Toxicity assessment of exposure to four short- and ultra-short PFAS using *Daphnia magna* and *Hydra vulgaris* as models

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Running title: Toxicity assessment of short- and ultra-short PFAS in aquatic invertebrates

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Abstract

Short- and ultra-short per- and polyfluoroalkyl substances (PFAS) such as perfluorobutanoic acid (PFBA, 4C), perfluorobutane sulfonic acid (PFBS, 4C), trifluoroacetic acid (TFA, 2C) and trifluoromethane sulfonic acid (TFMS, 1C) were detected in various environmental matrices but studies addressing the effects attributed to exposure are still scarce. This study aimed to (1) investigate acute toxicity of these emerging PFAS using *Daphnia magna* (immobility, mortality, reproduction, and body size) and *Hydra vulgaris* (morphological changes and reproduction) and (2) assess the sub-chronic/chronic toxicity of TFA in both species. Glutathione S-transferase (GST) activity was also assessed in both species after long-term exposure to TFA. Chemical analysis confirmed presence and stability of 4 PFAS in exposure media. For all tested PFAS, endpoints examined after acute tests were not markedly affected by exposure and LC_{50} values were > 1000 mg/L. However, this toxicity was significantly affected by media acidification induced by the tested chemicals (LC_{50} = 316 and 31,6 mg/L for *D. magna* and *H. vulgaris,* respectively). Long-term exposure to TFA did not significantly induce any effect on both species and GST levels were not altered. Overall, results suggest lower toxicity of ultra/short-chain PFAS to these species under selected exposure conditions. However, additional studies investigating multigenerational effects of these PFAS using realistic environmental concentrations are needed to overcome the significant gaps in our understanding of short /ultra-short PFAS-induced toxicity.

Introduction

Per- and polyfluoroalkyl substances (PFAS) form a large and heterogenous group of over 9000 fluorinated chemicals (Shittu et al., 2023). Owing to their oleophobicity, hydrophobicity, heat resistance, and persistence (Buck et al., 2011; Shittu et al., 2023), PFAS have been widely used since the 1940s in a variety of industrial and commercial applications including aerospace technologies, firefighting foam, and consumer products (Brennan et al., 2021) including clothes, outdoor textiles, carpets (Kotthoff et al., 2015) cosmetics, and insecticides (Valsecchi et al., 2017). A broad range of these substances were detected in various environmental and biotic samples worldwide (Ma et al., 2022; Sharp et al., 2021). Several investigations and reviews reported toxicity of PFAS in algae, aquatic invertebrates (crustaceans, bivalves, gastropods, platyhelminthes, annelids) and vertebrates (amphibia, fish) (Ankley et al., 2021; Ma et al., 2022; 2023; Valsecchi et al., 2017). In addition, investigators noted that the toxicity of carboxylic acids was correlated to the carbon chain length of these chemicals and that sulfonic acids exhibit more toxicity than carboxylic acids for the same carbon chain length (Buhrke et al., 2013; Gaballah et al., 2020; Ulhaq et al., 2013; Zheng et al., 2012). A few PFAS (PFOS; PFOA; perfluorohexane sulfonate, PFHxS) and their respective salts and related compounds were listed under the Stockholm Convention as Persistent Organic Pollutants (POPs) (UNEP, 2019). Long-chain perfluorocarboxylic acids with carbon chain lengths from 9 to 21 carbons, their salts and related compounds are currently under review by the Stockholm Convention (Stockholm Convention, pops.int).

The regulation and phase-out of longer chain PFAS resulted in a significant shift in the industry towards fluorinated alternatives such as short- (C = 4-7) and ultra-short (C = 2-3) chain PFAS (Ateia et al., 2019; Xing et al., 2023), which are presumed to display less propensity to

bioaccumulate (Conder et al., 2008; Martin et al., 2003; Olsen et al., 2009) and induce toxic effects (Wang et al., 2013). However, it has been found recently that physicochemical properties and environmental fate of several substitutes were similar to long chain molecules (Gomis et al., 2015; Militao et al., 2021). Short PFAS were measured and detected in surface water of rivers and estuaries (Allinson et al., 2019; Zhao et al., 2016), groundwater (Gao et al., 2019), tap water (Gao et al., 2019; Schwanz et al., 2016), Arctic environment (sediment and lake and ocean water) (Stock et al., 2007; Yamashita et al., 2008) and wildlife (Gebbink et al., 2016). Due to their high solubility in water and low/moderate sorption to sediments, short-chain homologs constitute a concern for aquatic environments (Ateia et al., 2019).

Among dominant short PFAS, 4 carbon chain molecules such as perfluorobutanoic acid (PFBA) and perfluorobutane sulfonic acid (PFBS), were widely detected in the environment (see Table S1 for a compilation of studies). However, few studies investigated PFAS-initiated toxicity on aquatic invertebrates, and some chronic testing was reported for these contaminants. Kadlec et al. (2024) found no marked effects after short-term exposure of *Hyalella azteca* and *Chironomus dilutus* to PFBA and PFBS and after chronic exposure of *Ceriodaphnia dubia* to these compounds. However, sublethal exposure to PFBA led to metabolic perturbations in *Daphnia magna* via disruption of the arginine and proline metabolism pathways (Labine et al., 2023). In fish such as *Oryzias melastigma* and *Danio rerio*, toxicological studies indicated that PFBS exposure impaired reproduction, embryonic development, pancreatic organogenesis, and energy homeostasis (Chen et al., 2019; Sant et al., 2018). In addition, Hagenaars et al. (2011) noted that exposure to PFBS, but not PFBA, induced malformations of the zebrafish embryos head, and only 3000 mg/L PFBS resulting in alterations of embryo heart rates after 48hr and 72hr exposure.

Ivantsova et al. (2024) suggested that several biological responses are involved in the toxicity attributed to exposure to PFBS and PFBA in different systems (cardiac, immune, hepatic, and reproductive) of fish and indicated that understanding of the mechanisms underlying these effects is limited by non-relevance of tested concentrations and lack of published reported studies.

To date, ecotoxicology research focused on a few PFAS and little was noted on ultra-short-chain (C1-C3) PFAS (Björnsdotter et al., 2019). Trifluoroacetic acid (TFA, C2), the most studied amongst the ultra-short chains (Björnsdotter et al., 2019), may be formed via degradation of hydrochlorofluorocarbons (HCFCs), hydrofluorocarbons (HFCs), and hydrofluoroolifines (HFOs) (Sidebottom and Franklin, 1996). Several chemicals frequently employed in pharmaceutical and agricultural applications might also act as TFA precursors and contribute to its environmental input (Freeling and Björnsdotter, 2023). In addition to TFA, trifluoromethanesulfonic acid (TFMS, C1) was found by Zhou et al (2020) to be a POPs and mobile organic chemical which was detected at concentrations up to μg/L in water samples such as urban effluent, surface water, ground water and drinking water) collected from European countries (Montes et al., 2017; Zahn et al., 2016). Despite global distribution and presence in many water samples including rivers, lakes, canals, reservoirs, estuaries, tributaries, drainage basins, snowmelt, and precipitation, only two studies determined the effects of TFA in aquatic species (Berends et al., 1999; Boutonnet et al., 1999). Understanding the potential toxicity of these substances is therefore important to better support their identification, evaluation and management.

In this context, it has been reported that PFAS interact with antioxidant proteins such as glutathione-S-transferase activity, involved in antioxidant defense system (Rajak et al., 2024; Hamid et al., 2024), and in detoxification processes via conjugating glutathione (GSH) to a variety of hydrophobic and electrophilic compounds facilitating their solubilization and excretion (Hamid

et al., 2024). It was also reported that the mRNA level of GST was reduced in RTL-W1 exposed to perfluoroethylcyclohexane sulphonate (PFECHS) at environmental concentrations (Mahoney et al., 2023) and positively correlated to PFBA concentrations in coelomic fluid of sea cucumbers (Cocci et al., 2025). Due to the marked increase in GST activity in the digestive gland of *M. galloprovincialis* exposed *in vitro* to PFBS, Cunha et al. (2025) suggested that there is an enhanced cellular response aiming to detoxify this pollutant or its potentially reactive metabolites.

The objectives of the present study were to investigate the acute and chronic/sub-chronic toxicity of 4 short- and ultra-short-chain PFAS (TFA, TFMS, PFBA, and PFBS) utilizing two aquatic invertebrates (*Daphnia magna* and *Hydra vulgaris*). These relevant model species are widely distributed in the natural environment, easy to maintain and manipulate in the lab, possess short life cycles, rapid reproduction, and are sensitive to a broad spectrum of contaminants (Ahmed, 2023; Kovačević et al., 2024). In addition to mortality and physiological endpoints, GST activity was also determined in both species as its regulation has been considered as a proxy for cellular antioxidant and xenobiotic defense mechanisms. We hypothesize that although those short PFAS are presumed to display less propensity to bioaccumulate than long chain PFAS, they potentially have the capacity to elicit oxidative or electrophilic stress as suggested by Cunha et al. (2025). To our knowledge, this study is the first to focus on the biological effects of short/ultra-short-chain PFAS, particularly for TFA and TFMS in aquatic invertebrates.

Material and methods

Chemical reagents and stock solutions

PFBA (heptafluorobutyric acid, purity 98%), PFBS (nonafluorobutane-1-sulfonic acid, purity 97%) and TFA (trifluoroacetic acid, purity 99%) were purchased from Sigma Aldrich (Oakville,

ON, Canada), and TFMS (trifluoromethane sulfonic acid, 95%) was purchased from Toronto Research Chemicals (Toronto, ON, Canada) (Table S2). Stock solutions (1 g/L) were prepared prior to exposure by dissolving different PFAS in the corresponding exposure media and stored refrigerated (4°C) until testing. Since all tested PFAS markedly acidified the stock solutions (pH less than 3), two acute tests with and without adjusting the pH of the stock solutions with sodium hydroxide were carried out for each species. The final pH of the adjusted stock solutions was similar to the pH of daphnid and hydra culture media. In addition, as evidence indicated that pH of working solution is a key factor in accurately interpreting results (Wasel et al, 2021), physico-chemical properties (dissolved oxygen, conductivity, temperature and pH) of exposure media were measured at the beginning and at the end of exposure (except conductivity) using standard methods (Environment Canada, 1990; Environment and Climate Change Canada, 2020), in at least one replicate per concentration (see Table S3 and S4). Nominal concentrations for *D. magna* (0.01, 1, 10, 100, and 1000 mg/L) and *H. vulgaris* (0, 0.0001, 0.001, 0.01, 1, 10, 100, and 1000 mg/L) testing were selected based upon results from previous PFAS studies using congeners of similar structures with aquatic species (Barmentlo et al., 2015; Jeong et al., 2016; Wasel et al., 2021). The concentrations were selected to include environmentally relevant levels and higher doses to determine LC_{50} and observe long-term effects.

Test organisms, quality assurance and quality control

Genetically homogenous *Daphnia magna* obtained from the Quebec Laboratory for Environmental Testing of Environment Canada (Qc, Canada) were cultured in growth chamber following Environment Canada's method (Environment Canada, 1990). Cultures were kept at 20 ± 2 °C with a 16:8hr light:dark photoperiod in beakers in medium composed of reconstituted water, 0.02 g/L B_{12} vitamins (100 µL/L), at pH between 6.5 and 8.5. The daphnids were fed daily with

the green algae *Raphidocelis subcapitata* (3.5×10^5 cells/ml) and YCT preparation (0.0125 g/L of yeast-cerophyll-trout chow) and the medium was renewed three times per week**.** Key water parameters, such as pH and dissolved oxygen, were monitored weekly during routine water changes. Neonates were used for the different experiments if the following criteria were met: the % mortality of adult daphnia ≤ 25% during the 7day preceding exposures, first hatching ≤ 12 day and mean of neonates /adults ≥ 15 (Environment Canada 1990). In order to confirm the sensitivity of the tested organisms and suitability of experimental conditions in detecting potential toxicity, a reference toxicant test was conducted using NaCl. The determined 48hr-CL_{50} (5.37 g/L; 95% confidence interval (CI) = 4.98 - 5.8) was within the warning limit of the chart established as reported in Environment Canada's procedure method (Environment Canada 1990).

Hydra vulgaris used in this study were obtained from the Quebec Laboratory for Environmental Testing of Environment Canada (Qc, Canada). The polyps were cultured in covered crystallization bowels under standard methods (Environment and Climate Change Canada, 2020). Hydra were reared in a medium composed of 0.15g $CaCl_2$, 2 H_2O, and 0.1 g N-tris hydroxymethyl 1-2- aminoethanesulfonic acid buffer at pH 7. Polyps were fed daily with iodine-disinfected brine shrimps, *Artemia salina,* hatched within 24hr and medium was renewed approximately every 3-5hr after hydra feeding. Key water parameters, such as pH and dissolved oxygen, were monitored weekly during routine media changes. A reference test was also conducted using $ZnSO_4$ $7H_2O$ and EC/LC_{50} (0.22 mg/L; 95% CI = 0.16-0.3 and 0.81 mg/L; 95% CI = 0.64-1.02, respectively) which was within control limits as reported in Environment Canada's procedure method (Environnement et Changement Climatique Canada, 2025).

Daphnia magna: acute and sub-chronic testing

For acute and sub-chronic exposures, neonates were maintained under 20 ± 1 °C with a 16:8hr light: dark. Acute toxicity (48hr) was tested using two replicates of 5 neonates (< 24hr) for each experiment with and without pH adjustment following Environment Canada's standard procedure (Environment Canada, 1990). Neonates obtained from adult daphnia (20-28 days old) after approximately 9-12 days were transferred into 100 ml polypropylene beakers filled with 75 ml culture medium alone (control) or containing increasing concentrations of tests solutions (0.01, 1, 10, 100, or 1000 mg/L). The exposure concentrations were obtained by serial dilution in reconstituted water from the highest concentration made from the stock solution of 1g/L (with or without adjusted pH). pH was adjusted to 8 ± 0.5 using 1N NaOH. Endpoints of immobilization and death were monitored at the end of two acute exposures. An organism was considered immobile when no swimming could be observed within 15sec after stirring and a cardiac activity could be recorded.

For sub-chronic exposure, 4 replicates of 12 neonates (< 24hr) were exposed for 14 days. Currently, no standardized sub-chronic exposure tests exist for *D. magna*. Therefore, this test is a compromise between the acute (Environment Canada, 1990) and chronic bioassays from the OECD guideline (OECD, 2012). The sublethal doses (0.01, 1, 10, or 100 mg/L) of TFA were selected based on the LC_{50} (Table S5) determined after 48hr acute exposure. Untreated *D. magna* exposed only to the reconstituted water were used as control. The tested solutions were prepared similarly to those for acute toxicity. Organisms kept in culture media were used as a control group. Daphnids were fed daily with algae and YCT and media renewed every 48hr. Temperature, pH, and dissolved oxygen were monitored at 48-hr intervals before and after each media change. Endpoints of immobility and mortality were monitored every day. The number of offspring was also recorded daily and were removed from the vessel. At the end of the exposure, body length,

defined as the distance from the top of daphnia head to the base of the tail spine, was determined for a subset of three surviving organisms in each treatment and measured under a digital image analyzing system (Leica M165c Stereomicroscope, Wetzlar, Germany). The remaining organisms were collected and stored at -80 °C for biomarker analyses.

Hydra vulgaris: acute and chronic testing

Acute and chronic exposures were carried out in multi-well microplates covered with parafilm to prevent evaporation and kept in an incubator at 20 °C with 16:8hr light-dark cycle during the exposure time. The selection of healthy (morphology score of 10 based upon the Wilby scale) (Wilby, 1988) non-budding hydra polyps of similar size was done randomly using a microscope. As for *D. magna*, acute toxicity (96hr) of PFBA, PFBS, TFA, and TFMS was assessed with and without adjusting pH (Environnement et Changement Climatique Canada, 2025). Exposures were conducted, without feeding, in a 12-well microplate (3 individuals per well in triplicate; N = 9) and 3 ml/well of test solutions (0.0001, 0.001, 0.01, 1, 10, 100, or 1000 mg/L) or hydra medium only for the control groups were added. Tested concentrations up to 100 mg/L were obtained by serial dilution in hydra medium from the highest concentration (1000 mg/L) used as a stock solution. pH was adjusted at 7 ± 0.5 using 1N NaOH.

For chronic exposure (7 days), only sub-lethal doses of TFA (0.0001, 0.001, 0.01, 1 or 10 mg/L) selected based upon LC_{50} results (Table S5) along with untreated control hydra exposed to hydra medium only were used to assess chronic effects of this ultra-short PFAS without adjusting pH. For each concentration, 6 non-budding polyps were randomly placed in a well of 6 multi-well microplates containing 8 ml test solutions (4 replicates for each concentration). The exposure was performed in triplicate (N=72 polyps) and polyps were fed every 3 days, 2hr before media renewal. At the end of the acute and chronic exposure, the morphological changes of the individual polyps

were observed by microscopic examination. Five classical morphological stages were examined based upon the Wilby scale (Wilby , 1988): healthy polyps were normal and exhibit an extended body and tentacles (score 10); first sign of intoxication was characterized by presence of clubbed tentacles (Score 8), late phase of intoxication was marked by the presence of shortened tentacles and body (Score 6); tulip stage was characterized by polyps exhibiting markedly shortened tentacles and body (score 5) and animal death was marked by polyps disintegration (score 0). Scores ≥ 6 are reversible and sub-lethal, while scores <6 are irreversible and lethal.

The population growth and reproduction rates of *H. vulgaris* were also determined during chronic testing. Following the 7day exposure period, the mean population growth rate (K) and budding rates (K_{tot}) were determined following the methodology outlined by Holdway et al. (2001). The value K reflects the growth rate based solely upon the count of detached buds and is calculated as follows: $K = \frac{ln\ (nt7) - \ln(nt0)}{7}$ where nt_7 is the total number of hydras produced during the assay (including detached buds and initial polyps). Similarly, the budding rate value K_{tot}, which accounts for both attached buds and new polyps, was calculated as $Ktot = \frac{\ln(ntot) - \ln(nt0)}{7}$, where n_{tot} represents the sum of the number of attached (forming) buds and the number of detached polyps as indicator of reproduction.

GST activity

To better understand the potential sublethal effect of TFA, glutathione-S-transferase (GST) activity, a marker of oxidative stress, was quantified in both species after 14 and 7d for daphnia and hydra, respectively. Daphnids (4-5 organisms; N = 8) and hydra (pooled from 2 wells / concentration; N = 6) were homogenized using buffer solution consisting of 100 mM NaCl, 25mM HEPES-NaOH, 0.1mM dithiothreitol and 1 µg/L aprotinin at pH 7.4. The homogenates were

centrifuged at 15,000 g for 20 min at 4 °C. GST activity was determined according to the method described by Gagné (2014). The reaction mixture consisted of 10 mM HEPES pH 6.5, 1mM GSH, 1mM 1-chloro-2-4-dinitrobenzene, 125 mM NaCl. The conjugation of glutathione (GSH) to 1-chloro-2, 4-dinitrobenzenem was evaluated by monitoring the increase in absorbance at 340 nm, using the Synergy 4 microplate reader (BioTek, Winooski, VT, USA). Data were expressed as nmole GSH/min/mg protein. Protein content was measured according to the method of Bradford (1976) using a standard solution of bovine serum albumin for calibration.

Chemical analysis

Concentrations of the three PFAS were measured in media at 0 and 48hr for *D. magna* and at 0 and 96hr for *H. vulgaris* as exposure media was changed at these time points. Samples were collected in 15 ml falcon tubes previously rinsed three times with methanol at 0h and 48h for *D. magna* and 0 h and 96h for *H. vulgaris* (0 to 1000 mg/L) and stored at -20 °C until the analyses. All TFA solutions (n = 24) and the non-adjusted pH media for PFBA, PFBS and TFMS were analyzed (N = 12 for each compound). Samples were filtered (glass fiber, 0.3 μm, 25 mm) and analyzed by on-line solid-phase extraction (SPE) coupled to hydrophilic interaction liquid chromatography (HILIC) interfaced to high-resolution mass spectrometry (HRMS) (A Q-Exactive Orbitrap - Thermo Scientific, Waltham, MA, USA). The on-line SPE and its connection to the HILIC-MS system were performed by a dual switching-column array consisting of 6-port and 10-port valves. In the first steps, the sample was injected into 1 ml injection loop and loaded onto the on-line SPE column (for sample pre-concentration. After sample loading, the on-line SPE mobile phase was left to flow to remove the matrix/salt. The samples were then eluted at 0.5 ml/min with HILIC-HRMS mobile phase, the analytes retention was conducted on a Thermo Scientific Acclaim™ Trinity Q1 LC column thermostated at 35 °C, starting from strong organic conditions

(90/10 A/B). The HILIC mobile phases were 25 mM ammonium acetate (A) and HPLC-water (B). Details the on-line SPE HILIC-HRMS acquisition method and chromatograms of the four studied PFAS analyzed are provided in Tables S6 and Figure 2S. Limits of detection for PFBA, PFBS, TFA and TFMS were 0.01, 0.01, 0.02, and 0.05 µg/L, respectively.

Statistical analysis

Statistical analyses were conducted using STATISTICA software package (version 7, France). All data were tested for normality using Shapiro Wilks test and homogeneity using Bartlett's test. All values were expressed as mean ± SD and were compared by using one-way ANOVA. When the data did not follow a normal distribution, the non-parametric method Kruskal-Wallis test was used to compare the difference between treatments and control of *D. magna* and *H. vulgaris*. The multiple comparison test was carried out using Tukey's post hoc test. Significance was set at $p \leq 0.05$. CETIS software version 1.9.7.10. (Tidepool Scientific Software, McKinleyville, CA, USA) was used to analyze lethal and sublethal ecotoxicity data. The 50% lethal concentration (LC_{50}) and the sublethal effects (EC_{50}) endpoints for acute exposures were determined using the binomial method (Stephan, 1977).

Results

Acute and chronic toxicity in D. magna

Data demonstrated that concentrations of 4 tested PFAS determined in the *D. magna* and *H. vulgaris* media at the beginning and the end of the exposure were generally close to nominal concentrations (Table S7). Variation between the analyzed and the expected concentrations in the media may be initiated by (1) differences in the standards used, (2) carryover cross-contamination for low concentrations, (3) manipulations made during dilutions for high concentrations and (4)

method uncertainties. In addition, results obtained for TFA showed that measured concentrations after each 48hr during sub-chronic exposure deviated slightly from the nominal values (for example, accuracy was of 95 ± 10% (± SD, n = 6) and 81 ± 20% (± SD, n = 6) for TFA at 100 mg/L at the beginning and the end of the exposure, respectively) (Table S8), in agreement with an observation reported by Xie et al. (2025) during their testing with PFBS.

Exposure of neonates to differing PFAS after adjusting the pH did not induce any marked effects (mortality, immobility) in *D. magna*. Only the higher exposure concentration (1000 mg/L) with non-adjusted pH led to 100% mortality for all tested substances (the pH was then below 4 and probably the main reason for the toxic effects). The EC_{50} and LC_{50} were estimated at 316 mg/L for all PFAS, while these values were estimated to be higher than 1000 mg/L with adjusted pH (Table S5). Similar to the acute testing, TFA 14day chronic exposure did not produce increase mortality rates in *D. magna* (Figure 1A). No marked alteration in reproduction was found, and no significant difference was observed in number of neonates between controls and TFA treatments (Figure 1B). Daphnids from two replicates of the 10 mg/L treatment had their first neonates at day 9, all other treatments and controls reproduced at day 10. The average cumulative reproductive output (number of neonates per female) after 14days for control (Figure 1C) was noted at 21 ± 2 and for tested concentrations this value varied from 20 ± 2 to 24 ± 1. Body measurements indicated similar body sizes between exposed organisms and controls at the end of exposure (Figure 1C). Analysis of GST activity in daphnids treated chronically with TFA did not exhibit any significant variation in its levels compared to control (Figure 2A).

Acute and chronic toxicity in H. vulgaris

Morphological alteration after acute (96hr) exposures to PFBA, PFBS, TFA, and TFMS indicated no increased mortality rates or sub-lethal effects for any treatment with adjusted pH by

comparison to controls (Table S5). However, in a non-adjusted pH media, no mortality was found for the 4 PFAS tested acutely up to concentrations of 10 mg/L (all hydra were normal), while the two highest concentrations (100 and 1000 mg/L) resulted in 100% mortality (hydra were at tulip and degenerated stages and were unable to recover) by comparison to controls (Figure 3A). The LC_{50} for PFBA, PFBS, TFA, and TFMS with non-adjusted pH after 96hr were determined to be 31.6 mg/L but exceeded 1000 mg/L when the pH was adjusted (Table S5); no significant differences were observed between EC_{50} and LC_{50} for the 4 PFAS. These concentrations were higher than environmental levels reported for these PFAS (exceeding the maximum concentrations detected in the environment by more than $7x10^3$fold higher) (Table S1). Regarding chronic exposure (Figure 3B), data demonstrated that all tested TFA concentrations did not induce morphological alteration on *H. vulgaris* compared to control.

Our results showed no significant differences in number of hydranths (hydranths develop on the main body of the hydra and eventually detach to become separate individuals, a budding structure is also considered a hydranth) after acute and chronic exposure between controls and treatments , No significant, change was observed after exposure to the two lowest concentrations of TFA for 7day (Figure 4A). In addition, data demonstrated that the growth (K) and budding rates ($K_{tot\text{-}7d}$) were not markedly affected by differing treatment (Figure 4B). Similarly to *D. magna*, GST activity was not significantly altered after chronic exposure to TFA for 7day by comparison to control (Figure 2B).

Discussion

The available literature on the toxicity of short- and ultra-short-chain PFAS is limited. To better understand their potential toxicity on aquatic organisms, 4 short-chain PFAS (PFBA, PFBS, TFA, and TFMS) were tested using *D. magna* and *H. vulgaris*. Our results showed that EC_{50}/LC_{50} were

317 significantly different between organisms exposed in media with (>1000 mg/L) and without pH
318 adjustment (316 and 31.6 mg/L for *D. magna* and *H. vulgaris*, respectively). This finding
319 suggested that toxicity was significantly affected by media acidification initiated by different
320 PFAS as previously reported (Wasel et al. 2021). The different LC_{50} values observed between
321 species may be related to specific hydra media which seems to be more susceptible to acidification
322 by PFAS than that of daphnids. The addition of 100 mg/L of PFAS surpassed the buffering
323 capacity of the hydra medium and produced a drop in pH, from 7 to < 4, while the daphnid media
324 pH remained close to that of control; only addition of 1000 mg/L of PFAS induced media
325 acidification. The concentrations used in the present study may have been too low to provide
326 substantial acute and chronic toxicity data and a toxicity rank for buffered PFAS. Further testing
327 with higher concentrations is needed to determine which of these two species is more sensitive to
328 short-chain PFAS.

329 To the best of our knowledge, the effects of most PFAS on hydra remain largely unknown.
330 Only one other study investigated the effects of PFOA and PFOS on *H. vulgaris* and Wang et al.
331 (2021) reported that the minimum effective dose estimated at 100 mg/L resulted in 100% mortality
332 after 92hr treatment. Comparing the 48hr-EC_{50}/LC_{50} values for PFBA found for *D. magna* in the
333 present study with literature data, demonstrated that acute value was in a similar range than that
334 reported by Ding et al. (2012) (180 mg/L without pH adjustment and 4260 mg/L with pH
335 adjustment) and Barmentlo et al. (2015) (48h-EC_{50} = 5250 mg/L) for this species. Barmentlo et al.
336 (2015) suggested that acute toxicity attributed to perfluoroalkyl acids in *D. magna* rose with
337 increasing carbon-chain length, as these investigators found that the 48 hr-EC_{50} values for PFHxA
338 (perfluorohexanoic acid, C6; 1048 mg/l) and PFOA (C8; 239 mg/L) were 5- and 21-fold lower
339 than that of PFBA, respectively. This observation indicating that chain length acts as a driver of

differential PFAS-initiated toxicity was also corroborated by mortality rates obtained with *in vivo* and *in vitro* zebrafish models (larvae and embryonic fibroblast cells) (toxicity of PFOA > PFHxA > PFBA) (Wasel et al., 2021). It was also stated that toxicity was elevated with sulfonate group, since potassium perfluorobutane sulfonate K-PFBS (C4; 96 hpf-LC_{50} =1,394 mg/L) exhibited higher toxicity than PFBA (C4; 96 hpf-LC_{50} = 9,703 and 83.6 mg/L for buffered and unbuffered PFBA, respectively) (Wasel et al., 2021). In agreement with our findings, these outcomes confirm that buffered PFBA and PFBS display toxicity at concentrations higher than in 1000 mg/L. However, the role of chain length in the toxicity of PFAS was not supported by the study of Ulhaq et al. (2013) indicating that the order of toxicity (144 hr-EC_{50}/LC_{50}) for zebrafish embryo was as follow: PFBS (144 hr-EC_{50}/LC_{50}= 450 /1500 mg/L) > TFA (144 hr-EC_{50}/LC_{50}= 700 / >3000 mg/L) > PFBA (144 hr-EC_{50}/LC_{50}= 2200 / >3000 mg/L). This was also in agreement with Wang et al. (2014) in freshwater rotifer, *Brachionus calyciflorus*, reporting that acute effects (24hr-LC_{50}) of TFA (2C; 70 mg/L), PFPrA (perfluoropropionic acid, 3C; 80 mg/L), PFBA (4C; 110 mg/L), PFPeA (perfluopentanoic acid, 5C; 130 mg/L) and PFHxA (perfluorohexanoic acid, 6C; 140 mg/L) were negatively correlated to the carbon chain length due to higher water solubility of shorter PFAS. However, these findings need to be considered with caution since the observed effects were related to the acidification of exposure media. A lower 24hr-EC_{50} (55 mg/L) was also estimated by Rhône-Poulenc 1995 (cited in Boutonnet et al., 1999) for *D. magna* exposed to TFA and this was attributed to the pH effect. It was also noted that 1200 mg/L sodium trifluoroacetate (corresponding to 1000 mg/L trifluoroacetate) did not markedly induce any effects on *D. magna* after exposure for 48hr (Berends et al., 1999; Boutonnet et al., 1999), and on *Brachydanio rerio* (Boutonnet et al., 1999) and *Danio rerio* (Berends et al., 1999) after 96hr of treatment.

Overall, discrepancies observed in the data reported may be explained, at least in part, by variations in culture conditions, experimental design, such as the lack of adjusting solutions to neutral pH, and by differences in organism sensitivity. Although it is difficult to draw a conclusion on the most toxic unbuffered PFAS (LC_{50} >1000 mg/L for all tested PFAS), the available results suggest limited impacts for these individual short- and ultra-short chain PFAS to freshwater organisms, as the estimated LC_{50} values were far higher than PFAS concentrations detected in the environment. The PFAS tested in the present study may be considered as not toxic according to the EU Directive 93/97/EEC classification of toxic contaminants (CEC, 1996) as the acute LC_{50} values were all above 100 mg/L. Additional acute toxicity research is needed to further address potential ecotoxicity of short- and ultra-short chain PFAS across different aquatic species.

Since toxicity generally tends to rise with increasing exposure time, chronic exposure to TFA was carried out using *D. magna* and *H. vulgaris*. Our sub-chronic results demonstrated that 14day exposure of *D. magna* to TFA, up to the maximum dose of 100 mg/L, did not significantly induce any effects on survival, body size, and number of neonates. In addition, data demonstrated that exposure to 10 mg/L TFA did not markedly alter reproduction or growth rate of hydra after 7day treatment. To the best of our knowledge, there is no apparent published information on chronic exposure of aquatic organisms to TFA. A study that examined the combined effects of TFA salt and trichloroacetic acid salt in *Myriophyllum spicatum* and *Myriophyllum sibiricum* noted that this mixture did not induce marked effects in these aquatic plants after 35day exposure (Hanson et al., 2002). However, although no marked effects were observed under the present tested experimental conditions, the findings need to be considered with caution. Previously, Xie et al (2025) found that toxicity of PFBS in *D. magna* increased over 6 generations; the 21day-LC_{50} for F0 and F3 were >1470 and 483 mg/L, respectively, and the F0-EC_{50} for reproduction (856 mg/L) and growth (886

mg/L) decreased respectively to 470 and 499 mg/L for F3 in relation with elevated PFBS bioaccumulation in subsequent generations. Exposure over multiple generations may therefore affect daphnia resistance and ultimately affect survival, reproduction, and population growth (Xie et al., 2025).

Given the paucity of published literature, more information is needed regarding the chronic toxicity and mechanisms of action of short-and ultra-short chain PFAS to better understand the potential effects of these chemicals. Several investigators reported that the mechanisms of long-chain PFAS toxicity in aquatic organisms acted via a series of reactions in cells, including oxidative stress which subsequently induced modulation in antioxidant defense system such as GST activity (Jeong et al., 2016). An increase in GST activity was observed in offspring of *D. magna* exposed to 10 mg/L of PFOS (Jeong et al., 2016), while a reduction in GST activity was detected in *D. magna* chronically exposed to 8 mg/L of PFOS for 10 and 21days (Liang et al., 2017). Huang et al. (2022) reported a significant rise in GST activity after exposure of zebrafish for 28days to 100 µg/L of a mixture of 5 PFAS (chlorinated polyfluorinated ether sulfonate (F-53B), PFOS, sodium p-perfluorous nonenoxybenzenesulfonate (OBS), PFHxS, and PFBS, suggesting that GST played a role in the PFAS detoxification. The absence of GST induction in the present study suggested that tested concentrations of TFA were not sufficient to trigger stress in daphnids and hydra. However, it should be noted that one cannot attribute oxidative stress to the actions of PFAS as only one parameter (GST activity) was examined. This constitutes a limitation as future studies need to also determine carbonyl and MDA levels to assess oxidative stress.

Conclusions

Overall, acute and sub-chronic/chronic results from this study indicated a lack of toxicity of the tested short- and ultra-short chain PFAS to *D. magna* and *H. vulgaris.* Findings also indicated that assessing the pH of media is essential to the accurate interpretation of PFAS toxicity results. Given the low toxicity observed for PFBA, PFBS, TFMS, and TFA relative to the concentrations reported in aquatic environments, it may be important to consider longer exposure (months, years, whole life cycle) of aquatic organisms to better assess their toxicity at biochemical, molecular, cellular, and physiological levels. It is also crucial to consider realistic environmental exposure and multigeneration scenarios to overcome the significant gaps in our understanding of short /ultra-short PFAS toxicity. Further toxicity tests of individual and combined PFAS across different aquatic species are needed to better determine their environmental impact. In this context, and with growing concerns regarding short- and ultra-short PFAS, *in vitro* methods may provide a rapid screening tool for their toxic potential and help in elucidating their mechanisms of action.

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Disclosure statement

The authors report there are no competing interests to declare.

Data availability statement

Data are available on the Environment and Climate Change open government portal (https://open.canada.ca/en).

CRediT authorship contribution statement

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Marie Houédé: Methodology, formal analysis, writing - original draft, writing - Review and editing, visualization.

Geneviève Farley: Methodology, formal analysis, review and editing.

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Sébastien Sauvé: Supervision, review and editing.

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Figure captions

Figure 1. Effect of **sub-chronic** exposure to different TFA concentrations on survival (A), hatching (B), number of neonates per female (C) and body length (D) of *D. magna* after 14d of exposure. Data are expressed as mean ± SD.

Figure 2. Changes in GST activity level in A) *D. magna* after **14d** and B) *H. vulgaris* after 7d of exposure to TFA. Data are expressed as mean ± SD.

Figure 3. Stacked bar chart showing the number of *H. vulgaris* with sublethal (normal, clubbed and shortened tentacles) and lethal (tulip and disintegrated) morphological changes after A) 96h of exposure to short- and ultra-short PFAS and B) after 7d of exposure to TFA.

Figure 4. Mean number of (A) hydranth after acute and chronic exposure (96h and 7d) and (B) population growth rate (K) and budding rate (Ktot) after 7d of exposure to TFA. Data are expressed as mean ± SD.

Figure 1

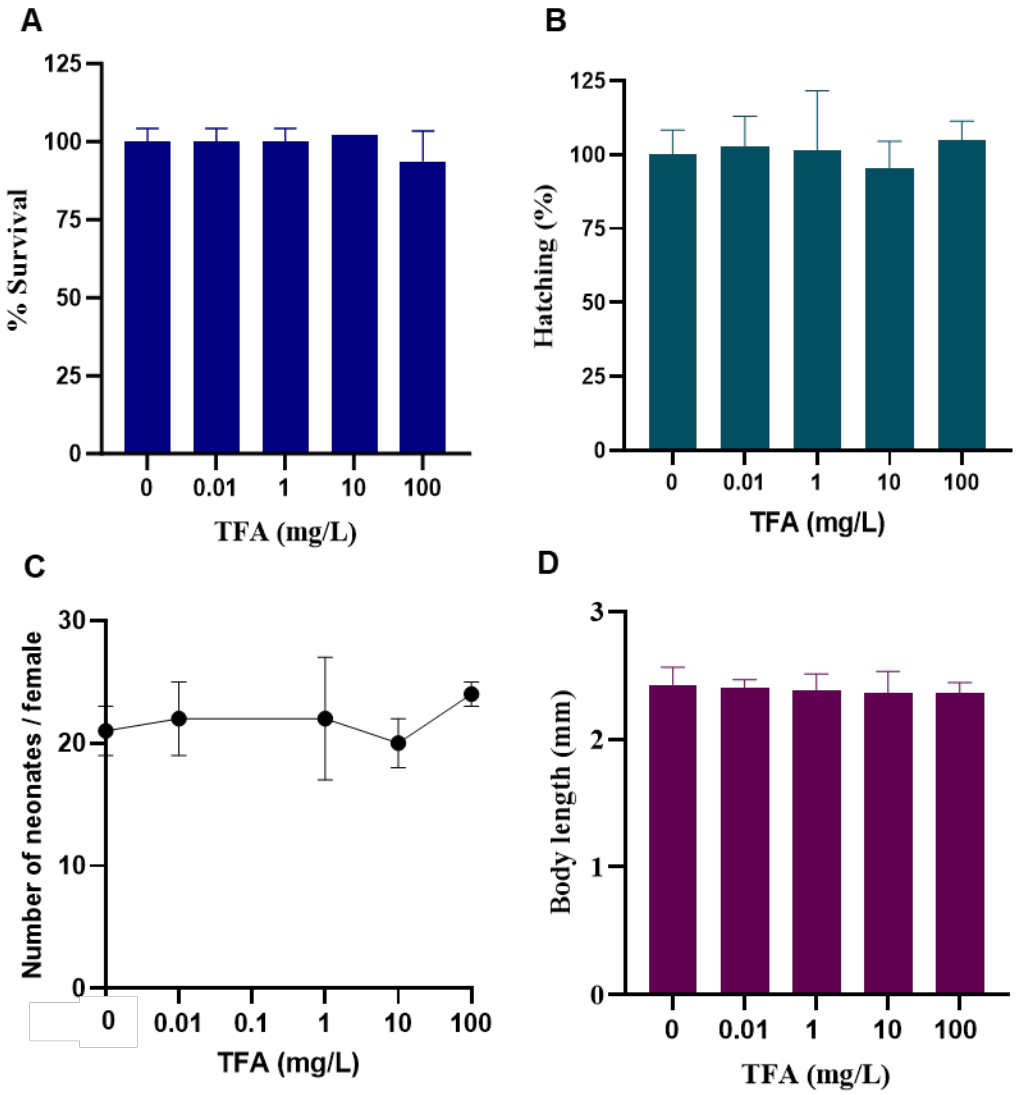

A
% Survival
125
100
75
50
25
0
0
0.01
1
10
100
TFA (mg/L)
B
Hatching (%)
125
100
75
50
25
0
0
0.01
1
10
100
TFA (mg/L)
C
Number of neonates / female
30
20
10
0
0
0.01
0.1
1
10
100
TFA (mg/L)
D
Body length (mm)
3
2
1
0
0
0.01
1
10
100
TFA (mg/L)

Figure 2

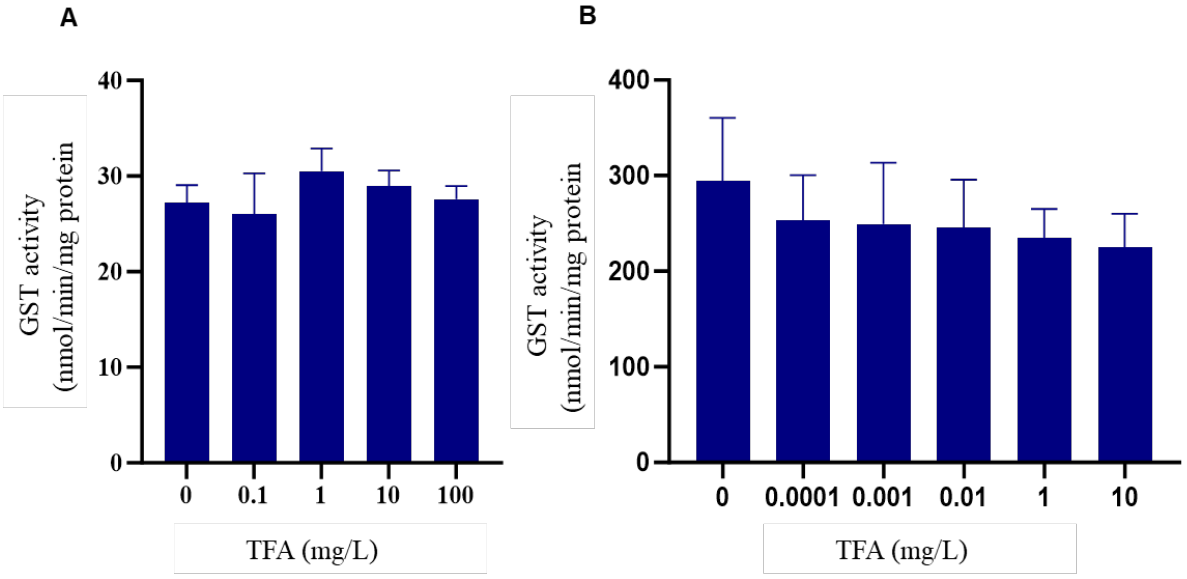

A
GST activity (nmol/min/mg protein
40
30
20
10
0
0
0.1
1
10
100
TFA (mg/L)
B
GST activity (nmol/min/mg protein
400
300
200
100
0
0
0.0001
0.001
0.01
1
10
TFA (mg/L)

Figure 3

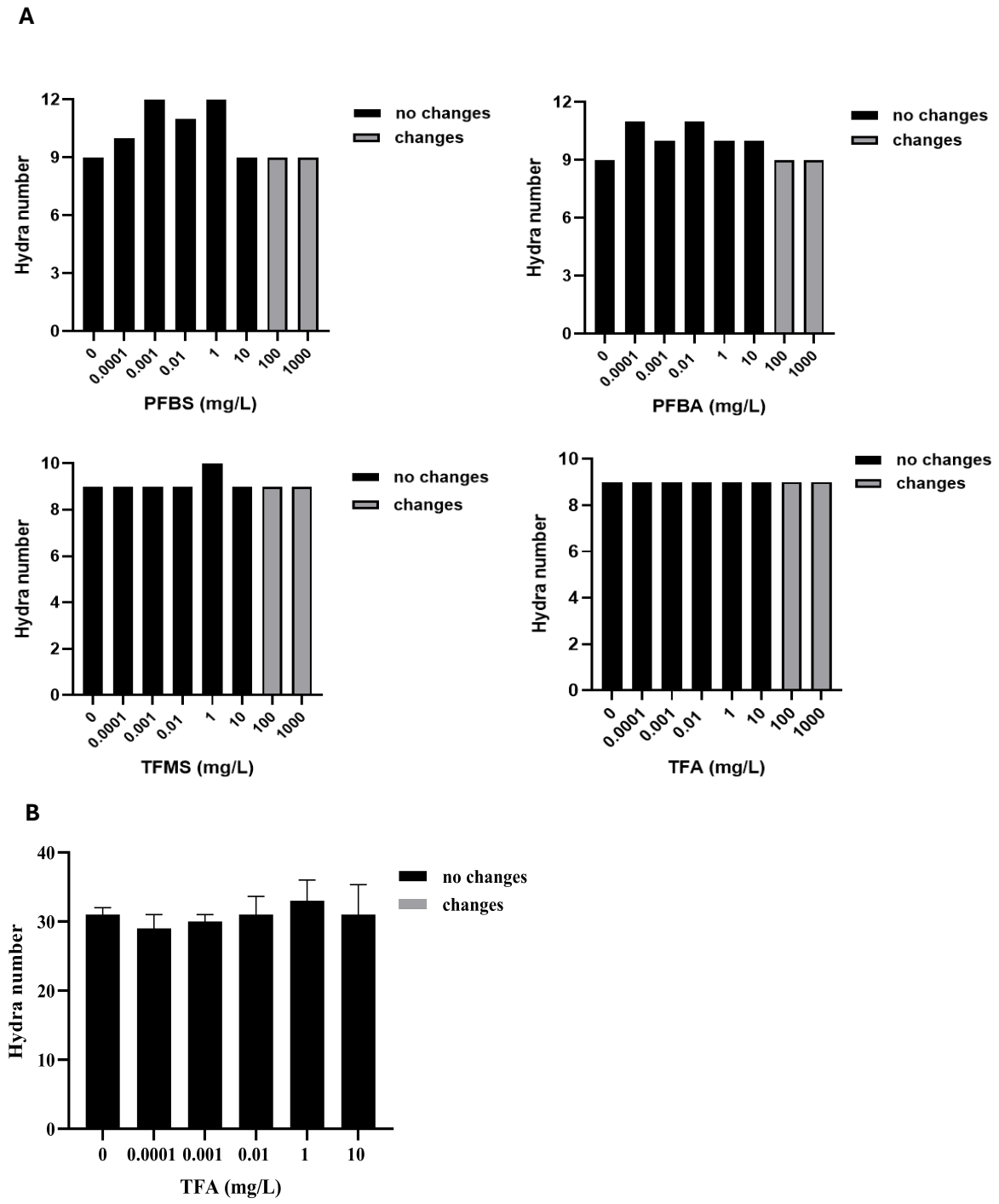
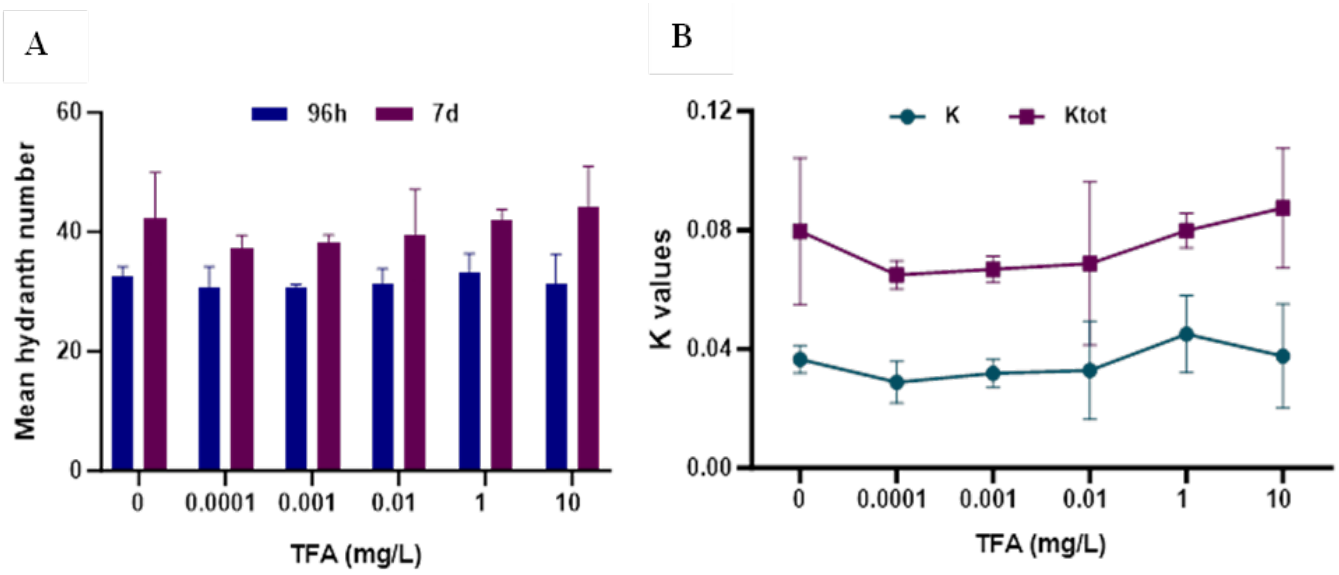

A
Hydra number
no changes
changes
0
0.0001
0.001
0.01
1
10
100
1000
PFBS (mg/L)
PFBA (mg/L)
TFMS (mg/L)
TFA (mg/L)
B
TFA (mg/L)

Figure 4


A
96h
7d
Mean hydranth number
60
40
20
0
0
0.0001
0.001
0.01
1
10
TFA (mg/L)
B
K
Ktot
K values
0.12
0.08
0.04
0.00
0
0.0001
0.001
0.01
1
10
TFA (mg/L)

Supplementary Material

Toxicity assessment of exposure to four short- and ultra-short PFAS using *Daphnia magna* and *Hydra vulgaris* as models

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Table S1. Concentrations (ng/L) of TFA (2C), TFMS (1C), PFBA (4C), and PFBS (4C) in environmental waters reported in the literature after 2000. See Jordan and Frank (1999) and Zhang et al. (2005) for reviews of TFA concentrations reported in waters before 2000. *Review of PFBA and PFBS reported in China surface waters between 2006-2022. nd = not detected.

Location	Year of collection	Media	TFMS	TFA	PFBA	PFBS	Reference
Canada	1997	Lakes		(<0.5-360)			Scott et al. 2000
Switzerland	1996-1997	Rivers		87 (12-328)			Berg et al. 2000
		Midland lakes		119 (37-204)			
		Mountain lakes		141 (46-360)			
Canada/United States	1998	Lakes, river		18-150			Scott et al. 2002
Africa		Lake		1-5			
China	2001	Lakes		(6.8-98)			Zhang et al. 2005
		Rivers		(10-221)			
	1999-2001	Seawater		(6-190)			
Canada	2001	Lakes		(61-170)			Scott et al. 2006
Germany	2007	River				1.7-3.4	Ahrens et al. 2009
India	2008	Rivers				nd	Yeung et al. 2009
Netherlands	2008	Rivers, seawater			(0.22-335)	(<0.08-181)	Möller et al. 2010
France	2010	River				1.3 (0.6-2.6)	Labadie and Chevreuil 2011

Faroe Islands (Kingdom of Denmark)	2012	Lakes			(<0.04)	(<0.04-1.2)	Eriksson et al. 2013
Norway	2006	Lake, river, seawater			(0.06-1.2) [a]		Kwok et al. 2013
Japan	2019	Rivers, seawater			(nd-18)	(nd-49)	Takemine et al. 2014
Germany	2013-2014	River, estuary, coast			(<0-3.7)	(1.4-40)	Heydebreck et al. 2015
Netherlands		River			(<0-1.9)	(0.8-3.6)	
China		River			(4.8-4770)	nd	
Spain	2010	Rivers			20 (0.1-111)	0.3 (0.4-4.1)	Campo et al. 2015
Italy		Rivers			5 (0-52)	11 (0-66)	Castiglioni et al. 2015
Vietnam	2013	Rivers, canals			nd		Duong et al. 2015
France	2012	Lakes, rivers			(<0.17-11)	(<0.02-29)	Munoz et al. 2015
China	2012	Landscape waters		643 (345-828)			Zhai et al. 2015
India	2014	River			(<0.004-1)	<0.04–10.2	Sharma et al. 2016
Germany	2016-2017	River, streams		(<50-140 000)			Scheurer et al. 2017
China and South Korea	2016	Rivers, seawater			(<1.3-35)	(<0.13-1.5)	Zhou et al. 2018
Australia	2012	River, estuary			(1.7-11)	(0.4-7)	Allinson et al. 2019
Finland	2016-2017	Rivers			1 (max 5.3)	0.23 (max 1.5)	Junttila et al. 2019
Canada	2008-2017	Lakes			max 4.9	< limits of detection	Gewurtz et al. 2019

Norway	2014-2016	Lakes, rivers, seawater			(<0.08-2)	<0.003	Skaar et al. 2019
Philippines	not found	Lake	(<0.06-22.1)		(0.5-5.15)	(<0.03-1.2)	Guardian et al. 2020
Thailand	not found	River, dams	(<0.06-30.7)		(0.4-6.5)	(<0.03-2.4)	
South Korea	2017-2018	Lake				17 (1.4-43.6)	Lee et al. 2020
United States	2016	River			(0.9-1.4)	(0.5-2.5)	Penland et al. 2020
Sweden	2019	Streams, lake	(0.11−15)	(30-820)	(<0.1-12)	(<0.1-22)	Björnsdotter et al. 2022
United States	2020	Rivers				(nd-79)	Viticoski et al. 2022
Canada	2019	River			(3.6-87)	(0.42-0.52)	Munoz et al. 2022
Canada	2013 to 2020	Lakes, rivers			max 73	max 138	Lalonde and Garron 2022
Canada	2012-2015	Proglacial rivers, creeks			(0.6-6.2)	(<0.002-0.3)	MacInnis et al. 2022
China	2018	River estuaries			17 (0.3-283)	12 (nd-575)	Du et al. 2022
Tibet	2020-2021	Rivers, Tibetan plateau			(0.09-2.8)	(0.04-4.0)	Ren et al. 2023
Portugal	2017-2018	Rivers			(<0.040-23)	(<0.020-7.1)	Barbosa et al. 2023
China	2002-2022	Lakes, rivers, reservoirs			(0.03-3720)	(0.03-3720)	Zhang et al. 2023*

[a] Range of means

Table S2. Chemical information for the four studied PFAS.

Compound	Acronym	Structure	Mass	Internal standard used
Trifluoroacetic acid	TFA	CF_3COO^-	112.98449	TFA-$^{13}C2$
Perfluorobutanoic acid	PFBA	$CF_3CF_2CF_2COO^-$	212.9781	PFBA-$^{13}C4$
Trifluoromethane sulfonic acid	TFMS	$CF_3SO_3^-$	148.95148	PFBA-$^{13}C4$
Perfluorobutane sulfonic acid	PFBS	$CF_3CF_2CF_2CF_2SO_3^-$	298.94189	PFBS-$^{13}C3$

Table S3. Physico-chemical parameters of *Daphnia magna* and *Hydra vulgaris* exposure media without pH adjustment.

Daphnia magna													
Nominal concentration (mg/L)	**Time (h)**	**PFBS**						**PFBA**					
		0	**0.01**	**1**	**10**	**100**	**1000**	**0**	**0.01**	**1**	**10**	**100**	**1000**
Dissolved oxygen (%)	0	96.8	96.9	98.2	97.3	98.1	99.0	97.6	98.4	99.0	98.3	98.7	98.7
	48	104.8	105.4	105.3	105.7	105.3	107.7	104.5	105.7	104.5	102.0	102.6	105.7
Temperature (ºC)	0	20.3	20.4	20.5	20.1	20.4	20.3	20.6	20.5	20.6	20.5	20.4	-
	48	19.5	19.6	19.7	19.6	19.7	19.8	19.7	19.5	19.6	19.7	19.7	20.1
pH	0	8.03	8.05	8.01	7.79	6.92	2.98	8.01	8.04	8.02	7.71	6.82	2.82
	48	7.81	7.86	7.85	7.73	7.13	2.90	7.88	7.92	7.92	7.75	7.05	2.85
Conductivity (uS/cm)	-	317	317	317	317	313	1007	317	318	318	318	313	1469
Nominal concentration (mg/L)	**Time (h)**	**TFMS**						**TFA**					
		0	**0.01**	**1**	**10**	**100**	**1000**	**0**	**0.01**	**1**	**10**	**100**	**1000**
Dissolved oxygen (%)	0	97.6	97.7	98.2	98.2	98.3	100.5	97.2	98.0	96.8	97.0	98.0	99.8
	48	101.6	101.0	101.1	101.3	101.2	102.7	99.9	100.6	100.1	99.4	100.7	101.3
Temperature (ºC)	0	18.9	18.9	19.1	19.1	19.1	19.4	19.0	19.1	19.1	19.2	19.2	19.3
	48	19.8	19.7	19.7	19.6	19.7	19.7	20.1	19.9	19.9	19.9	19.9	20.1
pH	0	8.02	8.05	7.99	7.57	6.41	2.60	8.04	8.03	7.94	7.45	5.94	2.50
	48	7.90	7.97	7.94	7.74	6.89	2.72	7.85	7.84	7.85	7.60	6.34	2.58
Conductivity (uS/cm)	-	317	317	317	317	317	2220	317	317	317	317	315	3240

Hydra vulgaris																	
Nominal concentration (mg/L)	**Time**	**PFBS**								**PFBA**							
		0	**0.0001**	**0.001**	**0.01**	**1**	**10**	**100**	**1000**	**0**	**0.0001**	**0.001**	**0.01**	**1**	**10**	**100**	**1000**
Dissolved oxygen (%)	0	95.0	97.9	97.8	98.9	98.3	97.6	97.6	99.0	95.4	97.2	98.7	98.4	100.5	97.7	99.8	99.6
	96	104.7	103.5	104.4	104.8	102.3	103.5	102.7	102.3	104.8	104.4	103.9	103.2	103.5	103.7	104.5	103.7
Temperature (ºC)	0	19.7	19.6	19.7	19.5	19.5	19.5	19.5	19.4	20.1	20.1	20.2	20.1	20.1	20.1	20.0	20.0
	96	20.4	20.2	20.1	20.1	20.1	20.1	20.0	20.0	20.1	19.9	19.7	19.7	19.6	19.6	19.6	19.6
pH	0	7.04	7.02	7.03	7.03	7.02	6.90	3.85	2.88	7.04	7.02	7.00	7.03	7.00	6.80	3.61	2.72
	96	6.81	6.84	6.85	6.88	6.88	6.75	3.84	2.84	6.82	6.87	6.85	6.87	6.86	6.64	3.55	2.78
Conductivity (uS/cm)	0	266	269	269	269	269	269	323	1405	266	268	268	268	268	268	371	1847
	96	265	269	269	269	269	269	322	1390	265	269	269	268	269	269	369	1839
Nominal concentration (mg/L)	**Time (h)**	**TFMS**								**TFA**							
		0	**0.0001**	**0.001**	**0.01**	**1**	**10**	**100**	**1000**	**0**	**0.0001**	**0.001**	**0.01**	**1**	**10**	**100**	**1000**
Dissolved oxygen (%)	0	96.6	98.0	96.6	96.6	98.0	98.0	96.6	96.7	93.0	96.7	98.1	92.5	96.7	95.5	96.3	98.0
	96	107.2	106.8	106.1	108.3	107.7	107.1	106.6	107.2	109.1	107.5	106.7	107.5	107.5	106.9	107.1	103.9
Temperature (ºC)	0	18.6	18.4	18.2	18.0	18.1	18.0	18.0	18.1	17.8	17.6	17.6	17.6	17.7	17.5	17.4	17.5
	96	21.2	20.9	20.8	20.7	20.7	20.6	20.6	20.5	20.8	20.5	20.4	20.4	20.3	20.3	20.2	20.3
pH	0	7.14	7.14	7.13	7.14	7.08	6.65	3.36	2.64	7.18	7.15	7.15	7.15	7.11	6.82	3.48	2.72
	96	6.98	6.93	6.98	7.01	6.96	6.62	3.48	2.81	6.97	6.95	6.97	6.98	6.92	6.51	3.34	2.58
Conductivity (uS/cm)	0	268	262	269	269	269	271	529	3170	268	270	269	270	270	272	462	2770
	96	267	271	270	270	270	272	354	2690	267	271	269	269	278	271	521	3080

Table S4. Physico-chemical parameters of *Daphnia magna* and *Hydra vulgaris* exposure media with pH adjustment.

Daphnia magna													
Nominal concentration (mg/L)	**Time (h)**	**PFBS**						**PFBA**					
		0	**0.01**	**1**	**10**	**100**	**1000**	**0**	**0.01**	**1**	**10**	**100**	**1000**
Dissolved oxygen (%)	0	97.7	96.9	97.7	97.8	98.1	98.4	99.0	99.3	99.3	99.0	99.2	100.2
	48	105.6	106.6	106.1	105.7	104.7	106.2	104.0	105.5	106.3	105.3	104.9	105.1
Temperature (ºC)	0	20.6	20.6	20.5	20.1	20.5	20.7	20.6	20.5	20.5	20.5	20.6	20.7
	48	19.5	19.5	19.5	19.6	19.4	19.5	19.8	19.8	19.8	19.8	19.8	19.8
pH	0	8.07	8.07	8.08	7.79	8.07	7.99	8.05	8.06	8.07	8.07	8.04	7.77
	48	7.83	7.88	7.89	7.73	7.89	7.87	7.82	7.92	7.88	7.85	7.76	7.64
Conductivity (uS/cm)	-	316	316	317	317	339	530	317	317	317	320	350	639
Nominal concentration (mg/L)	**Time (h)**	**TFMS**						**TFA**					
		0	**0.01**	**1**	**10**	**100**	**1000**	**0**	**0.01**	**1**	**10**	**100**	**1000**
Dissolved oxygen (%)	0	98.2	99.1	98.5	99.1	98.5	99.8	96.8	97.1	98.2	97.1	97.1	98.3
	48	103.4	104.1	103.9	104.0	104.0	104.3	101.4	99.9	100.0	100.2	100.4	101.3
Temperature (ºC)	0	19.0	18.9	18.9	19.0	19.1	19.4	19.0	18.8	18.9	18.9	19.0	19.1
	48	20.0	19.9	20.0	20.0	19.9	19.9	19.7	19.6	19.7	19.8	19.6	19.6
pH	0	8.02	8.03	8.04	8.05	8.11	8.68	8.01	8.05	8.05	8.03	7.88	7.23
	48	7.88	7.92	7.93	7.95	7.96	8.41	7.80	7.86	7.87	7.86	7.80	7.34
Conductivity (µS/cm)	-	318	317	318	323	371	840	318	317	317	324	383	959

Hydra vulgaris																	
Nominal concentration (mg/L)	**Time (h)**	**PFBS**								**PFBA**							
		0	**0.0001**	**0.001**	**0.01**	**1**	**10**	**100**	**1000**	**0**	**0.0001**	**0.001**	**0.01**	**1**	**10**	**100**	**1000**
Dissolved oxygen (%)	0	96.2	101.7	102.6	102.0	102.4	101.5	102.2	102.1	96.8	101.8	102.0	101.3	102.2	101.0	102.4	102.6
	96	103.4	103.9	104.2	104.2	104.6	105.3	103.7	104.2	105.5	103.6	103.6	104.4	104.3	103.4	103.9	103.4
Temperature (ºC)	0	19.6	19.6	19.5	19.4	19.4	19.4	19.4	19.6	20.0	19.9	19.9	19.8	19.7	19.7	19.6	19.8
	96	19.9	19.9	19.9	19.9	19.9	19.8	19.7	19.7	20.0	19.7	19.6	19.6	19.5	19.5	19.5	19.5
pH	0	7.07	7.05	7.06	7.04	7.03	7.02	7.02	7.02	7.00	7.03	7.03	7.03	7.01	7.03	7.06	7.32
	96	6.87	6.91	6.92	6.90	6.91	6.88	6.84	6.85	6.89	6.98	6.97	6.99	6.95	6.95	6.96	7.17
Conductivity (uS/cm)	0	270	269	268	269	269	271	287	456	272	267	267	267	268	271	302	596
	96	270	269	268	269	270	271	288	455	274	268	268	268	268	272	302	594
Nominal concentration (mg/L)	**Time (h)**	**TFMS**								**TFA**							
		0	**0.0001**	**0.001**	**0.01**	**1**	**10**	**100**	**1000**	**0**	**0.0001**	**0.001**	**0.01**	**1**	**10**	**100**	**1000**
Dissolved oxygen (%)	0	93.0	91.5	95.0	94.7	95.1	94.4	94.8	95.6	94.5	96.6	97.6	96.5	96.2	96.0	95.9	96.3
	96	108.6	107.6	108.0	108.4	107.0	107.3	107.2	106.3	102.6	108.6	108.7	108.4	107.7	107.8	108.1	106.8
Temperature (ºC)	0	18.4	18.6	18.5	18.5	18.5	18.4	18.4	18.5	18.3	18.5	18.5	18.4	18.4	18.3	18.4	18.6
	96	20.4	20.4	20.4	20.4	20.4	20.4	20.4	20.4	20.5	20.1	20.1	20.1	20.2	20.1	20.1	20.1
pH	0	7.18	7.19	7.19	7.18	7.17	7.15	7.15	7.15	6.97	7.02	7.04	7.07	7.10	7.11	7.11	7.07
	96	6.84	6.96	6.98	6.99	7.00	7.01	7.01	6.99	6.91	6.98	6.97	6.98	6.95	6.92	6.94	6.89
Conductivity (µS/cm)	0	270	268	268	268	268	274	327	825	265	268	268	268	269	277	344	1008
	96	267	269	268	268	269	274	326	818	271	269	268	268	270	277	344	1000

Table S5. EC_{50} and LC_{50} (mg/L) of short- and ultra-short PFAS tested in the current study. CL= Confidence Level.

		Daphnia magna		***Hydra vulgaris***	
Chemicals	**pH**	**48h-EC_{50} (95% CL)**	**96h-LC_{50} (95% CI)**	**96h-EC_{50} (95% CI)**	**96h-LC_{50} (95% CI)**
TFA	Non-adjusted for pH	316 (100-1000)	316 (100-1000)	31.6 (10-100)	31.6 (10-100)
	pH adjusted	> 1000	> 1000	> 1000	> 1000
PFBA	Non-adjusted for pH	316 (100-1000)	316 (100-1000)	31.6 (10-100)	31.6 (10-100)
	pH adjusted	> 1000	> 1000	> 1000	> 1000
TFMS	Non-adjusted for pH	316 (100-1000)	316 (100-1000)	31.6 (10-100)	31.6 (10-100)
	pH adjusted	> 1000	> 1000	> 1000	> 1000
PFBS	Non-adjusted for pH	316 (100-1000)	316 (100-1000)	31.6 (10-100)	31.6 (10-100)
	pH adjusted	> 1000	> 1000	> 1000	> 1000

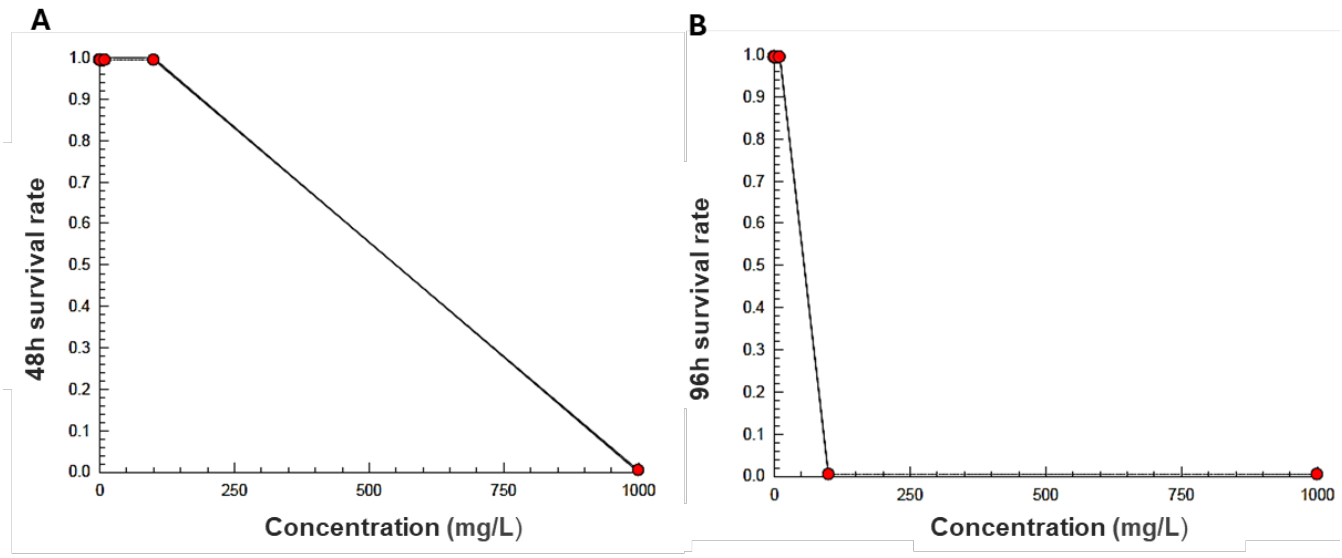

Figure S1. Example of concentration-response curves analyzed by the CETIS software after acute exposure of *D. magna* neonates (A) and *H. vulgaris* (B) to the different tested PFAS without pH adjustment.

Table S6. Details on the on-line SPE HILIC-HRMS method for media analysis.

Injection Volume	1000 µL
SPE Column	Biotage S Isolute ENV Online SPE cartridge (30 mm x 2.1 mm, 20- 80 µm particle size)
On-line SPE mobile phases	A: 0.0125% FA in HPLC-water, B: Acetonitrile
On-line SPE gradient	Time (min) / % A / % B / Flow rate µL/min 0.0 / 100 / 0 / 2500 1.6 / 100 / 0 / 2500 1.7 / 0 / 100 / 1500 10.5 / 0 / 100 / 1500 10.6 / 100 / 0 / 1000 19.0 / 100 / 0 / 1000 20.0 / 100 / 0 / 2500
HILIC -HRMS system	Dionex Ultimate 3000 UHPLC chain. heated electrospray ionization source (negative ion mode). Thermo Q-Exactive Orbitrap high-resolution mass spectrometer.
Separation column	Thermo Scientific Trinity Q1 column (100 × 2.1 mm. 3 µm particle size)
Ionization	Heated electrospray ionization source (HESI). negative ion mode
Column oven temperature	35 °C
HILIC mobile phases	A: 25mM AmAc in HPLC-water, B: Acetonitrile. Mobile phase flow rate 450 µL/min
HILIC gradient	Time (min) / % A / % B 0.0 / 10 / 90 2.6 / 10 / 90 9.6 / 70 / 30 13.6 / 70 / 30 16.6 / 10 / 90 20.0 / 10 / 90
Source	Sheath gas flow rate 50 a.u. Aux gas flow rate 20 a.u. Sweep gas flow rate 2 a.u. Capillary temperature (°C) 350. Vaporizer temperature (°C) 350. S-lens RF level 65.
Q-Exactive Orbitrap settings	*Full Scan MS mode.* Scan range (*m/z*) 150-600. Resolution 70.000 at *m/z* 200. AGC target 3e6. Max. Inject Time (ms) 100.

Table S7. Measured concentrations (mg/L, % nominal concentration) of the studied PFAS in exposure media of *D. magna* and *H. vulgaris* following acute exposure.

Daphnia magna								
Nominal concentration (mg/L)	**PFBA-0h (mg/L)**	**PFBA-48h (mg/L)**	**PFBS-0h (mg/L)**	**PFBS-48h (mg/L)**	**TFA-0h (mg/L)**	**TFA -48h (mg/L)**	**TFOH-0h (mg/L)**	**TFOH - 48h (mg/L)**
0	<LOD	<LOD	<LOD	0,011	<LOD	<LOD	<LOD	<LOD
0.01	0,01 (100%)	0,05 (500%)	0,01 (100%)	0,02 (200%)	0,01 (100%)	0,01 (100%)	0,01 (100%)	0,01 (100%)
1	1,17 (117%)	1,01 (101%)	1,25 (125%)	1,19 (119%)	1,12 (112%)	0,84 (84%)	1,30 (130%)	1,22 (122%)
10	11,48 (115%)	10,03 (100%)	11,03 (110%)	10,25 (103%)	9,40 (94%)	10,92 (109%)	11,17 (112%)	10,91 (109%)
100	93,62 (94%)	90,01 (90%)	105,56 (106%)	102,93 (103%)	84,73 (85%)	101,72 (102%)	111,73 (112%)	106,91 (107%)
1000	973,63 (97%)	789,90 (79%)	818,65 (82%)	994,55 (99%)	761,32 (76%)	781,34 (78%)	872,80 (87%)	856,19 (86%)
Hydra vulgaris								
Nominal concentration (mg/L)	**PFBA-0h (mg/L)**	**PFBA-96h (mg/L)**	**PFBS-0h (mg/L)**	**PFBS-96h (mg/L)**	**TFA-0h (mg/L)**	**TFA-96h (mg/L)**	**TFMS-0h (mg/L)**	**TFMS-96h (mg/L)**
0	< LOD	< LOD	< LOD	< LOD	0,003	0,003	< LOD	< LOD
1	0.93 (93%)	0.92 (92%)	1.02 (102%)	1.00 (100%)	1.51 (151%)	1.53 (153%)	1.00 (100%)	0.81 (81%)
1000	1425.89 (143%)	639.86 (64%)	1570.35 (157%)	752.28 (75%)	904.63 (90%)	1074.82 (107%)	1070.17 (107%)	836.07 (84%)

Table S8. Measured TFA concentrations (mg/L ± SD) in *D. magna* media following 48h interval during the sub-chronic assay.

TFA	Measured concentration (mg/L)	
Nominal concentration (mg/L)	**t=0h**	**t=48h**
0	0.02 ± 0.01	0.02 ± 0.01
0.01	0.02 ± 0.01	0.02 ± 0.00
1	0.86 ± 0.15	0.88 ± 0.22
10	8.75 ± 0.11	8.49 ± 1.21
100	95.05 ± 9.88	81.32 ± 20.10

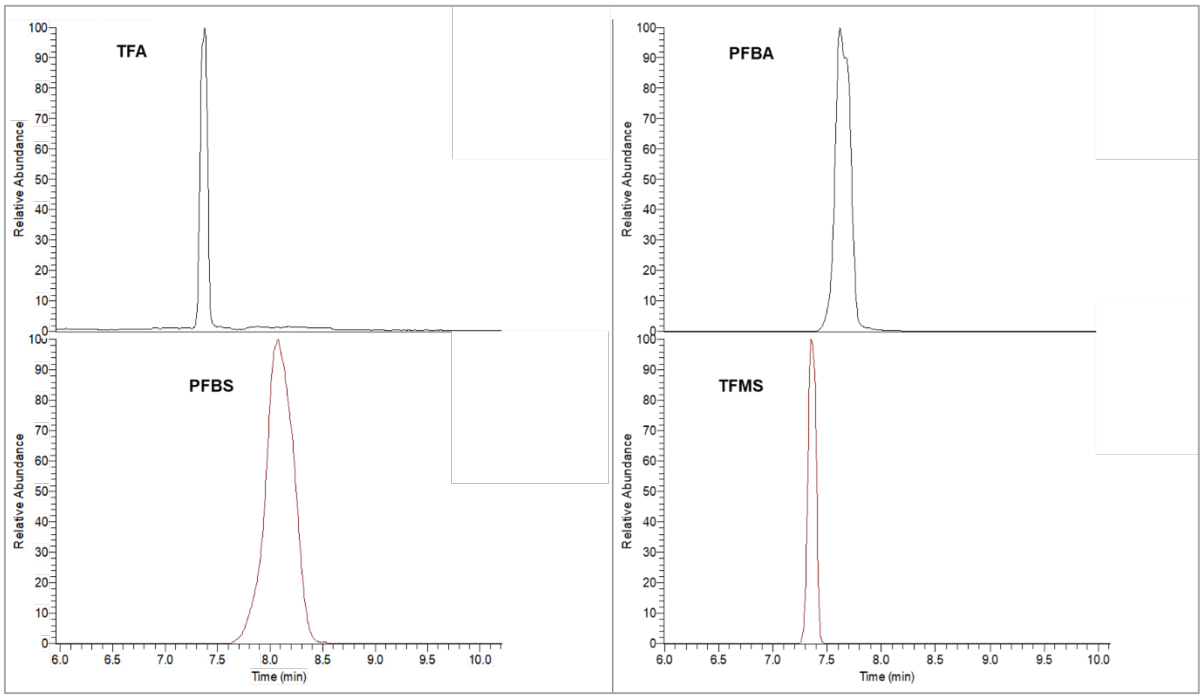

Figure S2. Chromatograms of the four studied PFAS analyzed by on-line solid-phase extraction (SPE) coupled to hydrophilic interaction liquid chromatography (HILIC) interfaced to high-resolution mass spectrometry (HRMS).

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