Empirical measurement of PFAS dosing within in vivo aquatic high throughput assays

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Introduction

- Understanding per- and polyfluoroalkyl substances (PFAS) toxicity is a priority of the US EPA, and high-throughput testing (HTT) has been developed as a rapid, low-cost alternative to traditional *in vivo* toxicity testing.
- The unique physicochemical properties of PFAS preclude accurately modeling free chemical concentrations using current *in vitro* disposition models.
- Characterizing chemical behavior and partitioning in HTT format is critical for comparison of HTT-derived points of departure with those from traditional aquatic toxicity assays.
- Here, 22 individual PFAS (Table 1) were screened for toxicity to juvenile *Daphnia magna* (see poster 4.21.P-Th175) or *Pimephales promelas* (poster 2.14.P-We058) following 24 h exposure in 96 well format.
- Final concentration of each PFAS in exposure media was empirically measured by LC-MS to accurately assess exposure conditions.

Methods

Chemical Exposures

- PFAS (Table 1) were acquired from EvoTec (South San Francisco, CA) solubilized in DMSO at a nominal concentration of 30 mM and 9.5 mM. Subsequent concentrations were made in a log dilution series from these two solutions for a total of 9 concentrations at ½ log steps. Nominal exposure concentrations ranged 0.03 – 100 µM for each PFAS.
- Organisms were exposed to 8 concentrations of each chemical (plus a control) in 1mL polypropylene 96-well plates (n=5 individuals per concentration) in a total volume of 700 µL Lake Superior water spiked with working stock (concentration of DMSO<0.33%).
- Working stocks were saved in 1:1 acetonitrile:media or 3:1 acetonitrile:media for chemical analysis.
- Exposures were performed for 24 hours at 20 °C (*D. magna*) or 25°C *(P. promelas)* with a 16:8 light:dark cycle. Following this, media was transfered from exposure plates into a clean well plate, diluted 1:1 or 3:1 with acetonitrile, and stored at -20°C.
- Three replicates per concentration were analyzed for each individual PFAS. For *P. promelas*, individual replicate wells from one exposure plate were measured while for *D. magna*, 5 replicate wells per concentration were pooled within 3 replicate exposure plates. The lowest concentration of each PFAS was not measured due to concentrations consistently below or near detection limits (n = 14-21 wells per chemical).

Chemical Verification

- Chemical stocks used to prepare exposure plates and media from post-exposure plates were analyzed by LC-MS using a Vanquish LC system coupled to a TSQ Altis tandem MS.
- One method based on EPA Method 1633 (method details given below) was used for quantification of most target analytes. Individual methods were developed for 6:1 FTOH, PFPB, and PFTP.
- A stable mass labeled internal standard was used where possible to account for potential matrix effects and analyte loss during sample preparation.
- For compounds without a matched internal standard, a stable labeled surrogate with the nearest retention time was used for quantification.

Thermo Scientific Vanquish LC system

- Column: StableBond C18, 2.1 x 50mm, 1.8µm
- Column Temp: 40°C
- Injection volume: 5 µL
- Mobile phase: A = H2O+5mM ammonium acetate
  B = MeOH
  Flow rate: 0.4 mL/min
- Gradient: 10% B to 55% B at 2.5 min, to 90% B at 9 min, to 100% B at 9.5 min, held for 2 min, return to 10% B.
- Total run time: 14 min

Thermo Scientific TSQ Altis

- Ionization mode: H-ESI, negative
- Gas Temp: 300°C
- Ion Transfer Tube: 325°C
- Sheath Gas : 60
- Aux Gas: 10
- Sweep Gas: 1
- Capillary voltage: 2500 V

Vanquish LC system coupled to a TSQ Altis tandem MS

Results and Discussion

Table 1. List of 22 PFAS tested in the current study.

DTXSID	Chemical Name	Abbreviation	Class	Molecular weight	Molecular Formula
DTXSID6062599	Perfluoropentanoic acid	PFPeA	carboxylate	264.0	C5HF9O2
DTXSID1037303	Perfluoroheptanoic acid	PFHpA	carboxylate	364.1	C7HF13O2
DTXSID8031865	Perfluorooctanoic acid	PFOA	carboxylate	414.1	C8HF15O2
DTXSID8031863	Perfluorononanoic acid	PFNA[a]	carboxylate	464.1	C9HF17O2
DTXSID8047553	Perfluoroundecanoic acid	PFUdA[a]	carboxylate	564.1	C11HF21O2
DTXSID90868151	Perfluorotridecanoic acid	PFTrDA[a,b]	carboxylate	664.1	C13HF25O2
DTXSID3059921	Perfluorotetradecanoic acid	PFTeDA[a]	carboxylate	714.1	C14HF27O2
DTXSID3037709	Potassium perfluorohexanesulfonate	PFHxS	sulfonate	400.1*	C6F13KO3S
DTXSID8037706	Potassium perfluorooctanesulfonate	PFOS	sulfonate	500.1*	C8F17KO3S
DTXSID70381090	1H,1H,8H,8H-Perfluoro-3,6-dioxaoctane-1,8-diol	FC8DOD[b]	diol	294.1	C6H6F8O4
DTXSID30396867	1H,1H,8H,8H-Perfluorooctane-1,8-diol	FC8diol[b]	diol	362.1	C8H6F12O2
DTXSID50369896	1H,1H,10H,10H-Perfluorodecane-1,10-diol	FC10diol[b]	diol	462.1	C10H6F16O2
DTXSID70191136	Perfluoro-3-methoxypropanoic acid	PFMPA[b]	fluoroether	230.0	C4HF7O3
DTXSID60663110	Perfluoro-4-isopropoxybutanoic acid	PFPBA[b]	fluoroether	380.1	C7HF13O3
DTXSID70276659	Perfluoro-(2,5,8-trimethyl-3,6,9-trioxadodecanoic) acid	HFPO-TeA[a,b]	fluoroether	662.1	C12HF23O5
DTXSID30891564	2-(Perfluorobutyl)-1-ethanesulfonic acid	4:2 FTS	fluorotelomer sulfonate	328.2	C6H5F9O3S
DTXSID6067331	6:2 Fluorotelomer sulfonic acid	6:2 FTS	fluorotelomer sulfonate	428.2	C8H5F13O3S
DTXSID00192353	8:2 Fluorotelomer sulfonic acid	8:2 FTS[a]	fluorotelomer sulfonate	528.2	C10H5F17O3S
DTXSID50469320	Perfluorohexanesulfonamide	FHxSA[a,b]	sulfonamide	399.1	C6H2F13NO2S
DTXSID1032646	N-Ethylperfluorooctanesulfonamide	N-EtFOSA[a,b]	sulfonamide	527.2	C10H6F17NO2S
DTXSID70379295	3H-Perfluoro-2,2,4,4-tetrahydroxypentane	PFTP[b]	alcohol	262.1	C5H5F7O4
DTXSID00190950	6:1 Fluorotelomer alcohol	6:1 FTOH[b]	alcohol	350.1	C7H3F13O

*Molecular weight of PFHxS and PFOS given as the free acid.
[a]Solutions stored in 3:1 ACN:media for chemical analysis.
[b]Mass labeled internal standard not available, nearest retention time surrogate used for quantification.

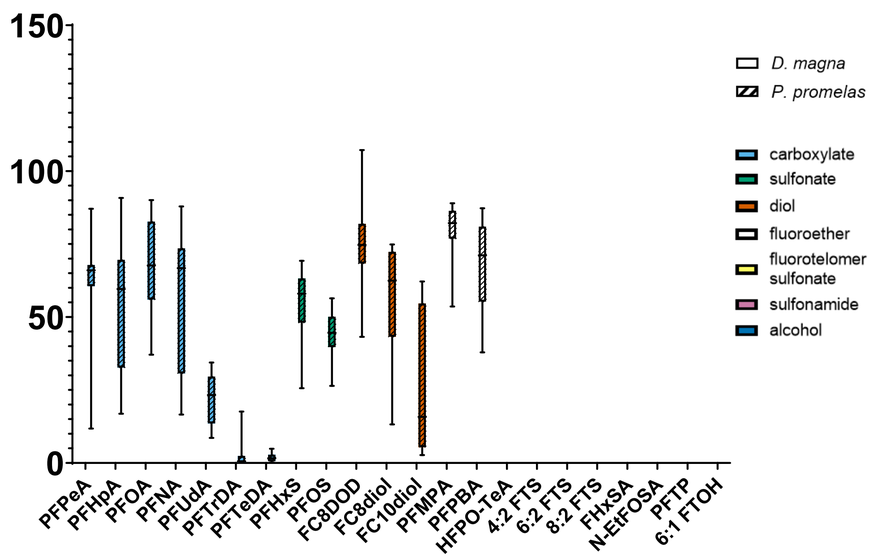

Figure 1. Percent recovery of individual PFAS (color coded by chemical class) relative to nominal concentration after 24 h exposure to *D. magna* (unfilled boxes) or *P. promelas* (hashed boxes) in 96 well plates. Within a chemical class, chemical molecular weight increases from left to right. Boxes represent the inner quartile range with a line at the median, and bars represent min/max. n = 3 replicates per concentration; 6 to 7 concentrations per chemical.

- PFAS concentrations varied widely across tested compounds, with median concentrations ranging near 100% of nominal for PFTP to as low as 5.4% for N-EtFOSA (Figure 1).
- In general, longer chain compounds showed lower recoveries, regardless of PFAS class. Most compound classes follow a linear decrease in recovery with increasing molecular weight (Figure 2; mol wt. used here as an approximation of carbon chain length).
- Class specific differences were observed (Figure 1, Figure 2). Sulfonamides showed lower recovery relative to other classes of similar molecular weight.
- The fluorotelomer alcohol, 6:1 FTOH, was not detected in media after 24 h exposures, indicating potential loss to volatilization during exposure or during sample preparation steps. HFPO-TeA initially showed no recovery in *P. promelas* exposures but was also not present in DMSO stocks supplied from EvoTec. This compound class is known to degrade in DMSO, so new exposure stocks were prepared in methanol for the exposure conducted with *D. magna*.
- Recovery between test species was generally within the same range, but several compounds (8:2 FTS, FHxSA, FC10diol) had widely different recovery across species, suggesting issues in dosing for one or more exposure.
- To examine whether PFAS stock preparation is a large contributor to concentration variability, time 0 stocks were analyzed for the series of carboxylates. Comparing post-exposure (24 h) to pre-exposure (0 h) solutions (Figure 3) showed reduced variability for most compounds. Median recovery of C6 – C9 carboxylic acids was >75%, while longer chain carboxylates had greatly reduced median recovery <25%.

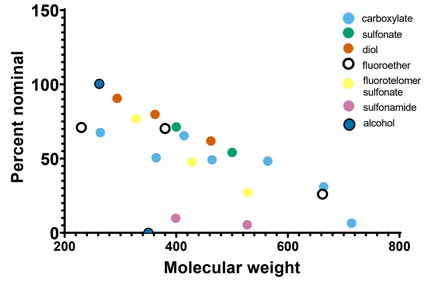

Figure 2. Apparent median recovery of individual PFAS from *D. magna* exposures relative to molecular weight (a surrogate of chain length). Compounds are color coded by chemical class.

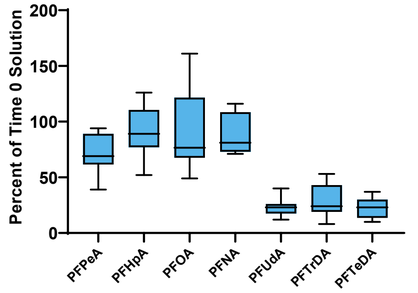

Figure 3. Recovery of individual carboxylates after 24 h exposure relative to their concentration at exposure start (time 0). Relative to Figure 1, variability was reduced for most compounds. Boxes represent the inner quartile range with a line at the median, and bars represent min/max.

Conclusions

- Empirical measurement of PFAS dosing provides a much more accurate representation of exposure conditions and captured variability across compound classes and chain lengths.
- Most compounds of 10-carbon chain length and less showed between 50-100% recovery, and median recovery shows a strong relationship with fluorinated chain length.
- In the context of deriving points-of-departure (POD) for PFAS effects in HTT systems, using nominal values for exposure would have negligible impact on POD accuracy for many PFAS in this assay. However, some longer chain compounds showed drastically reduced concentration relative to nominal. This highlights the need for compound specific consideration of potential HTT assay performance.
- Specific PFAS properties must be considered prior to HTT testing to ensure compounds are not degraded (e.g., HFPO-TeA degrading in DMSO) or compounds to be likely lost during exposure (e.g., 6:1 FTOH).
- Dosing and/or liquid handling errors may have a large impact on variability, as seen when comparing concentration at 24 h to nominal versus empirical time 0 concentrations (see Figure 1 and Figure 3).
- This dataset provides a baseline for beginning to model PFAS disposition within *in vivo* HTT exposure systems.

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