 3, except PFPeS, were exposed in parallel and have shared DMSO control data. PFPeS was synthesized for this study and tested separately, with an experiment-specific DMSO control.

Chemical Exposures

At 0 dpf, zebrafish embryos were bleached as previously described (Tal et al. 2017). A single embryo at the dome-to-epiboly stages (Kimmel et al. 1995) was placed into each individual well of a 96-well plate containing a 40-μm nylon mesh filter (Millipore,

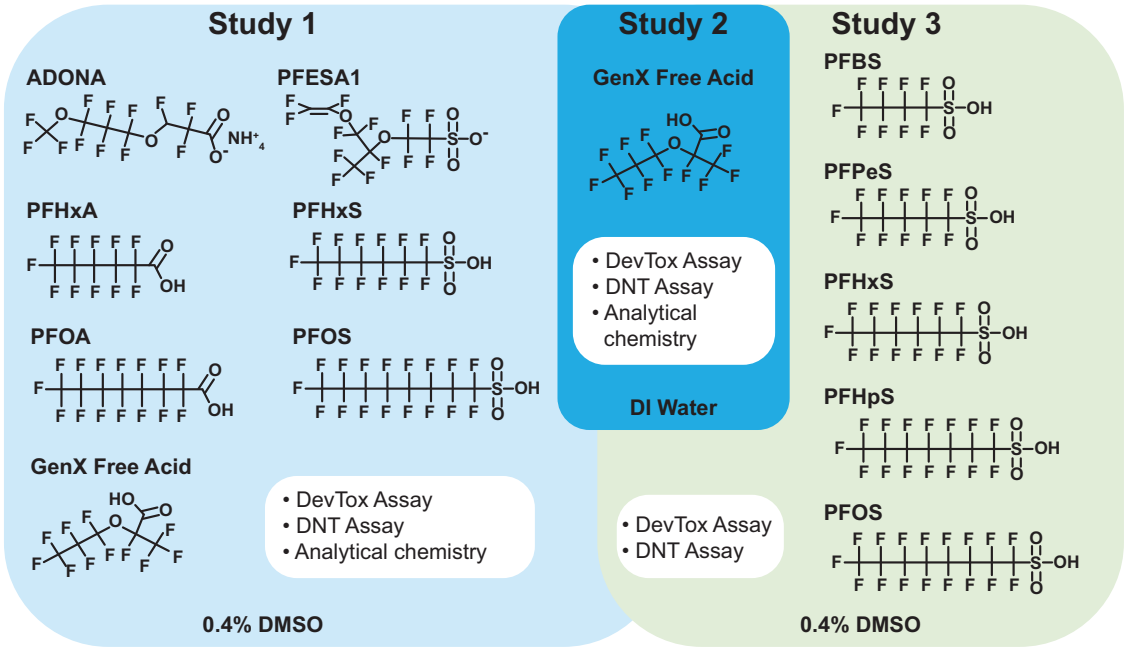

Figure 1. Study design. Zebrafish were semi-statically exposed to test PFAS daily, from 0–5 dpf. At 6 dpf. developmental toxicity, developmental neurotoxicity, and PFAS tissue concentrations were assessed. Test PFAS included in Study 1, solubilized in DMSO (final concentration 0.4% DMSO), are highlighted in light blue. Because GenX Free Acid was not stable in DMSO, the compound was retested in all three assays using DI water as a diluent in Study 2 (highlighted in blue). In Study 3, a set of sulfonic acid aliphatic PFAS solubilized in DMSO were tested in the DevTox and DNT assays (shown in green). Note: ADONA, 4,8-dioxa-3H-perfluorononanoate; DevTox, developmental toxicity; DI, deionized; DMSO, dimethyl sulfoxide; DNT, developmental neurotoxicity; dpf, days post fertilization; GenX Free Acid, perfluoro-2-propoxypropanoic acid; PFAS, per- and polyfluoroalkyl substances; PFBS, perfluorobutanesulfonic acid; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHpS, perfluoroheptanesulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid; PFPeS, perfluoropentanesulfonic acid.

Catalog No. MANMN4010) with 400 μL of 10% Hanks' balanced salt solution (HBSS) per well. Filter inserts containing zebrafish embryos were transferred to 96-well culture trays (Millipore, Catalog No. MAMCS9610) containing 250 μL of 10% HBSS (Westerfield 2007) and 1 μL of 250× working solutions per well. A final concentration of 0.4% DMSO was used for all exposure groups and as a vehicle control. In the case of GenX Free Acid in Study 2 (Figure 1), DI water was used as a vehicle control. Daily, from 1–5 dpf, plates underwent 100% media changes to refresh chemical dosing solutions by blotting (Brandel; Catalog No. FPXLR-196) and transferring mesh inserts containing zebrafish to new bottom plates (Millipore; Catalog No. MAMCS9610). To minimize evaporation, plates were sealed (Biorad; Catalog No. MSA5001) and wrapped with parafilm. Plates were maintained on a 14 h:10 h light cycle at 26.0°C and scored daily for death, malformations, hatching, and swim bladder inflation. At 6 dpf, plates were evaluated by two independent observers and DevTox or DNT assays were performed or media and tissue were collected for analytical chemistry analyses as described below.

Developmental Toxicity Assay

In Study 1 (Figure 1), zebrafish were exposed, as described in the "Chemical Exposures" section, to 0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, or 80.0 μM PFOS, PFOA, PFHxS, PFHxA, or ADONA, or 0.4% DMSO. Six 96-well plates were tested with a single chemical concentration included on each microtiter plate. Subsequently, as part of Study 1, zebrafish were exposed to 0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, or 80.0 μM PFESA1, or 0.4% DMSO. The number of biological replicates per study and additional experimental details are shown in Table 2. In Study 2, GenX Free Acid diluted in DI water was tested by exposing zebrafish to 0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, or 80.0 μM of the compound or DI water. In a follow-up study to assess the toxicity of aliphatic sulfonic acid PFAS (Study 3), a higher starting concentration was used to increase the likelihood of observing both malformations with shorter-chain compounds and malformations at multiple test concentrations. Zebrafish were exposed to 1.7, 3.1, 5.5, 9.8, 17.6, 31.4, 56.0, or 100.0 μM of PFBS, PFHxS, PFHpS, or PFOS or 0.4% DMSO. Subsequently, zebrafish were exposed to 1.7, 3.1, 5.5, 9.8, 17.6, 31.4, 56.0, or 100.0 μM PFPeS, or 0.4% DMSO. Chlorpyrifos was used as positive control for malformations (8.0 μM) and lethality (80.0 μM) (Padilla et al. 2012; Tal et al. 2017). To conduct DevTox assay assessments, at 6 dpf, two independent observers evaluated zebrafish larvae for survival, hatching, swim bladder inflation, and malformations, including curved body axis, shortened trunk, pericardial edema, yolk sac edema, necrotic yolk sac, pectoral fin abnormalities and head/jaw

Table 2. Study-specific metrics.

Study	Name	Diluent and/or vehicle	Assay	Concentrations tested (μM)	Exposure replicate[a] (*n*)	Control replicate[a] (*n*)	96-well plates chemicals tested across[b] (*n*)
1	ADONA	DMSO	DevTox	0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, 80.0	6	216	6
			DNT	4.4, 7.9, 14.0, 25.1, 44.8, 80.0	24	394	17
			Chemistry	25.1, 44.8, 80.0	5	5	3
1	GenX Free Acid	DMSO	DevTox, DNT, Chemistry	Not stable in DMSO; results not reported; see Study 2			
1	PFESA1	DMSO	DevTox	0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, 80.0	6	44	1
			DNT	4.4, 7.9, 14.0, 25.1, 44.8, 80.0	24	339	7
			Chemistry	25.1, 44.8, 80.0	4	5	3
1	PFHxA	DMSO	DevTox	0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, 80.0	6	216	6
			DNT	4.4, 7.9, 14.0, 25.1, 44.8, 80.0	24	394	17
			Chemistry	25.1, 44.8, 80.0	4	4	2
1	PFHxS	DMSO	DevTox	0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, 80.0	6	216	6
			DNT	4.4, 7.9, 14.0, 25.1, 44.8, 80.0	24	394	17
			Chemistry	14.0, 25.1, 44.8	4	4	2
1	PFOA	DMSO	DevTox	0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, 80.0	6	216	6
			DNT	4.4, 7.9, 14.0, 25.1, 44.8, 80.0	24	394	17
			Chemistry	25.1, 44.8, 80.0	4	4	2
1	PFOS	DMSO	DevTox	0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, 80.0	6	216	6
			DNT	0.2, 0.3, 0.6, 1.0, 1.8, 3.1	24	394	17
			Chemistry	1.0, 1.8, 3.1	4	4	2
2	GenX Free Acid	DI water	DevTox	0.04, 0.1, 0.4, 1.1, 3.1, 9.3, 27.2, 80.0	6	44	1
			DNT	4.4, 7.9, 14.0, 25.1, 44.8, 80.0	24	161	4
			Chemistry	25.1, 44.8, 80.0	4	4	2
3	PFBS	DMSO	DevTox	1.7, 3.1, 5.5, 9.8, 17.6, 31.4, 56.0, 100.0	10	140	6
			DNT	5.5, 9.8, 17.6, 31.4, 56.0, 100.0	25	327	10
3	PFPeS	DMSO	DevTox	1.7, 3.1, 5.5, 9.8, 17.6, 31.4, 56.0, 100.0	6	44	1
			DNT	3.1, 5.5, 9.8, 17.6, 31.4, 56.0	24	186	4
3	PFHxS	DMSO	DevTox	1.7, 3.1, 5.5, 9.8, 17.6, 31.4, 56.0, 100.0	10	140	6
			DNT	3.1, 5.5, 9.8, 17.6, 31.4, 56.0	25	327	10
3	PFHpS	DMSO	DevTox	1.7, 3.1, 5.5, 9.8, 17.6, 31.4, 56.0, 100.0	10	140	6
			DNT	1.7, 3.1, 5.5, 9.8, 17.6, 31.4	25	327	10
3	PFOS	DMSO	DevTox	1.7, 3.1, 5.5, 9.8, 17.6, 31.4, 56.0, 100.0	10	140	6
			DNT	0.5, 1.0, 1.7, 3.1, 5.5, 9.8	25	327	10

Note: ADONA, 4,8-dioxa-3H-perfluorononanoate; DevTox, developmental toxicity; DI, deionized; DMSO, dimethyl sulfoxide; DNT, developmental neurotoxicity; GenX Free Acid, perfluoro-2-propoxypropanoic acid; PFBS, perfluorobutanesulfonic acid; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHpS, perfluoroheptanesulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid; PFPeS, perfluoropentanesulfonic acid.
[a]Replicate numbers indicate single animals except for chemistry samples comprising pools of 10 larvae.
[b]Indicates total number of 96-well microtiter plates assessed for each study and/or assay. For Study 1 DevTox and DNT assays, ADONA, GenX Free Acid, PFESA1, PFHxA, PFHxS, PFOA, and PFOS were tested in parallel and shared the same 0.4% DMSO control samples. PFESA1 was obtained subsequently and had unique, experiment-specific control data. In Study 1, GenX Free Acid diluted in 0.4% DMSO was determined to be unstable, resulting in a null data set. In Study 2 (Figure 1), zebrafish were exposed to GenX Free Acid diluted in DI water and evaluated in the DevTox and DNT assays. Measured media and tissue doses were also obtained. Study 3 (Figure 1) examined the ability of PFBS (4-carbon), PFPeS (5-carbon), PFHxS (6-carbon), PFHpS (7-carbon), or PFOS (8-carbon) exposure to cause developmental toxicity or developmental neurotoxicity. All chemicals tested in Study 3, except PFPeS, were exposed in parallel and have shared DMSO control data. PFPeS was synthesized for this study and tested separately, with an experiment-specific DMSO control.

abnormalities. Directly after assessments, data were reviewed and, in the case of discrepancies, consensus calls were reached. Toxicity values were assigned to descriptive data (i.e., normal = 0, abnormal = 20, severely abnormal = 50, and dead = 100), modified from a previously described approach (Padilla et al. 2012). Briefly, animals with a single malformation were scored as abnormal, whereas animals with ≥2 malformations were scored as severely abnormal. A study inclusion criterion based on a previously published study (Padilla et al. 2012) was applied where microtiter plates with >15% abnormal or dead DMSO or DI water control larvae were excluded (one plate from Study 3 was excluded).

Developmental Neurotoxicity Assay

To increase the likelihood of observing behavioral effects in morphologically normal larvae, the highest concentration evaluated in the DNT assay was the lowest observed effect concentration (LOEC) determined in DevTox assay. Zebrafish were exposed in parallel to 4.4, 7.9, 14.0, 25.1, 44.8, or 80.0 μM PFOA, PFHxS, PFHxA, or ADONA or 0.2, 0.3, 0.6, 1.0, 1.8, or 3.1 μM PFOS or 0.4% DMSO. Subsequently, as part of Study 1, exposure to 4.4, 7.9, 14.0, 25.1, 44.8, or 80.0 μM PFESA1, or 0.4% DMSO was evaluated. In Study 2, zebrafish were exposed to 4.4, 7.9, 14.0, 25.1, 44.8, or 80.0 μM GenX Free Acid, or DI water. In Study 3, to increase the likelihood of observing malformations with shorter-chain compounds at multiple test concentrations, the highest concentration evaluated was 100.0 μM. Zebrafish were exposed to 5.5, 9.8, 17.6, 31.4, 56.0, or 100.0 μM PFBS; 3.1, 5.5, 9.8, 17.6, 31.4, or 56.0 μM PFHxS; 1.7, 3.1, 5.5, 9.8, 17.6, or 31.4 μM PFHpS; or 0.5, 1.0, 1.7, 3.1, 5.5, or 9.8 μM PFOS, or 0.4% DMSO. Subsequently, as part of Study 3, zebrafish were exposed to 3.1, 5.5, 9.8, 17.6, 31.4, or 56.0 μM PFPeS, or 0.4% DMSO. Chemical exposures and assessments were performed daily, as described above. To evaluate swimming behavior in a light/dark behavior test, microtiter plates were placed in a dark, temperature-controlled behavior testing room set to 26.0°C for at least 2 h prior to testing. At the time of testing, microtiter plates were placed on a Noldus tracking apparatus. Locomotor activity was recorded (30 frames/s) for a total of 60 min consisting of a 20-min dark acclimation period (0 lux) that was not analyzed followed by a 40-min testing period consisting of a 20-min light period (5.0 lux) and 20-min dark period (0 lux). Videos were analyzed using Ethovision software (version 3.1; Noldus Information Technology) as previously described (Jarema et al. 2015). Locomotor activity was collected for each individual fish for each 2-min period (minimum distance moved, set to 0.135 cm). Thus, for a 40-min test, 20 data points were collected per larvae. Based on microtiter plate inclusion criterion (i.e., <15% abnormal or dead control larvae), two plates from Study 1 were excluded. Four additional criteria for inclusion of individual larvae were applied. One, all larvae that were identified as abnormal, severely abnormal, or dead were excluded. Two, larvae with uninflated swim bladders were removed from analyses. Three, individual larvae that moved <2 cm in either 10-min dark period were removed. Four, concentrations of test PFAS with fewer than 13 animals remaining (i.e., >50% death or malformations within the test group) were excluded from behavior analyses. Also as part of the DNT assay, media samples were collected. At 6 dpf, 150-μL media samples ($n = 3$/chemical concentration) were collected and stored at −80°C until further analysis.

Tissue Sample Preparation for Analytical Chemistry

Zebrafish were exposed to 25.1, 44.8, or 80.0 μM ADONA, PFOA, PFESA1, or PFHxA, 14.0, 25.1, or 44.8 μM PFHxS, or 1.0, 1.8, or 3.1 μM PFOS, or 0.4% DMSO. The highest concentration evaluated was the no observed effect concentration (NOEC) determined in Study 1. In Study 2, zebrafish were exposed to 25.1, 44.8, or 80.0 μM GenX Free Acid or 0.4% DMSO. According to the previously described microtiter plate inclusion criterion, 15/15 plates were included in the study. Larvae were anesthetized by rapid cooling in chilled 10% HBSS. Groups of 10 anesthetized larvae were pooled to comprise one biological replicate ($n = 4$) in 500 μL of 10% HBSS, flash frozen in liquid nitrogen, and stored at −80°C. In the case of ADONA, $n = 5$.

Analytical Chemistry

PFAS native standards were obtained from SynQuest and Sigma-Aldrich. ^{13}C-labeled PFAS standards were obtained from Wellington Laboratories. Stock solutions of the PFAS were prepared in 95% methanol with 5% aqueous 2.5 M sodium hydroxide and stored at room temperature in plastic. Intermediate standards were prepared daily in methanol or acetonitrile.

Exposure Media Analysis

Exposure media samples (10% HBSS) and quality control (QC) samples (10% HBSS) at microgram-per-milliliter concentrations were diluted and fortified with a surrogate [i.e., perfluorononanoic acid (PFNA)] and internal standards. Calibration standards were prepared at nanogram-per-milliliter concentrations in aqueous 2.5 mM ammonium acetate with 20% methanol. Standards and samples were analyzed with ACQUITY ultra-high-performance liquid chromatography (UPLC) system and Quattro Premier XE triple quadrupole mass spectrometer (Waters Corporation) operated in negative electrospray ionization (ESI) mode. ESI source conditions were optimized for the $[M\text{-}H]^-$ ion of PFAS as follows: capillary voltage −1.97 kV, source temperature 150°C, desolvation temperature 350°C, cone gas flow 2 L/h, and desolvation gas flow 350 L/h. Compound-specific tandem mass spectrometry (MSMS) parameters were used to collect two multiple reaction monitoring (MRM) transitions for the $[M\text{-}H]^-$ ion of each target analyte (Table 3). UPLC separation was achieved using ACQUITY UPLC BEH C18 Column, 130Å, 1.7 μM, 2.1 mm × 50 mm (Waters Corp P/N 186,002,350) at 50°C with the gradient elution at a flow rate of 500 μL/min using 2.5 mM ammonium acetate in methanol and water with a 50-μL injection. Data collection, integration, calibration, and quantitation were performed using MassLynx software (version 4.1; Waters Corporation). Concentration for each PFAS analyte was determined by internal standard technique using isotopically labeled internal standards and calibration standards prepared in solvent. Qualitative identification was based on relative retention time and peak abundance ratio of two MRM transitions. Exposure media analysis was verified and evaluated using blanks, calibration standards, and QC standards prepared at three concentrations across the methods range. Batch results for exposure media were evaluated based on the following criteria: the calibration curve used a minimum of seven standards with a correlation coefficient of >0.99, standards accuracy tolerance <20% (30% at LLOQ), QC standard accuracy tolerance <20% (30% PFOS), QC standard precision expressed as percent relative standard deviation (%RSD) < 20%, >75% of QC standards satisfied accuracy criteria. Exposure media analysis method performance characteristics are listed in Excel Tables S1 and S2.

Tissue Analysis

Tissue samples were prepared by protein precipitation. Flash frozen samples, consisting of pools of ten 6-dpf zebrafish larvae, were homogenized using ∼100 mg of 1.0-mm diameter zirconia/silica beads and a Fast-Prep-24™ homogenizer (MP Biomedicals) in 100 μL 0.1 M formic acid fortified with the surrogate (i.e., PFNA). Protein was precipitated from the homogenate with 400 μL of acetonitrile containing internal standards and separated by centrifugation at

Table 3. Compound-specific Quattro Premier XE MSMS parameters.

Compound name	Parent (m/z)	Daughter (m/z)	Cone (V)	Collision (V)
GenX 1°	329.07	284.06	10	5
GenX 2°	329.07	184.72	10	23
$^{13}C_3$ GenX IS	332.00	287.06	10	5
PFHxA 1°	312.91	268.81	15	9
PFHxA 2°	312.91	118.64	15	25
$^{13}C_2$ PFHxA IS	315.00	269.81	15	9
PFHxS 1°	398.85	98.57	55	37
PFHxS 2°	398.85	79.62	55	41
$^{13}C_3$ PFHxS IS	401.85	79.62	55	41
PFOA 1°	412.93	368.84	15	11
PFOA 2°	412.93	168.63	15	21
$^{13}C_4$ PFOA IS	417.00	372.00	15	11
PFOS 1°	499.00	98.57	60	41
PFOS 2°	499.00	79.62	60	45
$^{13}C_4$ PFOS IS	503.00	98.57	60	41
ADONA 1°	377.02	250.83	15	13
ADONA 2°	377.02	84.70	15	29
PFESA1 (Nafion, BP 1) 1°	442.98	146.69	35	29
PFESA1 (Nafion, BP 1) 2°	442.98	262.79	35	19
PFNA 1°	463.00	418.90	15	13
PFNA 2°	463.00	218.84	15	17
$^{13}C_5$ PFNA IS	468.00	423.00	15	13

Note: 1° denotes the primary multiple reaction monitoring (MRM) transition for a compound used for quantitative analysis and 2° denotes the secondary MRM transition for a compound used for qualitative identification confirmation. ADONA, 4,8-dioxa-3H-perfluorononanoate; GenX Free Acid, perfluoro-2-propoxypropanoic acid; MSMS, tandem mass spectrometry; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid; PFPeS, perfluoropentanesulfonic acid; Quattro Premier XE, triple quadrupole mass spectrometer.

14,000 rpm for 15 min at 4°C. Fifty microliters of the extract was diluted with 200 µL aqueous 0.4 mM ammonium formate in the LC vial for analysis. Standards and samples were analyzed with Vanquish UPLC and Orbitrap Fusion mass spectrometer (Thermo Electron) operated in negative ESI mode. ESI source conditions were optimized for the $[M\text{-}H]^-$ ion of PFAS as follows: spray voltage −3.5 kV, sheath gas 25 au (arbitrary units), aux gas 6 au, sweep gas 0 au, ion transfer tube temperature 300°C, and vaporizer temperature 30°C. High-resolution accurate mass (HRAM) MS1 scans were collected with the following parameters: detector type orbitrap, orbitrap resolution 30,000 full width at half maximum (FWHM), normal mass range, Use quadrapole isolation = true, scan range 70–700 m/z, radio frequency (RF) lens 60%, automatic gain control (AGC) target 4.00×10^5, max injection time of 50 ms, 1 microscan, data type set to profile, negative polarity, and source fragmentation disabled. Data-dependent orbitrap MSMS (ddMS2-OT) scans were collected for the $[M\text{-}H]^-$ ion $[M\text{-}H\text{-}CO_2$ for GenX$]^-$ using a target mass list for the eight PFAS and PFNA surrogate for identity confirmation. The apex detection was set to an expected peak width of 2 s at FWHM and desired apex peak window of 30%. The dynamic exclusion parameters were set to exclude after a one-time, 60-s exclusion duration, low and high mass tolerances of 10 ppm, and exclude isotope was set to true. The intensity threshold for collecting an MSMS scan was set to 2.5E+04. Fragmentation was done by high-energy collisional dissociation (HCD). The ddMS2-OT scans were collected with the following parameters: orbitrap isolation mode, isolation window of 1.6 m/z, isolation offset off, stepped HCD collision energy of 30% ± 10%, scan range of auto mass-to-charge ratio normal, orbitrap resolution of 30,000 FWHM, first mass 75 m/z, max injection time of 54 s, AGC target 5.00×10^4, inject ions from available parallelizable time set to true, max injection time of 54 ms, 1 microscan, and data type set to centroid. UPLC separation was achieved using ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µM, 2.1 mm × 50 mm (Waters Corp P/N 186,002,350) at 50°C with gradient elution at a flow rate of 300 µL/min using 0.4 mM ammonium formate in acetonitrile and water with a 10-µL injection. Data collection, integration, calibration, and quantitation were performed using Xcalibur software (version 4.1; Thermo Electron). Concentration for each PFAS analyte was determined by internal standard technique using isotopically labeled internal standards and matrix-matched calibration standards. Quantitative analysis was performed using high-resolution MS1-extracted ion chromatograms. Qualitative identification was based on relative retention time, MS1 peak abundance ratio of $[M\text{-}H]^-$ to a source decomposition product, and ddMS2-OT spectra. Tissue analysis was verified and evaluated using blanks, matrix-matched calibration standards, and matrix-matched QC standards prepared at three concentrations across the method's range. Batch results for exposure media were evaluated based on the following criteria: the calibration curve used a minimum of seven standards with a correlation coefficient of >0.99, standards accuracy tolerance <20% (30% at LLOQ), QC standard accuracy tolerance <20%, QC standard precision expressed as %RSD <20%, >75% of QC standards satisfy accuracy criteria. The tissue analysis method performance characteristics are listed in Excel Tables S3 and S4.

Statistics

For the DevTox assay, a Kruskal-Wallace nonparametric one-way analysis of variance (ANOVA) with a Dunn's multiple comparison test was used to detect differences between exposure groups ($^*p < 0.05$, $^{**}p < 0.0001$) and LOEC values were determined. If a test for linear trend was significant ($p < 0.05$), with developmental toxicity observed at the highest concentration tested, nonlinear regression was performed with a Hill slope curve fitting for half maximal effective concentration (EC_{50}) value determinations.

For DNT assay results shown in Figures 3, 6, and 8, a repeated measures ANOVA analysis was used to detect differences in swimming behavior between exposed and control larvae (Catron et al. 2019b; Irons et al. 2013; Phelps et al. 2017; Stevens et al. 2018). These analyses were performed using SAS (version 9.4; SAS Institute Inc.). Group means and standard errors were calculated by SAS Proc Means for each 10-min period included in the 40-min testing period [i.e., light 1 (L1), light 2 (L2), dark 1 (D1), and dark 2 (D2)]. Given that individual activity values were collected for each larva for every 2-min period, there were five data points for each 10-min light or dark period. First, means were calculated by individual larva across the five time points, then concentration group means, and standard errors were calculated using the larval means. Parametric analysis of locomotor data was conducted using SAS Proc Mixed. For each test chemical, a mixed-effects repeated measures model was run separately for each light or dark period (i.e., L1, L2, D1, D2). Each larva was considered a subject and an autoregressive covariance matrix was estimated across the five time points within each 10-min light or dark period. The fixed effects included in the model were as follows: experiment, plate nested within experiment, concentration, time, and the two-way interaction concentration by time. If the concentration effect within each 10-min light or dark period was significant ($p < 0.0125$) (i.e., because the same larvae were tested for four 10-min time periods), then pairwise *t*-tests were computed, comparing each concentration group to the control group ($p < 0.05$). Dunnett's test was used to adjust for multiple comparisons ($p < 0.05$). The parametric mixed model analysis included the ability to model design variables (e.g., plate or day of test), the correlated structure of the repeated distance measurements, and relationships among fixed effects (e.g., concentration, time). These statistics are displayed in Figures 3, 6, and 8 and Excel Tables S5–S7. To conclude that a test chemical was positive for developmental neurotoxicity, significance must have been detected at either more than one concentration within a time period or at the same concentration across multiple time periods.

In addition to the evaluation of mean movement measures described above, linear mixed-effects models (SAS Proc Mixed)

were used to examine individual movement measures from individual zebrafish as a function of time, concentration, and time × concentration. Data from light and dark periods were evaluated separately, given the clear differences in time trends during each period. Nearly half of the light period data were at or below the limit of detection (LOD) (0.135 cm), whereas <5% of the dark period data were <LOD. The LOD was based on the minimum distance moved value, set to 0.135 cm in Ethovision. This means that individual larva must move, from one frame to the next, a minimum of 0.135 cm, to be considered in motion. All values <LOD were initially assigned a value of LOD divided by the square root of 2. Inspection of QQ-plots (see Figure S1A,D) indicated that both light and dark period data were not normally distributed (Pleil 2016b). A square root transformation was therefore performed to improve the shape of the upper end of each measurement distribution (see Figure S1B,E). Because square root transformation did not sufficiently alter the lower end of each distribution, a multiple value imputation strategy was used (Pleil 2016a). This strategy is analogous to robust regression on order statistics (ROS), a widely used imputation strategy for censored data. Briefly, for the light period data, ~50% of measurements were <LOD and values were therefore imputed for this ~50% (see Figure S1C). Zebrafish increased locomotor activity in the light period over time. An inverse trend between time and the percentage of behavior measurements <LOD was observed (see Figure S2A). For chemicals that caused light-phase hyperactivity (i.e., PFOS and PFHxS), a modest inverse relationship between chemical concentration and the percentage of measurements <LOD was also observed (see Figure S2B,C). No clear trend between chemical concentration and percentage of values <LOD emerged for compounds that were negative for light period hyperactivity (see Figure S2C–G). For the dark period data, ~5% of measurements were <LOD. Given the shape of the distribution (see Figure S1E), values were ultimately imputed for the lowest 30% of the data (see Figure S1F). This ROS imputation allowed the order of the raw data to be preserved in the final corrected distribution.

To carry out the ROS technique, for both light and dark period data sets, all measurements were ordered from smallest to largest. Values <LOD were all equivalent, and were therefore extracted, randomly sorted, and placed back into the main data sets. Next, the relative position (p_r) of each measurement was determined according to the equation: $p_r = (n - 0.5)/N$, where n is the rank for a given measurement and N is the total number of measurements. A z-score was then assigned to each measurement using the probit function in SAS (version 9.4). Here, the assigned z-score represents the quantile for a specific measurement, assuming it is from a standard normal distribution. In both data sets, the square root–adjusted measurements were regressed on z-scores where the regression was restricted to the upper 50% of the light period distribution and to the upper 70% of the dark period distribution. Regression equations were used to predict square root–adjusted measurements for the lower portions of the measurement distributions (see Figure S1C,F). The combined use of square root transformation and multiple value imputation allowed key regression assumptions (i.e., homoskedasticity and normality of residuals) to be met.

In all mixed models, the square root–transformed movement data were regressed on time, concentration, and the interaction of time × concentration. For the light period, measurements were considered between T = 04 and T = 20 min. For the dark period, measurements were considered between T = 24 and T = 40 min. Data at T = 02 (light period) and T = 22 (dark period) minutes were considered to reflect transition periods and were, therefore, excluded from the analysis. All mixed models included a random effect for zebrafish, thus allowing partitioning of measurement variance into that which was observed between and within (over time) individual organisms. A compound symmetry covariance matrix was used, which assumes constant correlated errors between time points within organisms. Observed p-values for time indicate whether the linear effect of time on movement is significantly different than 0. The p-values for concentration indicate whether the intercept for movement (at T = 04 min for the light period, and T = 24 min for the dark period) differs across concentration groups (with DMSO set as the reference group). Finally, the p-values for time × concentration indicate whether the linear relationship between time and movement changes as a function of concentration (with DMSO set as the reference). Mixed model results were used to estimate zebrafish movement at specific time points in the light and dark periods. Specifically, regression equations were used to estimate movement at T = 10 and T = 20 min in the light period, and at T = 30 and T = 40 min in the dark period [note: any estimates that were <LOD (occurring in the light period only) were assigned a value of LOD divided by the square root of 2]. The difference in estimated movement during each period was ultimately used to gauge the magnitude of concentration-related effects on movement across all study chemicals.

For targeted analytical chemistry, PFAS concentrations were measured across media ($n = 3$ replicates) and tissue samples ($n = 4$ replicates, each comprising 10 pooled larvae). Measured media samples that were below the method detection limit (<MDL) (see Excel Table S8) were replaced with the value MDL divided by the square root of 2. To control for heteroskedasticity, log-transformation was performed followed by linear regression of log (measured media concentration) on log(nominal media concentration). Linear regression did not consider concentration = 0 samples (i.e., <MDL). Linear regression therefore considered measurements >MDL and, further, met assumptions of normality and homoscedasticity. Significance indicates that a linear increase in measured concentration was observed in accordance with rising nominal concentrations ($p < 0.05$). Measured tissue samples <MDL (see Excel Table S9) were replaced with the value MDL divided by the square root of 2. Welch's ANOVA was performed followed by a Dunnett T3 test (Dunnet 1980) ($p < 0.05$). If a single concentration = 0 sample was >MDL, all four exposure groups were considered for multiple comparison testing (this occurred for ADONA and PFESA1). If concentration = 0 samples were at or below the MDL, only the top three exposure groups were considered in the Dunnett T3 test (this occurred for PFHxA, PFHxS, PFOA, and PFOS). Additional one-sample Student's t-tests were then performed to compare measured concentrations to the MDL divided by the square root of 2 or to the lowest measured value above the MDL for concentration = 0 ($p < 0.05$).

Data Availability

The data sets generated during the current study are available in Science Hub by searching for the manuscript title at https://sciencehub.epa.gov/sciencehub/.

Results

Developmental Toxicity Phenotypes in Larval Zebrafish Exposed to PFAS

To determine whether exposure to PFAS caused developmental toxicity in larval zebrafish, embryos were exposed to 0.04–80.0 μM PFOS, PFHxS, PFHxA, PFOA, ADONA, or PFESA1 or 0.4% DMSO daily from 0–5 dpf (Study 1) (Figures 1 and 2). At 6 dpf, morphological assessments revealed developmental exposure to PFOS caused failed swim bladder inflation and ventroflexion of the tail, relative to DMSO control larvae (Figure 2A) and the EC_{50} value for developmental toxicity was calculated to be

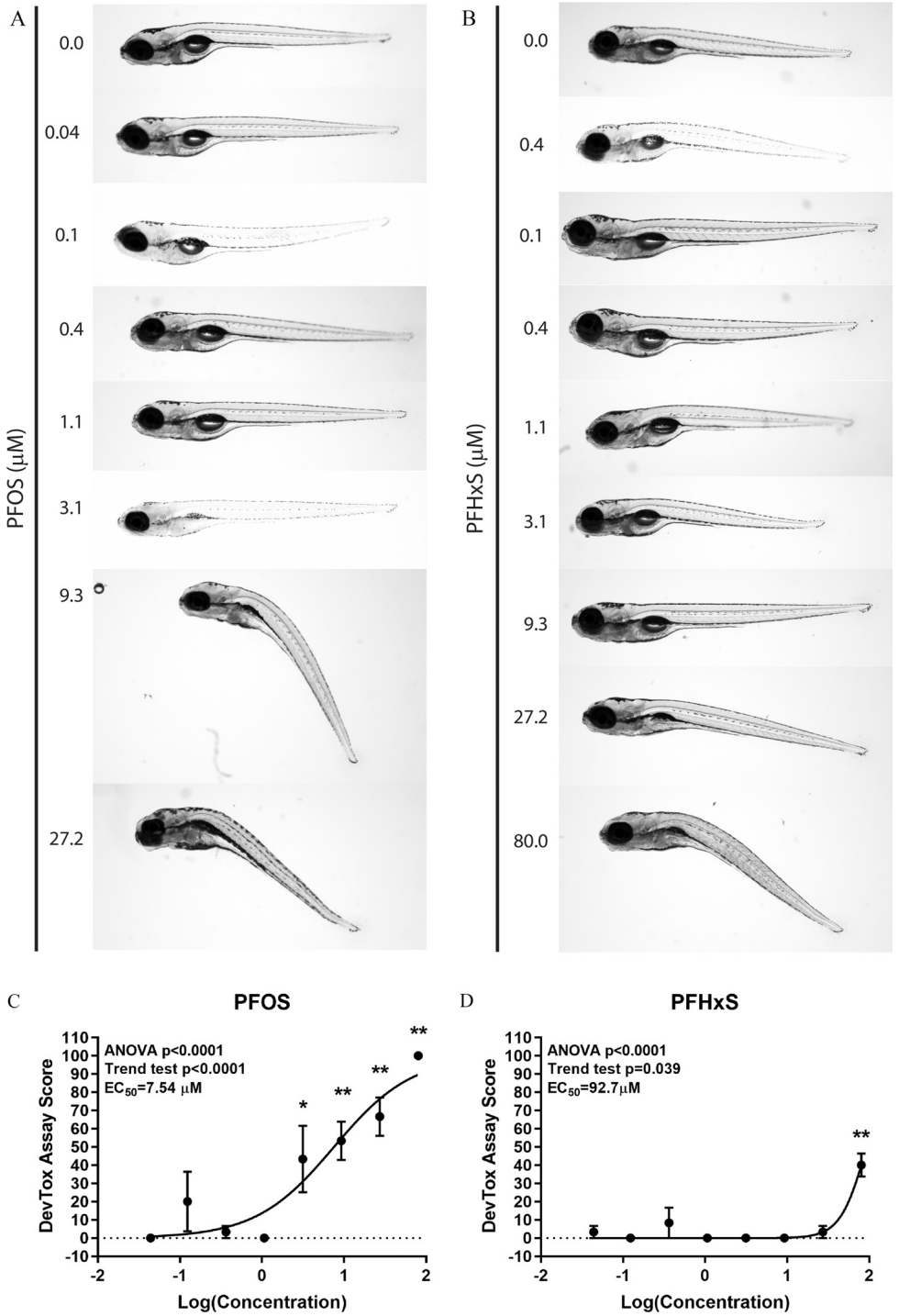

Figure 2. Measures of developmental toxicity in zebrafish exposed to PFAS. Zebrafish were semi-statically exposed to 0.04–80.0 μM ADONA, GenX Free Acid, PFESA1, PFHxA, PFHxS, PFOA, or PFOS daily, from 0–5 dpf. At 6 dpf, larvae were assessed for developmental toxicity. Representative images for (A) PFOS and (B) PFHxS are shown. DevTox assay scores for (C) PFOS, (D) PFHxS, (E) PFHxA, (F) PFOA, (G) ADONA, or (H) PFESA1 are shown. Significance relative to the 0.4% DMSO control was determined by a Kruskal-Wallis ANOVA with a Dunn's multiple comparison test ($^{*}p < 0.05$, $^{**}p < 0.0001$). If a test for linear trend was significant ($p < 0.05$), with developmental toxicity observed at the highest concentration tested, nonlinear regression was performed with Hill slope curve fitting for half-maximal EC_{50} value determinations. $n = 6$ larvae per concentration per chemical tested. Note: ADONA, 4,8-dioxa-3H-perfluorononanoate; DevTox, developmental toxicity; DMSO, dimethyl sulfoxide; dpf, days post fertilization; EC_{50}, half maximal effective concentration; GenX Free Acid, perfluoro-2-propoxypropanoic acid; PFAS, per- and polyfluoroalkyl substances; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid.

7.5 μM (Figure 2C). Exposure to PFHxS resulted in the same morphological phenotypes as PFOS (Figure 2B,D) but was less potent ($EC_{50} = 92.7$ μM), although this value was derived from a concentration–response curve with just a single positive concentration and, therefore, may not be entirely reliable. In comparison, exposure to PFHxA, PFOA, ADONA, or PFESA1 did not cause concentration-dependent effects on survival or development (Figure 2E–H).

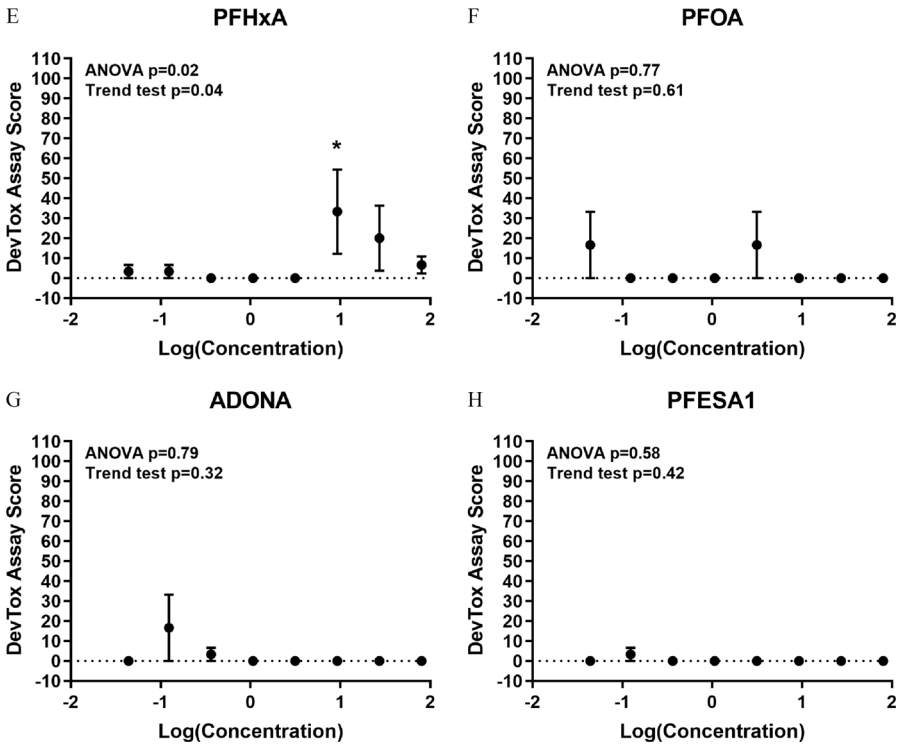

Figure 2. (*Continued.*)

Developmental Neurotoxicity Phenotypes in Larval Zebrafish Exposed to PFAS

To determine whether exposure to PFAS affect neurobehavioral development, zebrafish were exposed to 4.4–80.0 μM PFHxS, PFHxA, PFOA, ADONA, or PFESA1, 0.2–3.1 μM PFOS or 0.4% DMSO daily, from 0–5 dpf and locomotor activity was assessed at 6 dpf. Relative to DMSO, exposure to 1.0 μM PFOS caused hyperactivity in the L1 period and exposure to 0.6–1.8 μM PFOS caused hyperactivity in the L2 and D1 periods (Figure 3A,B). Like PFOS, developmental exposure to PFHxS caused hyperactivity in the L1 (14.0–25.1 μM), L2 (14.0 μM), and D1 (4.4, 14.0–25.1 μM) periods (Figure 3C,D). Finally, exposure to PFHxA resulted in hyperactivity relative to control in the L2 (25.1 μM) and D1 (14.0–25.1 μM) periods and, uniquely, in the D2 period (14.0–25.1 μM) (Figure 3E,F). Zebrafish developmentally exposed to PFOA (Figure 3G,H), ADONA (Figure 3I,J), or PFESA1 (Figure 3K,L) did not exhibit differences in locomotor activity at 6 dpf. The effect of PFAS exposures on the slope of the response to light or dark stimuli was also determined (see Figures S3 and S4). Significant differences in estimated movement over the testing period were detected for all test chemicals (see Figure S3). However, the difference in estimated movement during each period, used to gauge the magnitude of concentration-related effects on movement across all study chemicals, only revealed qualitatively pronounced changes in larvae exposed to PFHxS or PFOS (see Figure S4).

Bioaccumulation of PFAS in Larval Zebrafish

To measure media concentrations of PFAS, zebrafish were exposed to 4.4–80.0 μM PFHxS, PFHxA, PFOA, ADONA, or PFESA1 or 0.2–3.1 μM PFOS or DMSO daily, from 0–5 dpf and at 6 dpf, exposure media was collected from wells. For all test chemicals, a linear increase in measured media concentration was observed (Figure 4). To quantitate tissue concentrations of test PFAS, zebrafish were exposed to 25.1–80.0 μM PFHxA, PFOA, ADONA, or PFESA1, 14.0–44.8 μM PFHxS, or 1.0–3.1 μM PFOS or DMSO daily, from 0–5 dpf and parent PFAS were measured in pools of 10 larvae ($n = 4$) (Figure 5; see also Excel Table S9). PFOS was the most bioaccumulative compound with calculated bioconcentration factor (BCF) values ranging from 684 to 1,375, depending on test concentration (Table 4). Fluoroether PFAS (i.e., ADONA and PFESA1) and PFHxA were the least bioaccumulative chemicals assessed (Table 4).

Developmental Toxicity and Developmental Neurotoxicity Results in Larval Zebrafish Exposed to GenX Free Acid

Because GenX Free Acid was undetectable in media and zebrafish tissue at 6 dpf (data not shown) and, therefore, unstable in DMSO (see Figure S5), we retested the compound using DI water as a diluent. Daily exposure (0–5 dpf) to 0.0–80.0 μM GenX Free Acid was negative in the DevTox (Figure 6A) and DNT (Figure 6B,C; see also Figures S6 and S7) assays, relative to the DI water control. GenX Free Acid diluted in DI water resulted in detectable levels of the parent compound in media (Figure 6D; see also Excel Table S8) and larval zebrafish tissue (Figure 6E; see also Excel Table S9). Similar to other fluoroether PFAS assessed in the current study (i.e., ADONA and PFESA1) (Table 3), GenX Free Acid exposure yielded extremely low BCF values ranging from 0.29 to 0.49, depending on test concentration (Table 4).

Developmental Toxicity and Developmental Neurotoxicity Phenotypes in Larval Zebrafish Exposed to Alkyl Sulfonic Acid PFAS

Because exposure to structurally similar aliphatic sulfonic acid PFAS PFOS (8-carbon) or PFHxS (6-carbon) resulted in consistent morphological (Figure 2A–D) and behavioral (Figure 3A–D) phenotypes relative to the DMSO control, we hypothesized that sulfonic acid PFAS with perfluorinated alkyl chains would elicit the same toxicity outcomes. To test this hypothesis, zebrafish were

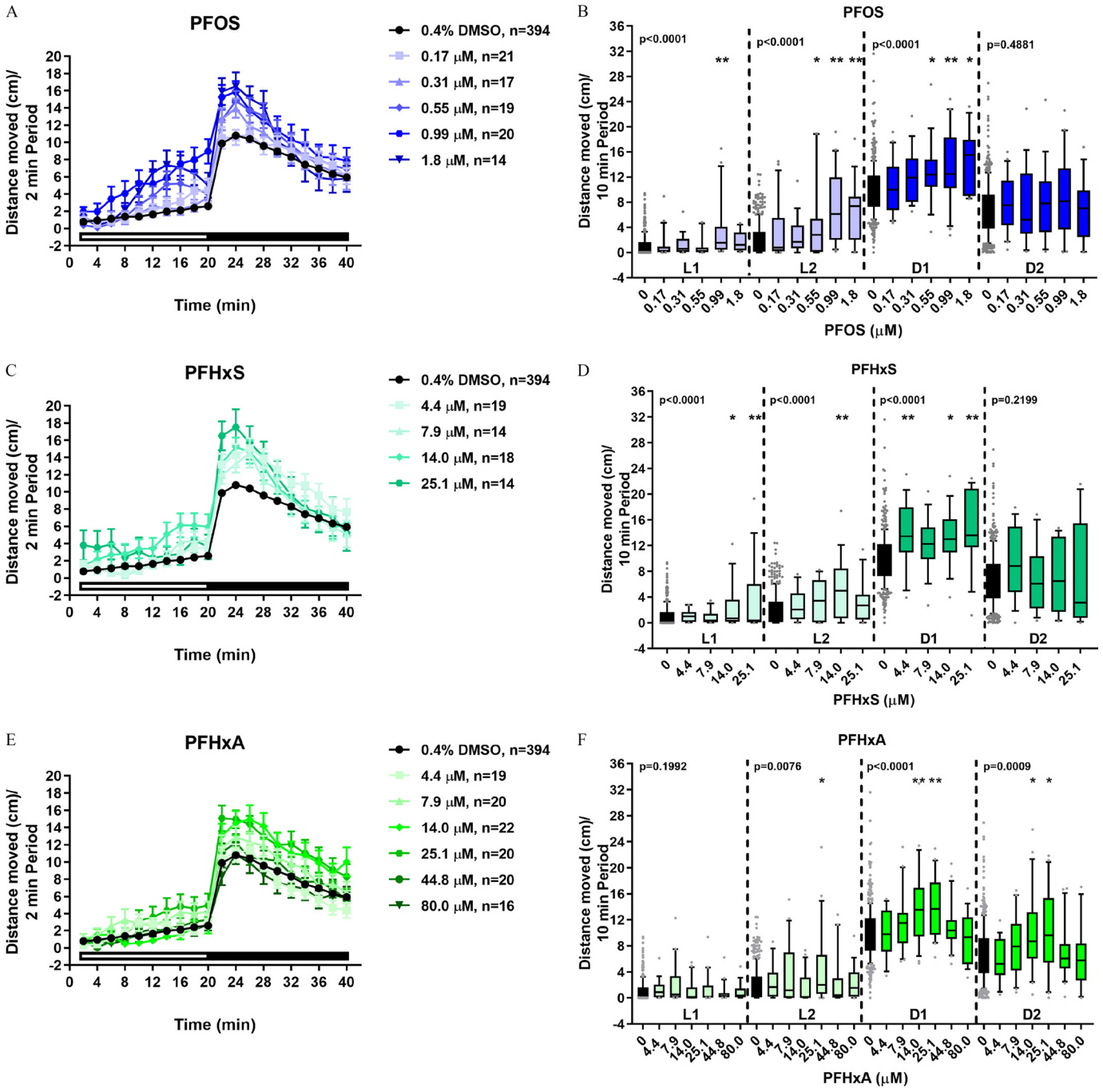

Figure 3. Locomotor activity in zebrafish developmentally exposed to PFAS. Zebrafish were semi-statically exposed to 4.4–80.0 μM ADONA, PFESA1, PFHxA, PFHxS, or PFOA, 0.2–3.1 μM PFOS, or 0.4% DMSO as a vehicle control daily from 0–5 dpf. At 6 dpf, larvae were assessed for developmental toxicity. Morphologically normal larvae with inflated swim bladders were subjected to behavioral testing. (A, C, E, G, I, K) Distance moved (cm) each 2-min period over the entire 40-min testing period are shown. (B, D, F, H, J, L) To make statistical comparisons, the mean distance moved during each 10-min light 1 (L1), 10-min light 2 (L2), 10-min dark 1 (D1), or 10-min dark 2 (D2) periods are shown. For all chemicals except PFESA1, 14–23 larvae were tested per chemical concentration and the same DMSO control larvae ($n = 394$) were used. PFESA1 was tested separately ($n = 35$–40 per chemical per concentration; 339 DMSO control larvae were evaluated). Repeated measures ANOVA models were run separately by period (L1, L2, D1, or D2). If a significant effect of concentration was detected ($p < 0.0125$), within-period pairwise comparisons to control were computed using *t*-tests with a Dunnett adjustment for multiple comparisons ($^{*}p < 0.05$, $^{**}p < 0.001$). Significance relative to period-specific DMSO controls are shown. Note: ADONA, 4,8-dioxa-3H-perfluorononanoate; ANOVA, analysis of variance; D, dark period; DMSO, dimethyl sulfoxide; dpf, days post fertilization; L, light period; PFAS, per- and polyfluoroalkyl substances; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid.

exposed to 0.0–100.0 μM PFBS (4-carbon), PFPeS (5-carbon), PFHxS (6-carbon), PFHpS (7-carbon), or PFOS (8-carbon) (Figure 1) daily, from 0–5 dpf and assessed for developmental toxicity at 6 dpf. PFBS was negative for developmental toxicity (Figure 7A). All other sulfonic acid PFAS resulted in significant developmental toxicity characterized by failed swim bladder inflation and ventroflexion of the tail (Figure 7B–E). Interestingly, PFPeS was quite potent for developmental toxicity with a calculated EC_{50} value of 48.8 μM (Figure 7B). The same five alkyl sulfonic acid PFAS shown in Figure 7 were also tested in the DNT

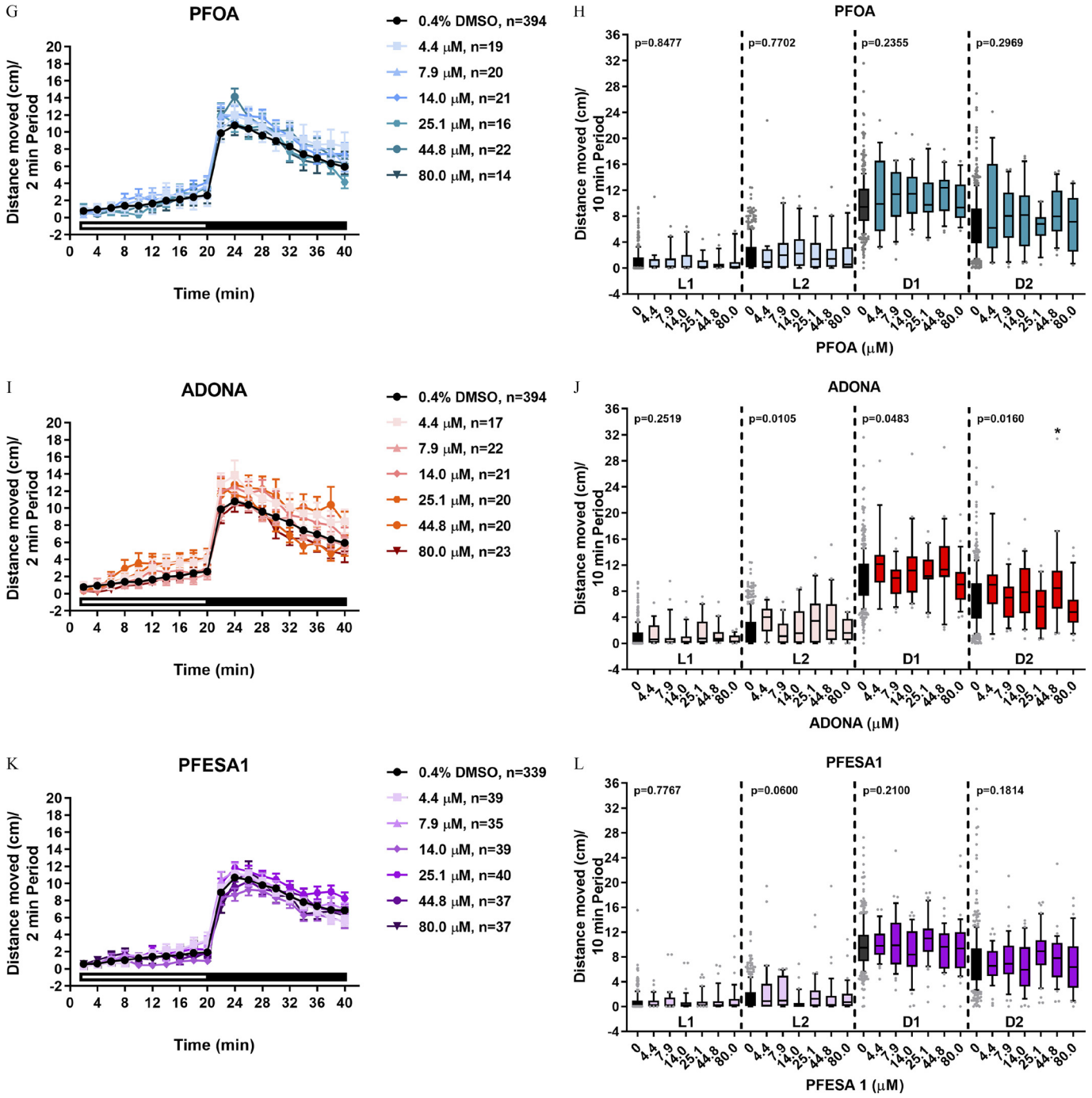

Figure 3. (*Continued.*)

assay. Exposure to PFBS did not result in significant locomotor effects (Figure 8A,B; see also Figures S8 and S9), whereas relative to DMSO alone, exposure to PFPeS resulted in hyperactivity in the L1 (5.5 μM) and L2 (3.1–5.5 μM) periods but had no effect in the dark periods (Figure 8C,D; see also Figures S8 and S9). In the case of PFHxS (Figure 8E,F; see also Figures S8 and S9), and like the results from Study 1 (Figure 3C,D), exposure caused hyperactivity relative to the DMSO control. However, the observed pattern of hyperactivity was modestly different, with hyperactivity detected in the L2 (17.6–31.4 μM), D1 (17.6 μM), or D2 (17.6 μM) periods (Figure 8E,F). Directly replicating the hyperactivity pattern observed in Study 1 (Figure 3A,B), developmental exposure to PFOS caused hyperactivity in the L1 (1.7–3.1 μM), L2 (1.7–3.1 μM), and D1 (1.0 μM) periods but no effect on the D2 period (Figure 8I,J; see also Figures S8 and S9). Similarly, exposure to PFHpS also triggered L1 (5.5 μM), L2 (3.1–5.5 μM), and D1 (3.1–5.5 μM) hyperactivity but no effect on the D2 period (Figure 8G,H; see also Figures S8 and S9).

Comparison of Toxicity Phenotypes in Zebrafish Developmentally Exposed to PFAS

Collective analysis of developmental toxicity and developmental neurotoxicity data sets revealed a shared toxicity phenotype for sulfonic acid PFAS that contain five or more fluorinated carbons (e.g., PFPeS, PFHxS, PFHpS, and PFOS) that was generally characterized

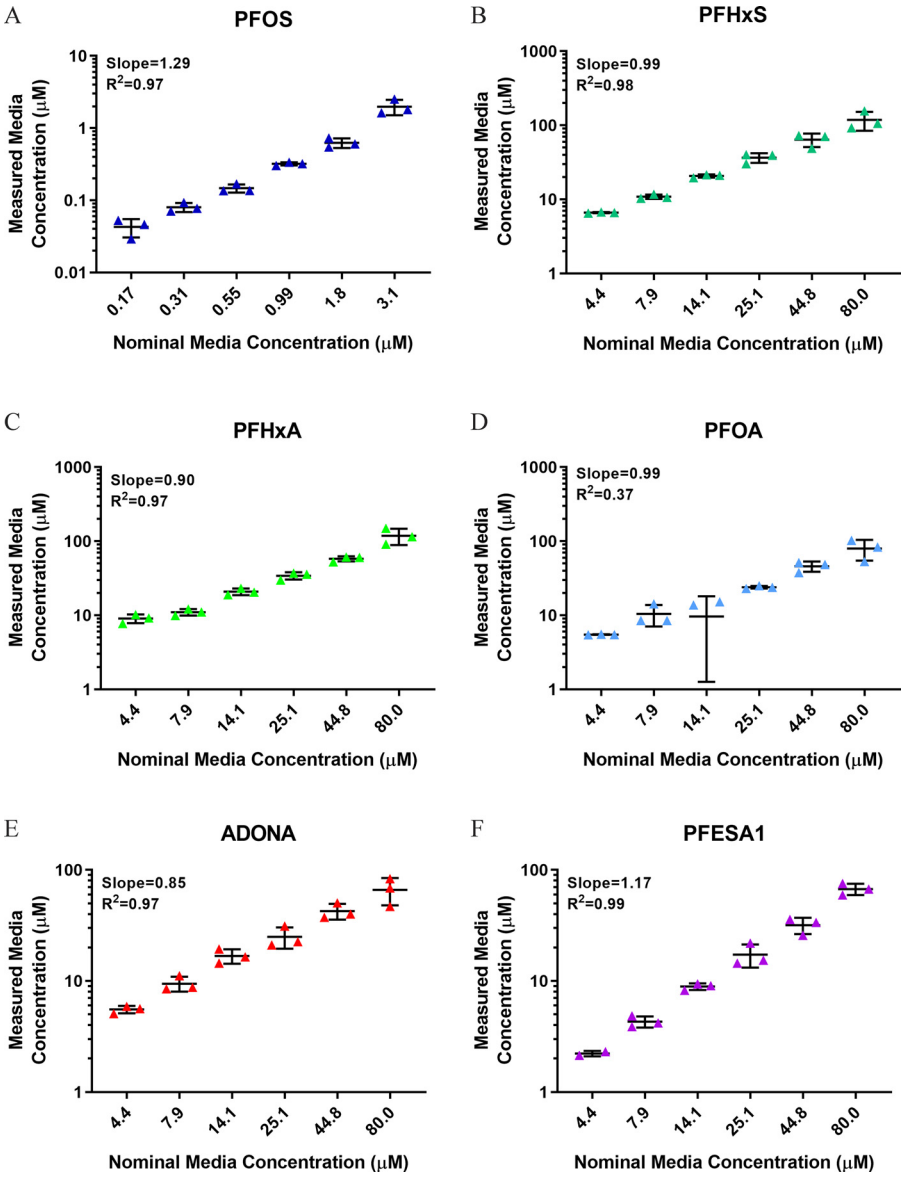

Figure 4. Media concentrations of test PFAS at 6 dpf. Zebrafish were semi-statically exposed to 4.4–80.0 μM PFHxS, PFHxA, PFOA, ADONA, or PFESA1 or 0.2–3.1 μM PFOS daily from 0–5 dpf. At 6 dpf, media was collected for targeted analytical chemistry ($n = 3$). Measured media concentrations for (A) PFOS, (B) PFHxS, (C) PFHxA, (D) PFOA, (E) ADONA, and (F) PFESA1 are shown. One observation for PFOA nominal media concentration 14.1 μM was <MDL and therefore not shown on the plot. However, it was included in the regression analysis using the value MDL/sqrt(2). Note: ADONA, 4,8-dioxa-3H-perfluorononanoate; dpf, days post fertilization; MDL, method detection limit; PFAS, per- and polyfluoroalkyl substances; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid; sqrt, square-root.

by abnormal ventroflexion of the tail and failed swim bladder inflation and, at nonteratogenic concentrations, hyperactivity in the L1, L2, and D1 periods (Figure 9A). Because PFPeS was more potent for developmental toxicity ($EC_{50} = 48.8$ μM; LOEC = 56.0 μM), relative to PFHxS ($EC_{50} = 227.9$ μM; Study 2 LOEC = 56.0 μM) and PFHpS ($EC_{50} = 168.1$ μM; LOEC = 31.4 μM), we did not identify a linear relationship between sulfonic acid carbon chain length and EC_{50} values for developmental toxicity ($R^2 = 0.027$) (Figure 9B). In the DNT assay however, sulfonic acid carbon chain length was correlated with Study 2 LOEC values for hyperactivity ($R^2 = 0.55$) (Figure 9C). These data also show that PFHxA has a unique toxicity phenotype consisting of hyperactivity in the L2, D1, and D2 periods with no observed developmental toxicity identified at the highest concentration tested (Figure 9A). Last, exposure to fluoroether PFAS (i.e., ADONA, GenX Free Acid, or PFESA1) failed to provoke developmental toxicity or developmental neurotoxicity in zebrafish.

Discussion

PFAS are a class of ubiquitous environmental contaminants. There is insufficient toxicity data for the majority of PFAS used in industry and consumer products (OECD 2018). The initial goal of this study was to evaluate the developmental toxicity and developmental neurotoxicity of seven PFAS, including compounds that have been phased out of use but are still widely detected in human serum (i.e., PFOA, PFOS, PFHxS, and PFHxA). In addition, the study included several emerging fluoroether compounds (e.g., ADONA) for which there are limited developmental toxicity data (Gordon 2011; Rushing et al. 2017). Last, we tested PFESA1, a by-product associated with the synthesis of polymer products.

One major finding of this work is that exposure to several PFAS resulted in developmental neurotoxicity characterized by hyperactivity. Epidemiological studies report both positive (Ghassabian et al.

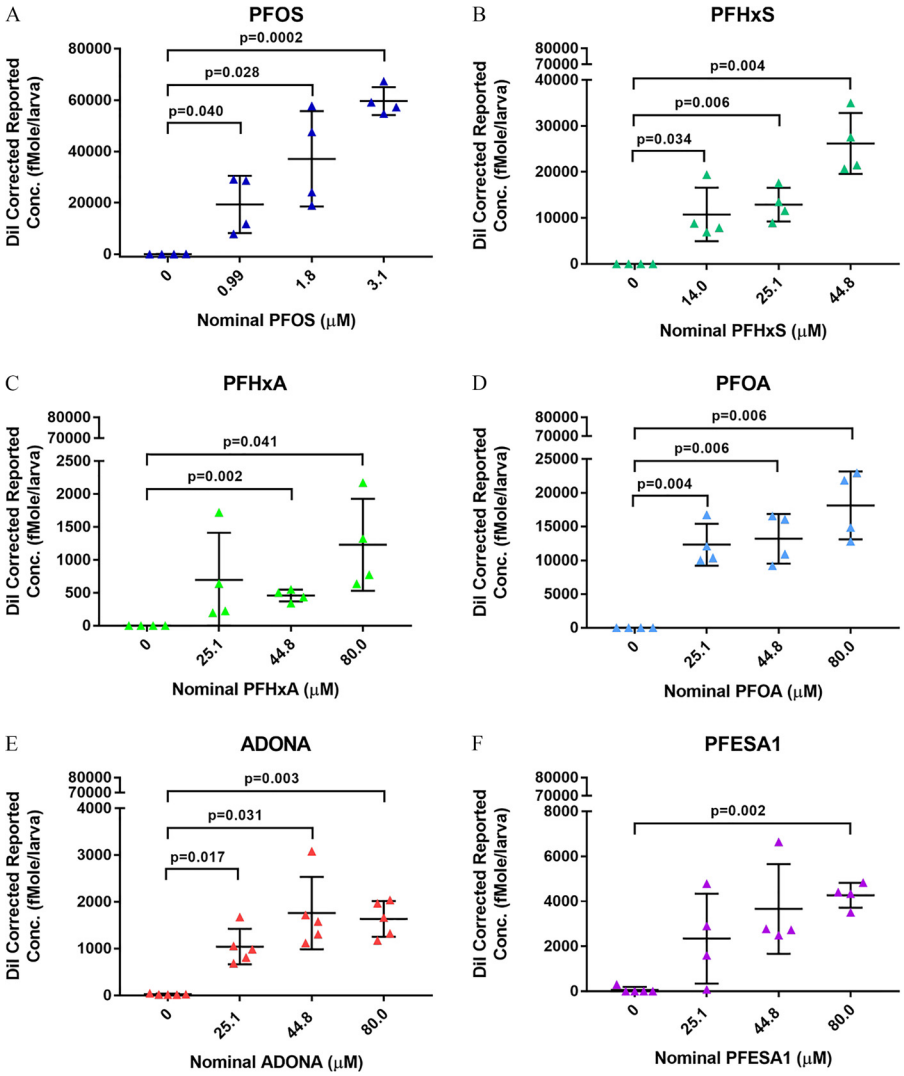

Figure 5. Internal tissue doses of test PFAS at 6 dpf. Zebrafish were semi-statically exposed to 25.1–80.0 μM ADONA, PFOA, PFESA1, or PFHxA, or 14.0–44.8 μM PFHxS, or 1.0–3.1 μM PFOS. At 6 dpf, larvae were pooled and flash frozen ($n = 4$ biological replicates with 10 pooled larvae per replicate) for targeted analytical chemistry. Measured internal tissue doses for (A) PFOS, (B) PFHxS, (C) PFHxA, (D) PFOA, (E) ADONA, and (F) PFESA1 are shown. Significance was determined by a Welch's ANOVA followed by a Dunnett T3 test ($p < 0.05$). Additional one-sample Student's *t*-tests were performed for PFHxA, PFHxS, PFOA, and PFOS ($p < 0.05$). Note: ADONA, 4,8-dioxa-3H-perfluorononanoate; ANOVA, analysis of variance; Dil, dilution; dpf, days post fertilization; PFAS, per- and polyfluoroalkyl substances; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid.

2018; Hoffman et al. 2010; Hoyer et al. 2015; Rappazzo et al. 2017) and negative (Lyall et al. 2018; Rappazzo et al. 2017; Stein and Savitz 2011; Stein et al. 2013) associations between PFAS exposures and neurodevelopmental outcomes. Similarly, exposure to PFAS has been reported to cause behavioral toxicity in some (Goulding et al. 2017; Johansson et al. 2008; Long et al. 2013; Sato et al. 2009; Wang et al. 2015), but not all (Butenhoff et al. 2009), animal studies. In those studies where a positive relationship was revealed, affected behavioral end points included hyperactivity (Goulding et al. 2017; Johansson et al. 2009), reduced habituation (Johansson et al. 2008), impairments in spatial learning and memory resulting from adult (Long et al. 2013) and prenatal exposures (Wang et al. 2015), and tonic convulsions in response to an ultrasonic stimulus (Sato et al. 2009). Molecular results obtained in animal studies suggest that PFAS exposures may disrupt dopaminergic and/or calcium signaling pathways during neurogenesis (Hallgren and Viberg 2016; Johansson et al. 2009; Lee and Viberg 2013; Liu et al. 2010a, 2010b; Zeng et al. 2011). Overall, because of contradictory evidence in human epidemiological and animal behavior studies, it remains unclear whether PFAS exposure is associated with adverse neurophysiological effects. To gain insight into this critical question, concentration-dependent automated behavioral data are needed to evaluate a variety of related and dissimilar PFAS structures. The zebrafish model represents an excellent alternative experimental system that can be used to address this growing research need because multiple chemicals can be evaluated in parallel using automated behavioral tests coupled with a powerful concentration–response design.

Legacy PFAS such as PFOA and PFOS have been extensively evaluated *in vitro* and in animal and epidemiological studies. However, PFAS are by no means a monolithic class of chemicals. They can be per- or polyfluorinated, straight or branched chained, and contain alkyl chains of varying lengths. PFAS may also contain ether linkages and either sulfonic acid or carboxylic acid R-group moieties. In the United States, there are 602 PFAS in active commercial use (U.S. EPA 2019) and the OECD has identified 4,730 PFAS structures included in various publicly accessible databases (OECD 2018), most of which lack adequate toxicity data. The

Table 4. Calculated bioconcentration factors (BCFs).

Compound name	Nominal concentration tested (μM)	Measured tissue dose (mg/kg)	Measured media dose (mg/L)	BCF_{dry} (L/kg)
ADONA	25.1	9.49 ± 3.1	9.98 ± 1.77	0.95
	44.8	16.0 ± 6.29	17.04 ± 2.24	0.94
	80.0	14.86 ± 3.11	26.52 ± 5.98	0.56
GenX Free Acid	25.1	2.48 ± 0.66	5.46 ± 1.15	0.45
	44.8	2.02 ± 0.27	7.06 ± 0.73	0.29
	80.0	5.11 ± 1.49	10.44 ± 1.57	0.49
PFESA1	25.1	23.67 ± 17.47	7.67 ± 1.46	3.09
	44.8	36.98 ± 17.44	14.11 ± 1.91	2.62
	80.0	43.16 ± 4.84	29.89 ± 2.87	1.44
PFHxA	25.1	4.97 ± 4.40	10.76 ± 0.98	0.46
	44.8	3.28 ± 0.56	18.22 ± 1.19	0.18
	80.0	8.77 ± 4.31	37.16 ± 7.53	0.24
PFOA	25.1	115.75 ± 25.19	9.91 ± 0.33	11.68
	44.8	124.13 ± 29.93	19.0 ± 2.45	6.52
	80.0	170.51 ± 41.12	33.01 ± 8.41	5.17
PFHxS	14.0	107.21 ± 50.09	9.07 ± 0.36	11.82
	25.1	128.55 ± 31.46	16.06 ± 1.97	8.01
	44.8	260.93 ± 57.16	28.04 ± 4.72	9.30
PFOS	1.0	220.10 ± 109.45	0.16 ± 0.007	1,374.89
	1.8	422.07 ± 182.86	0.31 ± 0.038	1,348.46
	3.1	677.86 ± 53.49	0.99 ± 0.194	684.03

Note: BCFs for ADONA, GenX Free Acid, PFESA1, PFHxA, PFHxS, PFOA, and PFOS in a 6-d zebrafish toxicity assay based on measured media and tissue concentrations reported in Excel Tables S8 and S9. BCF tissue doses (mg/kg) were calculated using a dry weight for 6 dpf larvae of 44 μg (Massei et al. 2015). ADONA, 4,8-dioxa-3H-perfluorononanoate; dpf, days post fertilization; GenX Free Acid, perfluoro-2-propoxypropanoic acid; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid.

Zürich Statement on PFAS lays out a strategy for tackling the huge gap in our understanding of PFAS toxicity (Ritscher et al. 2018). Given the large number of PFAS in commerce and rather than cataloging the effects of individual chemicals, the statement calls for action on grouping PFAS (Ritscher et al. 2018). One obvious way to achieve this is to group chemicals by their toxicological activities and, perhaps in doing so, identify structural features that provoke the same toxicity phenotypes *in vivo*. In the current study, we identified three groups of PFAS toxicity outcomes in zebrafish. Aliphatic sulfonic acid PFAS with greater than four fluorinated carbons resulted in similar morphological and behavioral phenotypes, characterized by failed swim bladder inflation, abnormal ventroflexion of the tail, and, at nonteratogenic concentrations, hyperactivity in the L1, L2, and D1 periods. The second phenotype was unique to PFHxA, an aliphatic carboxylic acid PFAS. Exposure to PFHxA was negative for developmental toxicity but caused pronounced hyperactivity in the L2, D1, and D2 periods. The third group of chemicals consisted of three fluoroether PFAS (i.e., GenX Free Acid, ADONA, and PFESA1), all of which were negative in both toxicity assays.

In addition to grouping PFAS based on attributes such as structure or biological activity, the Zürich statement also recommends amassing data on the toxicokinetics and toxicodynamics of PFAS exposures, particularly those for which little toxicity data exist, with the goal of identifying safer PFAS that can be prioritized for commercial use (Ritscher et al. 2018). Interestingly, we observed a trend of reduced BCFs with increasing nominal concentrations of several PFAS (i.e., ADONA, PFESA1, PFOA, and PFOS). These data replicate a recent study conducted in larval zebrafish that showed an inverse relationship between BCF values and increasing concentrations of perfluorobutanoic acid (PFBA), PFHxS, PFOA, and PFOS, which was suggested to result from the saturation of substrate binding sites (Vogs et al. 2019). Targeted analytical chemistry also revealed detectable levels of ADONA, PFESA1, and PFOA in fish tissue at 6 dpf, indicating that these chemicals are negative for developmental toxicity and developmental neurotoxicity in zebrafish (up to 80 μM).

To our knowledge, this is the first published report showing that GenX Free Acid, a branched fluoroether PFAS with a carboxylic acid group directly adjacent to an ether linkage, is not stable in DMSO. This is significant because DMSO is a commonly used solvent for zebrafish and high-throughput *in vitro* and biochemical toxicity screening studies. Assessment of GenX Free Acid diluted in DI water showed that, although this compound was detectable in zebrafish tissue at the end of the 6-d study period, it was negative for developmental toxicity and developmental neurotoxicity. Collectively, these results suggest that, at least for the types and concentrations tested in the current study, larger fluoroether compounds (i.e., ADONA, GenX Free Acid, and PFESA1) were nontoxic in zebrafish. More work should be performed to explore whether this finding can be extended to other large fluoroether replacement PFAS (Wang et al. 2013).

Compared with previously reported morphological and behavioral effects following exposure to PFBS, PFHxS, PFOS, or PFHxA (summarized in Table 5) (Hagenaars et al. 2011; Huang et al. 2010; Jantzen et al. 2016; Khezri et al. 2017; Padilla et al. 2012; Spulber et al. 2014; Truong et al. 2014; Ulhaq et al. 2013a, 2013b), this study expanded our understanding of aliphatic PFAS toxicity to include data on PFPeS and PFHpS for the first time and reported novel results for PFHxA and PFHxS. Although not systematically designed to test a specific PFAS R-group (i.e., sulfonic or carboxylic acids), Ulhaq et al. (2013a) tested 4-, 8-, 9-, and 10-carbon carboxylic acid aliphatic PFAS and 4- and 8-carbon sulfonic acid aliphatic PFAS in a zebrafish developmental toxicity assay and proposed the idea that carbon chain length may be a determinant of PFAS toxicity in zebrafish. The work presented here systematically tested the effects of aliphatic sulfonic acid PFAS with 4, 5, 6, 7, or 8 fluorinated carbons. Because of the 5-carbon compound PFPeS, carbon chain length was not correlated with malformations in the DevTox assay (PFOS > PFPeS > PFHpS > PFHxS; PFBS was negative; $R^2 = 0.35$). PFPeS was nearly as potent as PFOS, the most potent chemical evaluated in our study. In comparison, in the DNT assay, increasing carbon chain length was associated with increasing potency for hyperactivity (PFOS > PFHpS > PFPeS > PFHxS; PFBS was negative; $R^2 = 0.55$). Collectively, these data raise two important points. First, sulfonic acid aliphatic PFAS can be grouped based on their ability to cause the same morphological and behavioral toxicity phenotypes in zebrafish (i.e., failed swim bladder inflation, abnormal ventroflexion of the tail, and, at nonteratogenic concentrations, hyperactivity). Second, although carbon chain length generally increases PFAS potency, this dogma cannot be universally applied to all structurally similar PFAS, as exceptions to the rule exist (i.e., PFPeS).

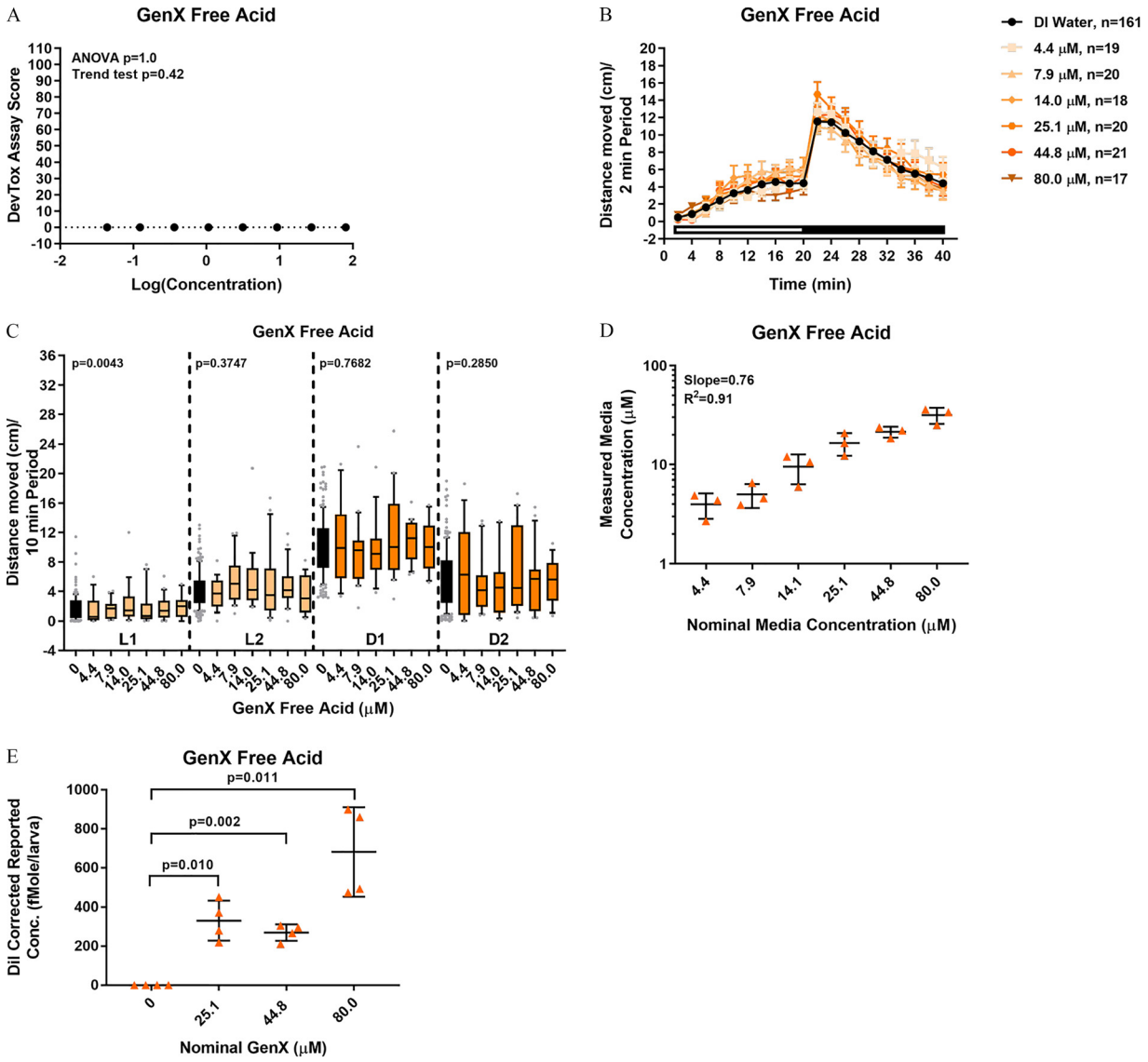

Figure 6. Developmental and behavioral assays and media and tissue concentrations in zebrafish exposed to GenX Free Acid diluted in DI water. (A) Developmental toxicity scores at 6 dpf obtained from zebrafish developmentally exposed to 0.04–80.0 μM GenX Free Acid diluted in DI water daily, from 0–5 dpf. Significance was determined by one-way ANOVA with a Tukey's multiple comparison test ($p < 0.05$). $n = 6$ larvae per concentration tested. For the DNT assay, zebrafish were exposed to 4.4–80.0 μM GenX Free Acid daily, from 0–5 dpf. At 6 dpf, locomotor activity was assessed. (B) Distance moved (cm) each 2-min period or (C) mean distance moved during the light 1 (L1), light 2 (L2), dark 1 (D1), or dark 2 (D2) 10-min periods are shown. Repeated measures ANOVA models were run separately by period (L1, L2, D1, or D2). If a significant effect of concentration was detected ($p < 0.0125$), within-period pairwise comparisons to control were computed using *t*-tests with a Dunnett adjustment for multiple comparisons ($^{*}p < 0.05$). $n = 17$–21 zebrafish per concentration and 161 DI water control larvae were assessed. (D) Media concentrations and (E) internal tissue dose at 6 dpf following daily exposure to 25.1–80.0 μM GenX Free Acid. $n = 3$ media replicates and $n = 4$ biological replicates each comprising 10 pooled larvae. Significance was determined by a Welch's ANOVA followed by a Dunnett T3 test ($p < 0.05$). Additional one-sample Student's *t*-tests were performed ($p < 0.05$). Note: ANOVA, analysis of variance; D, dark phase; DevTox, developmental toxicity; DI, deionized; Dil, dilution; DNT, developmental neurotoxicity; dpf, days post fertilization; GenX Free Acid, perfluoro-2-propoxypropanoic acid; L, Light period.

Here, we obtained DevTox and DNT Assay data for PFOS and PFHxS in two separate studies. This provided a unique opportunity to evaluate the consistency of observed toxicity effects across independent experiments. In the case of PFOS, EC_{50} values for developmental toxicity were similar, but not identical, with identified values of 7.5 μM in Study 1 and 28.2 μM in Study 3. Variability in calculated EC_{50} values was also observed in the PFHxS data set (92.7 μM in Study 1; 227.9 μM in Study 3). These discrepancies could reflect both inherent assay variability and the different concentrations ranges tested in Study 1 (0.04–80 μM) relative to Study 3 (1.7–100 μM). In addition, the EC_{50} value calculated from Study 1 was based on a single positive concentration and, therefore, may not be as reliable as the value determined in Study 3. Last, the OECD Fish Embryo Acute Toxicity Test (No. 236) indicates that 20 animals should be tested across five concentrations of test chemicals to evaluate developmental toxicity (OECD 2013). Although this study assessed developmental toxicity across a six-point concentration–response curve with a minimum of 44 replicates for the 0.4% DMSO control group, only 6–10 biological replicates per exposure group were used. Collectively, these design choices may have contributed to differences in detected EC_{50} values described above. However, these data are in line with previously published DevTox assay data with reported PFOS EC_{50} values of 42.3 μM (Padilla et al. 2012) and 3.5 μM (Truong et al. 2014) and PFHxS EC_{50} values of 116.5 μM (Padilla et al. 2012) and 114.7 μM (Truong et al. 2014). Given that the strain, rearing temperature, chemical source, exposure

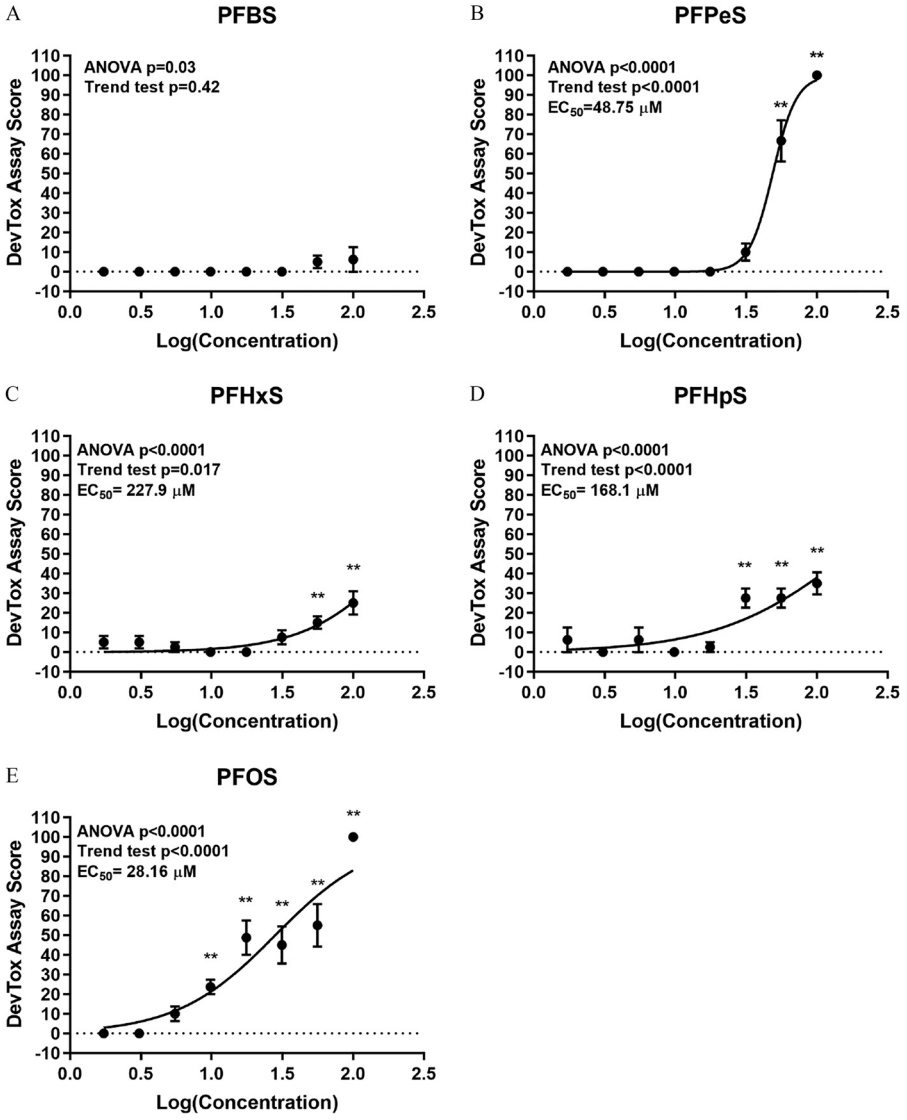

Figure 7. Measures of developmental toxicity in zebrafish exposed to alkyl sulfonic acid PFAS. Zebrafish were semi-statically exposed to 1.7–100.0 μM PFBS, PFPeS, PFHxS, PFHpS, or PFOS or 0.4% DMSO daily from 0–5 dpf. At 6 dpf, larvae were assessed for developmental toxicity. DevTox assay scores for (A) PFBS, (B) PFPeS, (C) PFHxS, (D) PFHpS, or (E) PFOS are shown. Significance relative to the 0.4% DMSO control was determined by a Kruskal-Wallis ANOVA with a Dunn's multiple comparison test ($^{**}p < 0.0001$). If a test for linear trend was significant ($p < 0.05$), with developmental toxicity observed at the highest concentration tested, nonlinear regression was performed with Hill slope curve fitting for half-maximal EC_{50} value determinations. $n = 8$ larvae per concentration per chemical tested. Note: ANOVA, analysis of variance; DevTox, developmental toxicity; DMSO, dimethyl sulfoxide; dpf, days post fertilization; EC_{50}, half maximal effective concentration; PFAS, per- and polyfluoroalkyl substance; PFBS, perfluorobutanesulfonic acid; PFHpS, perfluoroheptanesulfonic acid; PFHxS, perfluorohexanesulfonic acid; PFOS, perfluorooctanesulfonic acid; PFPeS, perfluoropentanesulfonic acid.

regimen (i.e., static vs. semi-static), and end point evaluation protocol varied across studies, these data are generally consistent and show that exposure to PFOS or PFHxS causes developmental toxicity in zebrafish. In the DNT assay, highly consistent LOECs were observed for PFOS (1.8 μM in Study 1; 3.1 μM in Study 3) and PFHxS (25.1 μM in Study 1; 31.4 μM in Study 3). However, although the pattern of the hyperactivity was identical across studies for PFOS with observed hyperactivity in the L1, L2, and D1 periods, it varied following exposure to PFHxS, where elevated locomotor activity was observed in the L1, L2, and D1 periods in Study 1 and the L2, D1, and D2 periods in Study 3. Regardless, in line with previously published work (Huang et al. 2010; Khezri et al. 2017; Spulber et al. 2014), exposure to nonteratogenic concentrations of PFOS or PFHxS consistently triggered behavioral hyperactivity. Although more work is needed to understand the biological relevance of disparate xenobiotic-induced locomotor activity phenotypes, this represents a powerful approach for grouping chemicals based on shared toxicity phenotypes.

From a developmental toxicity perspective, we and others have showed that exposure to sulfonic acid alkyl PFAS with at least five carbon atoms can cause developmental lethality and elicit conserved malformations at nonteratogenic concentrations consisting of swim bladder inflation failure and dorsoflexion of the tail (Hagenaars et al. 2011; Huang et al. 2010; Jantzen et al. 2016; Padilla et al. 2012; Truong et al. 2014; Ulhaq et al. 2013a). Similar to zebrafish, neonatal rodents exposed to PFOS die in the postnatal period (Conley et al. 2019; Grasty et al. 2005). Although humans do not have a swim bladder, the organ shares functional, structural, ontological, and transcriptional similarities with the human lung (Winata et al. 2009). In zebrafish, swim bladder inflation is used for buoyancy and functions as a site for gas interchange at the air–mucus interface, with surfactant composition similar to the lung (Agier et al. 2019;

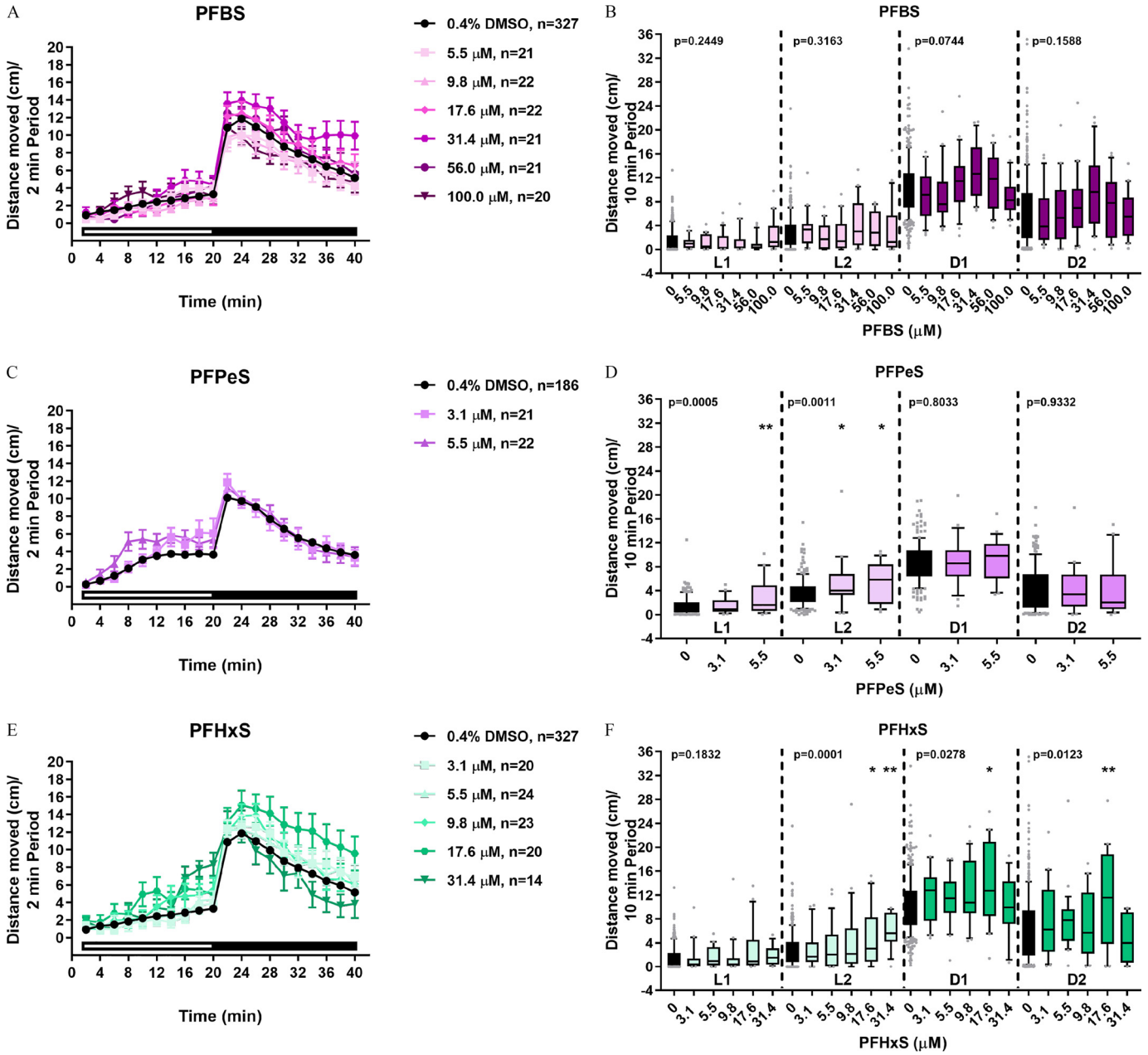

Figure 8. Locomotor activity in zebrafish exposed to alkyl sulfonic acid PFAS. Zebrafish were semi-statically exposed to 5.5–100.0 μM PFBS, 3.1–5.5 μM PFPeS, 3.1–31.4 μM PFHxS, 1.7–9.8 μM PFHpS, or 0.5–3.1 μM PFOS daily from 0–5 dpf. For all chemicals except PFPeS, 14–25 larvae were tested per chemical concentration and the same DMSO control larvae ($n = 327$) were used. PFPeS was tested separately ($n = 21$–22 larvae per concentration; 186 DMSO control larvae were evaluated). At 6 dpf, larvae were assessed for developmental toxicity. Morphologically normal larvae with inflated swim bladders were subjected to behavioral testing. (A, C, E, G, I) Distance moved (cm) each 2-min period or (B, D, F, H, J) mean distance moved during the light 1 (L1), light 2 (L2), dark 1 (D1), or dark 2 (D2) 10-min periods are shown. Repeated measures ANOVA models were run separately by period (L1, L2, D1, or D2). If a significant effect of concentration was detected ($p < 0.0125$), within-period pairwise comparisons to control were computed using *t*-tests with a Dunnett adjustment for multiple comparisons ($^*p < 0.05$). ANOVA, analysis of variance; D, dark phase; DMSO, dimethyl sulfoxide; dpf, days post fertilization; L, light period; PFAS, per- and polyfluoroalkyl substances; PFBS, perfluorobutanesulfonic acid; PFHpS, perfluoroheptanesulfonic acid; PFHxS, perfluorohexanesulfonic acid; PFOS, perfluorooctanesulfonic acid; PFPeS, perfluoropentanesulfonic acid.

Lapennas and Schmidt-Nielsen 1977; Robertson et al. 2007; Sullivan et al. 1998; Zeng et al. 2011). Toxicologically, neonatal rodents that died following prenatal exposure to PFOS presented with noted changes in lung histology (Grasty et al. 2005). In humans, epidemiologic evidence has shown that exposure to PFAS during pregnancy can result in decreased lung function in children (Agier et al. 2019). Although these similarities are intriguing, more work is needed to determine the relevance of zebrafish swim bladder defects for human health risk assessment.

Overall, this study rapidly assessed the developmental toxicity and developmental neurotoxicity of 10 PFAS, including some compounds that have never been previously tested in animal studies. However, limitations of the study warrant comment. In human NHANES data, mean PFAS levels are often higher in males relative to females (Calafat et al. 2007a, 2007b). We did not capture sex-specific outcomes in early life stage zebrafish. In addition, here we used locomotor activity in a light/dark behavior test as a functional readout of neurodevelopment. This is just one behavioral end point

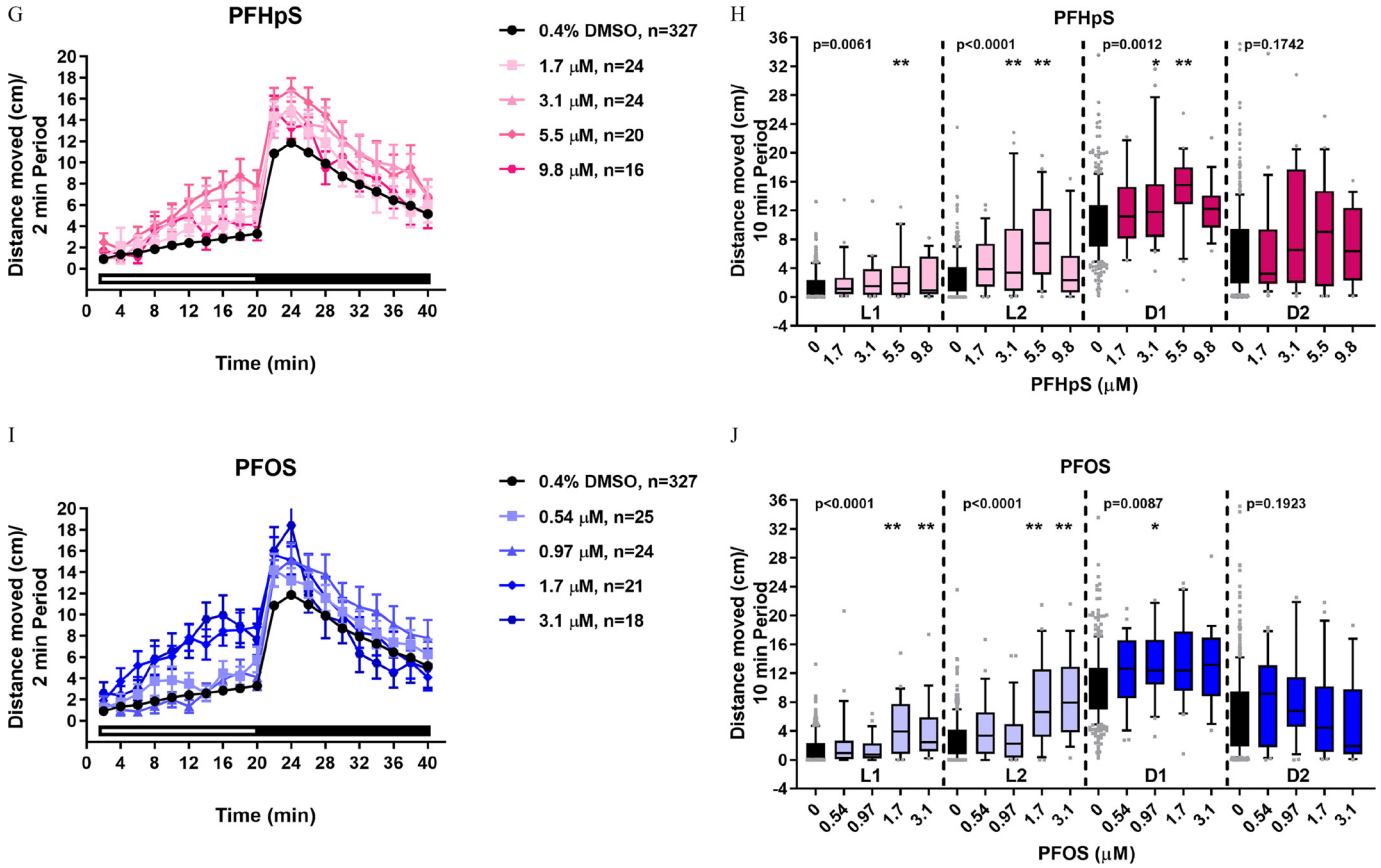

Figure 8. (*Continued.*)

among many. Future studies should examine the effect of PFAS on other zebrafish behavior end points such as habituation to further support the developmental neurotoxicity effects reported here. Here, 0.4% DMSO was used for all exposure groups, except GenX Free Acid which was diluted in DI water. This concentration of DMSO is commonly used in zebrafish chemical screens for developmental toxicity (Padilla et al. 2012) and does not affect the larval zebrafish photomotor response (Kokel and Peterson 2011) or light/dark swimming response (Teixidó et al. 2019). However, it should be noted that effects on the transcriptome (Turner et al. 2012) and metabolome (Akhtar et al. 2016) have been reported in zebrafish exposed to 0.1% DMSO. There is also evidence that elevated DMSO concentrations can facilitate increased uptake of chemicals into the perivitaline space (Kais et al. 2013). Therefore, although all chemical exposure data were statistically compared with a DMSO control, it is possible that the concentration of solvent used in the current study affected chemical uptake and the assessed end points.

These data raise some interesting questions for future research. Namely, do behavioral hyperactivity effects persist in older animals? One intriguing study observed hyperactivity in 14 dpf zebrafish developmentally exposed to PFOA or PFOS from 0–5 dpf (Jantzen et al. 2016), suggesting that early life perturbation of neuronal circuitry controlling the light/dark behavioral apparatus may persist at later life stages. Another key area that needs to be explored is the mechanism(s) by which PFAS cause locomotor hyperactivity in zebrafish. Last, humans and wildlife are exposed to a complex mixture of PFAS (Boiteux et al. 2016; Gebbink et al. 2017; Strynar et al. 2015), many of which may cause additive or synergistic disruption of neurodevelopmental signaling pathways (Khezri et al. 2017). Future research should consider testing groups of related PFAS in environmentally relevant mixtures. From a dosimetry perspective, we observed tissue doses of PFAS that have been measured in, for example, the Danish National Birth Cohort (Fei et al. 2007). To truly understand the relevance of zebrafish toxicity data for human risk assessment, there is an urgent need for physiologically based pharmacokinetic models that compare PFAS doses measured in whole-body zebrafish homogenates to maternal serum levels. Finally, analysis of larval behavior data is not standardized. Here, we opted to use a repeated measures ANOVA and parametric mixed model analysis to account for the complex data structure that contains multiple, repeated locomotor activity values for each animal over the 40-min testing period (Catron et al. 2019a; Irons et al. 2013; Phelps et al. 2017; Stevens et al. 2018). However, zebrafish behavior data are not normally distributed and may often be below the LOD. We therefore also analyzed the behavior data using a square root transformation and multiple value imputation strategy (Pleil 2016a, 2016b). Based on the LOD, a large percentage of individual values were imputed. Although the order of the raw data was preserved in the final corrected distributions, the large percentage of imputed values present in the light period data should be noted. However, the combined use of square root transformation and multiple value imputation allowed key regression assumptions (i.e., homoskedasticity and normality of residuals) to be met and the subsequent use of mixed-effects models further allowed the appropriate examination of repeated measures for individual zebrafish. Overall, the imputation-based analysis strategy was used to buttress findings generated from untransformed zebrafish behavior data while appropriately accounting for nonnormal distributions. Although both approaches revealed PFAS-dependent hyperactivity, standardized methods for the analysis of fish behavior data that account for repeat measurements and nonnormal data distributions are needed.

Taken together, and in keeping with recommendations by the Zürich Statement (Ritscher et al. 2018), we used zebrafish toxicity

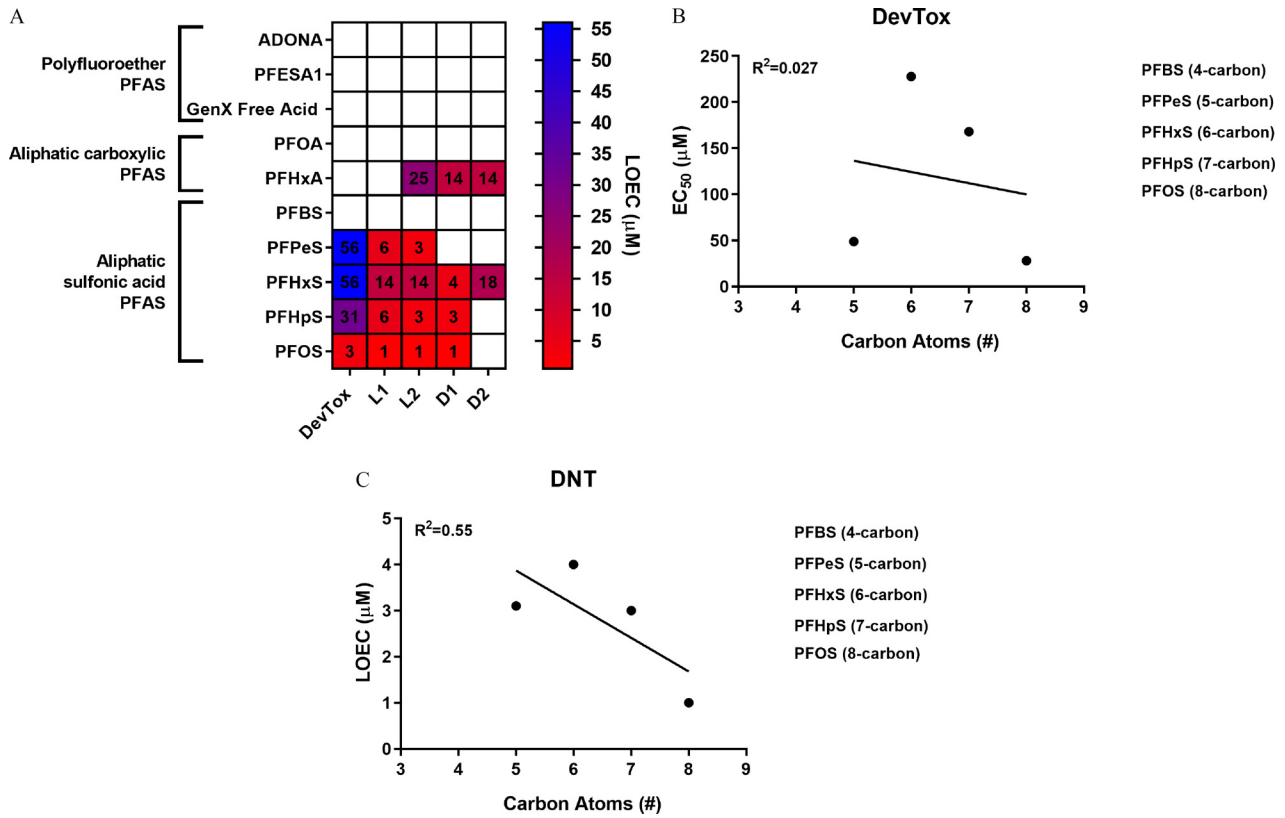

Figure 9. Identification of shared phenotypes between structurally similar PFAS. (A) Heatmap depicting LOEC values for the DevTox assay and significant hyperactivity in the L1, L2, D1, and/or D2 periods of the DNT assay (Studies 1, 2, and 3). If chemicals were replicated in Study 1 and Study 2, the lowest observed LOEC value was used. Linear regression of (B) Study 3 DevTox assay EC_{50} or (C) Study 3 DNT assay LOEC values for aliphatic sulfonic acid PFAS. Note: ADONA, 4,8-dioxa-3H-perfluorononanoate; D, dark period; DevTox, developmental toxicity; DNT, developmental neurotoxicity; EC_{50}, half maximal effective concentration; GenX Free Acid, perfluoro-2-propoxypropanoic acid; L, light period; LOEC, lowest observed effect concentration; PFAS, per- and polyfluoroalkyl substances; PFBS, perfluorobutanesulfonic acid; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHpS, perfluoroheptanesulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid; PFPeS, perfluoropentanesulfonic acid.

Table 5. Summary of key zebrafish toxicity data.

Compound	Class	DevTox assay phenotype	Ref	DNT assay phenotype	Ref
ADONA	Polyfluoroether	Negative[a]	This study	Negative[a]	This study
GenX Free Acid	Branched polyfluoroether	Negative[a]	This study	Negative[a]	This study
PFESA1	Branched polyfluoroether	Negative[a]	This study	Negative[a]	This study
PFHxA	Aliphatic carboxylic acid	Negative	This study; Truong et al. 2014	Hyperactivity[a]	This study
PFOA	Aliphatic carboxylic acid	Negative	This study; Padilla et al. 2012; Truong et al. 2014	Negative	This study; Khezri et al. 2017
		Positive	Hagenaars et al. 2011; Jantzen et al. 2016; Ulhaq et al. 2013a	Hyperactivity	Ulhaq et al. 2013b
PFBS	Aliphatic sulfonic acid	Negative	This study; Truong et al. 2014	Negative[a]	This study
		Positive	Hagenaars et al. 2011; Ulhaq et al. 2013a	Hyperactivity	Ulhaq et al. 2013b
PFPeS	Aliphatic sulfonic acid	Positive[a]	This study	Hyperactivity[a]	This study
PFHxS	Aliphatic sulfonic acid	Positive	This study; Truong et al. 2014	Hyperactivity[a]	This study
				Negative	Khezri et al. 2017
PFHpS	Aliphatic sulfonic acid	Positive[a]	This study	Hyperactivity[a]	This study
PFOS	Aliphatic sulfonic acid	Positive	This study; Hagenaars et al. 2011; Huang et al. 2010; Jantzen et al. 2016; Padilla et al. 2012; Truong et al. 2014; Ulhaq et al. 2013a, 2013b	Hyperactivity	This study; Huang et al. 2010; Khezri et al. 2017; Spulber et al. 2014
				Hypoactivity	Ulhaq et al. 2013b

Note: Data on PFAS evaluated in the present study and previous work. ADONA, 4,8-dioxa-3H-perfluorononanoate; DevTox, developmental toxicity; DNT, developmental neurotoxicity; GenX Free Acid, perfluoro-2-propoxypropanoic acid; PFBS, perfluorobutanesulfonic acid; PFESA1, perfluoro-3,6-dioxa-4-methyl-7-octene-1-sulfonic acid; PFHpS, perfluoroheptanesulfonic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexanesulfonic acid; PFOA, perfluoro-*n*-octanoic acid; PFOS, perfluorooctanesulfonic acid; PFPeS, perfluoropentaslufonic acid; Ref, reference.
[a]Indicates previously unreported findings.

data to group PFAS based on their ability to cause developmental toxicity and/or developmental neurotoxicity. We specifically identified aliphatic sulfonic acid PFAS as a particularly bioactive class of PFAS, thereby identifying relationships between chemical structures and *in vivo* phenotypes that may arise from putative shared mechanisms of PFAS toxicity. We also used analytical chemistry to reveal that GenX Free Acid is unstable in DMSO, a solvent widely used for zebrafish and *in vitro* screening studies. These data show that this emerging PFAS, in addition to other branched and/or fluoroether PFAS examined here, is negative for developmental toxicity and developmental neurotoxicity in zebrafish, possibly identifying a less bioactive group of PFAS (at least in the context of fish toxicology). Finally, this study supports the use of *in vivo* developmental neurotoxicity testing when evaluating this class of widely occurring environmental contaminants.

Acknowledgments

S.G. participated in study design, performed zebrafish experiments, analyzed data, prepared figures, and assisted with manuscript preparation. A.S. performed targeted analytical chemistry experiments. J.R.S. participated in study design, data interpretation, and analyzed behavior and analytical chemistry data. X.M.H. performed zebrafish experiments. J.S. performed behavior statistical analyses. T.C., J.M., E.H., and M.S. participated in study design and data interpretation. T.T. designed the study, assisted with zebrafish experiments, analyzed and interpreted data, and wrote and rebutted the manuscript. All authors reviewed the manuscript.

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