THE UPTAKE AND EFFECTS OF POLY- AND PERFLUOROALKYL SUBSTANCES ON LARVAL AND JUVENILE AMPHIBIANS

by

Sarah A. Vaughn

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THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Jason T. Hoverman, Chair

Department of Forestry and Natural Resources

Dr. Maria Sepúlveda

Department of Forestry and Natural Resources

Dr. Linda Lee

Department of Agronomy

Approved by:

Dr. Robert G. Wagner

Head of the Graduate Program

To my family, and friends that have become family

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ABSTRACT

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Committee Chair: Jason T. Hoverman

Poly- and perfluoroalkyl substances (PFAS) are ubiquitous contaminants across the globe, can bioaccumulate in aquatic taxa, and potentially biomagnify in food webs. Consequently, research examining the influence of PFAS on wildlife is warranted. Amphibians are sensitive to contaminants such as PFAS because of their porous skin and associations with aquatic habitats where contaminants accumulate. Because PFAS tend to bioaccumulate and can adversely affect the endocrine system, there is a need to examine uptake rates to inform ecotoxicology studies, as well as a need to examine sublethal effects. To address these knowledge gaps I conducted two experiments. First, I exposed larval northern leopard frogs (*Rana pipiens*), American toads (*Anaxyrus americanus*), and eastern tiger salamanders (*Ambystoma tigrinum*) to PFAS chemicals perfluorooctanoic acid (PFOA) or perfluorooctane sulfonate (PFOS) at concentrations of 10 or 1000 ppb for 10 days and sampled them every 48 hours during the exposure period. In the next experiment, I examined the effects of PFAS exposure via contaminated substrate on the survival and growth of post metamorphic amphibians of the same species. I found that, for all species, body burdens often reached steady state within 48 to 96 h of exposure. Steady-state body burdens of PFOA ranged from 3,819–16,481 ng/g dry weight among treatments and species (corresponding BCFs of 0.5 to 2.5), while PFOS body burdens ranged from 6,955–489,958 ng/g dry weight (corresponding BCFs of 47–259) among treatments and species. These data suggest that steady state is rapidly reached in larval amphibians exposed to PFAS, particularly regarding PFOS. This reflects a high potential for trophic transfer of PFAS within food webs because amphibians are often low in trophic position and are important prey for many aquatic and terrestrial species. In post-metamorphic amphibians, there was no influence of PFAS on survival or mass. However, significant effects on snout-vent length were observed in all species, and body condition differences were observed for two of my species. I found that all leopard frogs increased in scaled mass index (SMI) when exposed to a PFAS treatment, indicating an

increased body condition. Toads exhibited a more variable SMI pattern across treatments, with no outstanding trends, and tiger salamanders did not differ significantly across treatments. These data suggest that sublethal effects vary greatly depending on the species, possibly due to life history traits. Future research examining biomagnification potential is warranted to determine the influence of PFAS on food webs. Additionally, there is a need to determine the physiological mechanisms underlying the observed effects of PFAS exposure.

CHAPTER 1. LARVAL AMPHIBIANS RAPIDLY BIOACCUMULATE PFOA AND PFOS

1.1 Abstract

Poly- and perfluoroalkyl substances (PFAS) are ubiquitous contaminants across the globe, can bioaccumulate in aquatic taxa, and may biomagnify in food webs. Amphibians are particularly vulnerable to contaminants such as PFAS because of their porous skin, associations with aquatic habitats where contaminants accumulate, and sensitivity to endocrine disruptors such as PFAS during their aquatic larval stage. While knowledge of uptake rates is critical for the design of ecotoxicology studies, few studies have explored uptake rates of PFAS in amphibians. I examined uptake rates of two representative and regulated PFAS, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), by larval northern leopard frogs (*Rana pipiens*), American toads (*Anaxyrus americanus*), and eastern tiger salamanders (*Ambystoma tigrinum*) during a 10- d exposure period. Exposure concentrations were 10 and 1,000 µg/L and animals were sampled every 48 h. For each experimental unit and time point, I measured body burden and calculated bioconcentration factor (BCF). I found that for all species and treatments, body burdens often reached steady state within 48 to 96 h of exposure. Among treatments and species, PFOA steady-state body burdens ranged from 3,819–16,481 ng/g dry weight (corresponding BCFs of 0.5 to 2.5), while PFOS body burdens ranged from 6,955–489,958 ng/g dry weight (corresponding BCFs of 47–259). These data suggest that steady state occurs rapidly in larval amphibians exposed to PFAS, particularly for PFOS. This reflects a high potential for trophic transfer of PFAS within food webs because amphibians are often low in trophic position and are important prey for many aquatic and terrestrial species. Future research examining biomagnification potential is warranted for determining the influence of PFAS on food webs.

1.2 Introduction

Poly- and perfluoroalkyl substances (PFAS) are environmentally persistent compounds that have become prevalent globally (De Silva et al., 2016; Houde et al., 2011). Since the 1950s, PFAS have been used in numerous products including lubricants, adhesives, surface protectors, and fire-fighting foams (Bertin et al., 2014; Kissa, 2001). Historically, PFAS were heavily used

because of their ability to repel water and oil, and to resist degradation (Taniyasu et al., 2013). Restrictions on PFAS production have been enacted because of their potential mobility and toxicity (Hoover et al., 2017; U.S. Environmental Protection Agency, 2002, 2015). Despite these restrictions, their resistance to environmental degradation and widespread use has led to their frequent detection in terrestrial and aquatic habitats (Bertin et al., 2014; Houde et al., 2006; Zhu et al., 2014).

There are a growing number of studies documenting PFAS contamination in aquatic systems and several PFAS are of great concern to regulatory agencies because of their persistence and abundance in a variety of ecosystems (Prevedouros et al., 2006). For instance, PFAS levels in lakes in Albany, New York ranged from 0.009 – 0.036 µg/L (Kim and Kannan, 2007). In China, perfluorooctanesulfonic acid (PFOS) varied from 0.144–0.703 µg/L in river water samples (Pan and You, 2010). Near Tyndall Air Force Base close to Panama City, Florida, ground water contaminant levels were as high as 116 µg/L for perfluorooctanoic acid (PFOA), 2,300 µg/L for PFOS, and 920 µg/L for perfluorohexanesulfonic acid (PFHxS; Schultz et al., 2004). Collectively, these studies illustrate that PFAS are found in various environmental settings and can exist without a clear point source. Additionally, longer chain PFAS such as PFOS, PFOA, and PFHxS are generally more bioaccumulative and present in commercial products or terminal microbial metabolites resulting from probable perfluoroalkyl acid precursors leading to a higher frequency of detection in environmental samples (Liu and Avendaño, 2013; Salice, 2018; U.S. Environmental Protection Agency, 2003, 2012).

In addition to the raised awareness of PFAS contamination in aquatic systems, an increasing number of studies are documenting the potential for PFAS to bioaccumulate and potentially biomagnify in aquatic systems (Liu et al., 2018; McCarthy et al., 2017; Post et al., 2012). The majority of this work has focused on fish, as it has direct links to human consumption and potential health effects. Studies on common North American freshwater game fish, such as rainbow trout (*Oncorhynchus mykiss*) and European perch (*Perca fluviatilis*), determined bioaccumulation factors (BAFs) for PFOA, PFOS, and PFHxS with averages ranging from 4.0 to 6,400 (Ahrens et al., 2015; Martin et al., 2009a, 2009b). BAF is an organism's contaminant uptake from water as well as diet. Given this considerable accumulation, there is significant risk to higher trophic levels through biomagnification. For instance, biomagnification has been detected in bald eagles (*Haliaeetus leucocephalus*) and mink (*Mustela vison*) that consumed

contaminated salmon (*Oncorhynchus tshawytscha*) and carp, respectively (*Cyprinus* spp.; Houde et al., 2006; Kannan et al., 2002; Kannan et al., 2005). Collectively, these data indicate that PFAS are likely to bioaccumulate and biomagnify in aquatic species, yet relatively few amphibian species have been examined.

Data examining the bioaccumulation and impacts of PFAS on amphibians are sparse (Ankley et al., 2004; Cui et al., 2018; Hoover et al., 2017; Palmer and Krueger, 2001). Amphibians utilize aquatic and terrestrial habitats throughout their biphasic life cycle. Moreover, they have a high potential for contaminant uptake because of their thin, permeable skin, which is important for respiration and osmoregulation. These characteristics make them a species of environmental concern (Wake and Vredenburg, 2008). Although studies are limited on amphibians, there is evidence for bioaccumulation both in the field and in laboratory studies (Ankley et al., 2004; Cui et al., 2018). In addition, sublethal effects on northern leopard frog growth were confirmed after a 40-d exposure to PFOA, PFOS, PFHxS, and 6:2 fluorotelomer sulfonate (6:2 FTS) at various doses (Hoover et al. 2017).

Given that larval amphibians have thin, permeable skin and respiration occurs across the gills, steady state is expected to occur within a few days of exposure (Boyer and Grue, 1995; Greulich and Pflugmacher, 2004; Hall and Swineford, 1979; Zaga et al., 1998). Faster uptake rates could lead to greater biomagnification rates in food webs or greater toxicity during early developmental stages when larvae are most sensitive to contaminants (Bridges, 2000; Hoi et al., 1998). Amphibians experience hormone-regulated changes during their aquatic larval stage, and are therefore are sensitive to perturbations from endocrine disruptors such as PFAS during this period (Casals-Casas and Desvergne, 2011; Cheng et al., 2011; Hayes et al., 2006). In a previous study, PFAS steady state in amphibians appeared to be attained by the first sampling time of 10 days, but how early steady state was achieved is not known (Hoover 2017). To address this knowledge gap, I conducted experiments to assess PFAS uptake kinetics in larval amphibians during a 10-d exposure. I hypothesized that structural differences between PFOS and PFOA would result in difference in rates and magnitudes of bioaccumulation; PFOS has a longer chain length, and a sulfonate group, while PFOA has a shorter chain length and a carboxylate group. Based on these structural differences and previous studies, I predicted that PFOS would accumulate more rapidly than PFOA, and at higher concentrations (Conder et al., 2008; Liu et al., 2018).

1.3 Materials and methods

Chemicals and stock solution preparation

PFOA (SKU-Pack Size #171468-25G, 96% pure) and PFOS (SKU-Pack Size #77282-10G, ≥ 98% pure) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Primary stock solutions of 1,000-mg/L PFOA and 500 mg/L PFOS were prepared by dissolving PFOA and PFOS in UV-irradiated and filtered well water at room temperature using a stir plate. Complete dissolution took about 2 h for PFOA and 8 h for PFOS. I stored stock solutions in polypropylene (PP) bottles (Thermo Scientific Nalgene Economy Amber Wide-Mouth, Vernon Hills, IL) at room temperature prior to use.

Animal collection

My focal amphibian species were northern leopard frogs (*Rana pipiens*), American toads (*Anaxyrus americanus*), and eastern tiger salamanders (*Ambystoma tigrinum*), which were selected due to their broad distribution in North America. Moreover, results for the selected species should be broadly applicable to other North American amphibian species and study regions because they are representative of three widespread and diverse amphibian families in North America (Amystomatidae, Bufonidae, and Ranidae; Lannoo, 2005; Petranka, 1998). Recently laid egg masses of each species were collected from a temporary pond at the Purdue Wildlife Area (PWA), West Lafayette, IN. I collected tiger salamander eggs (n = 51 masses) early March 2017, leopard frog eggs (n = 9 partial masses) late March 2017, and toad eggs (n = 2 masses) early April 2017. Eggs and larvae of frogs and toads were reared outdoors in 200-L stock tanks filled with 150 L of aged well water and covered with 70% shade cloth to prevent the colonization of invertebrate predators. Larvae were fed TetraMin® Tropical Flakes or rabbit chow (Country Road Rabbit Pellets) *ad libitum* and conducted water changes approximately every 4 d. Initially, tiger salamanders were hatched and reared communally in 200-L tanks in the same conditions as anurans, and fed zooplankton *ad libitum*. Once salamander gape size was large enough to consume tank mates, I transferred them into individual holding containers following the methods of Tornabene and Hoverman (2018) to prevent cannibalism. Exposure experiments were started when tadpoles reached Gosner stage 26−28 (Gosner, 1960) and salamanders reached Harrison stage 46 (Harrison, 1969).

Experimental design and procedures

Experiments were conducted at the Purdue Wildlife Area-Animal Care Facility. The experimental design for each species consisted of a control (0 µg/L) and nominal PFOA and PFOS concentrations of 10 µg/L or 1,000 µg/L (five total treatments). I replicated anuran treatments three times for a total of 15 experimental units per species. The experimental units were 15-L Sterilite® plastic bins filled with 7.5 L of UV-irradiated, filtered well water. I randomly selected and assigned 22 tadpoles from my outdoor animal husbandry tanks to each experimental unit and allowed them to acclimate in control water to laboratory conditions (21°C with a 12:12 photoperiod) for 2 d prior to the start of the experiment. Because salamanders are cannibalistic, I modified the experimental design for this species by housing them individually in 300-mL plastic cups. I replicated each of the five treatments 42 times for a total of 210 experimental units. Salamanders were also randomly selected and assigned to the experimental units and acclimated to laboratory conditions in control water for 2 d prior to the start of the experiment. For each species, I also randomly selected and assigned 10 larvae to a bin to assess any mortality that may have occurred from handling stress. After 24 h, I counted living larvae in this group and euthanized them with a 0.4 g/L solution of buffered MS-222 (tricaine methanesulfonate) buffered with an equal mass of sodium bicarbonate. Survival was 100% in the handling group for all species. Experimental units were dosed with the stock solutions to reach the targeted concentrations. Water renewal was completed after 120 h of exposure, and fresh PFAS solutions were added. I fed TetraMin® to tadpoles and concentrated zooplankton (*Daphnia* spp.) collected from permanent ponds at PWA or black worms (*Lumbriculus variegatus*) to salamanders *ad libitum* daily during the experiment.

Temporal sampling of water and biota was conducted to assess actual exposure concentrations and examine changes in body burdens and bioconcentration factors (BCF) over time. BCF varies from BAF in that it applies to contaminant uptake in aquatic organisms from water, not including diet. Immediately after initial dosing and homogenization of water with PFAS stock solution in the anuran experiments, 2.5 mL of water was sampled from each experimental unit, placed in a 5-mL PP centrifuge tube and stored it at 4°C for later chemical analysis. For salamanders, I pooled water samples from two experimental units per treatment. Water sampling was repeated after approximately 120 h of exposure, immediately prior to a water change. I collected the first animal sample 5 h after the initiation of PFAS exposure

followed by additional animal samples every 48 h until day 10 (the last sampling period occurring at 240 h), when the remaining of the animals were removed. For anurans, I randomly selected three individuals from each experimental unit on each sample date. For salamanders, six experimental units were randomly selected per treatment on each sample date, until the final takedown on day 10. I euthanized, measured snout-vent length (SVL), and weighed (wet body weight) each individual (see Table S1 for treatment means). For anurans, three individual animals were pooled from an experimental unit into a single sample for PFAS analyses (n = 1 per experimental unit per sample date). Since salamanders were much larger, samples were kept as individuals (not pooled). Samples were placed into 5-mL PP centrifuge tubes and stored at -20°C for later body burden analysis.

Analytical procedure

Animal samples were lyophilized and homogenized approximately 48 hours before extracting PFAS analytes from the dried samples. Given the large size of the tiger salamander bodies, chemical analyses were conducted on a subsample after being lyophilized and homogenized. Samples were processed as described in Hoover et al. (2017) following a method modified from Luque et al. (2010). Extracts were analyzed using an automated Shimadzu (Nexera x2) ultra-HPLC (uPLC) with an AB Sciex Quadrupole Time of Flight (QTOF) 5600 mass spectrometer (MS) with a Phenomenex Kinetex 100 × 2.1 mm, 5 µm EVO C18 column. The mobile phase was a gradient of 0.15% acetic acid in water and 20 mM ammonium acetate in methanol. Instrument control was managed via Analyst TF1.7 software and Multiquant 3.0.1 was used to process data and determine body burdens (ng of PFAS /g of dry weight, dw).

For the control and 10 µg/L treatment water samples, I transferred 250 µL of subsamples to a 1.5-mL injection vial. For the 1,000-µg/L treatments, I transferred a 25-µL subsample and diluted it with ultrapure water to a factor of 10. I added 20 µL of an internal standard solution in methanol containing isotope-labeled compounds (250 ng/mL each of M2-6:2 FTS, M3PFHxS, M8PFOS, and M8PFOA) and 230 µL of methanol to each sample prepared, and stored these samples at 4°C prior to analysis. Water samples were then analyzed using the same methods as described for the animal samples. Calibration standards were prepared in 50/50 v/v MeOH/H_2O over a wide range of PFAS concentrations.

Body burden, BCF calculations, and data analysis

I calculated body burden values as the quantified PFOA or PFOS mass divided by dry weight (dw) of larval mass, after correcting measured concentrations for any background PFAS detected in laboratory blanks. I calculated BCF by multiplying dw body burden concentration by average percent dry matter (~0.1 for tadpoles, ~0.09 for salamanders) to calculate wet weight body burdens, then dividing this value by the 0.1 and 120 h water concentration average per species (n=3 per time period). For the water samples from the PFAS treatments, I ran a two-tailed paired t-test to determine if there was a significant change in water concentration between the first day of exposure, and the day of the water change 5 d later.

To examine temporal trends in body burdens changes, I separated my dataset and analyzed by treatment and did not make statistical comparisons between treatments or species. I $\log_{10}$ transformed body burden and BCF data to achieve normality. I used a linear mixed-effects model on each treatment separately (*lme* in *nlme* package; R Development Core Team, 2015) to test for differences in body burden or BCF among days, and included experimental unit as a random factor. If I detected a significant effect of time in the analysis, I used least square means (*lsmeans* in *lsmeans* package; R Development Core Team, 2015) using the Tukey method to determine where the differences occurred, and used compact letter display of pairwise comparisons (*cld* in *multcompView* package; R Development Core Team, 2015) to approximate which day steady state was reached. Finally, I used non-linear least squares regression with a self-starting model (R Development Core Team, 2015) to determine the steady state body burdens and BCFs. Using the calculated asymptote from the models, I back-transformed to determine steady state body burdens and BCFs for each species by chemical and concentration.

1.4 Results

Water Analysis

PFAS water concentrations across all treatments and species were not significantly different between days ($p \geq 0.073$; Table A1.2), and were close to targeted concentrations (Table 1.1). Therefore, I averaged the water concentrations across the sample days for use in BCF calculations among treatments. Nominal degradation was expected, as other studies have found this same trend (Hoover et al., 2017). Control water estimates were below the limit of

quantification (≤ 0.55 µg/L). Thus, I excluded the control animal samples from subsequent statistical analyses.

Body burdens and bioconcentration

PFAS uptake generally slowed after 48 h and steady state was reached between 48 to 144 h of exposure (Figure 1.1). For PFOS, I found that body burden trends were similar over time for all three species within both concentration treatments. Trends in steady state differed between concentrations and among species (Table 1.2, Fig. 1.1). Based on pairwise comparisons, body burdens were significantly lower after 5 h compared to other sample dates in all treatments (Table A1.3). Across all species body burdens for the PFOS 10 µg/L treatment ranged from 6,955 to 11,631 ng/g of dw and BCF ranged from 114 to 259 (Table 1.3, Fig. 1.2). For the PFOS 1,000 µg/L treatment at steady state, body burdens ranged from 336,511 to 489,958 ng/g of dw and BCF ranged from 47 to 70 at steady state. Controls were excluded from analyses because measured concentrations of PFAS in their water were below detection limits.

For PFOA, temporal patterns in body burdens were dependent on species and concentration treatment (Table 1.2, Fig. 1.1). Whole body burdens for the PFOA 10 µg/L treatment tended to increase over time for American toads and tiger salamanders, but leopard frog body burdens were higher at 5 h and 240 h exposure times compared to the other sampling times. Given the lack of a consistent directional trend for the leopard frogs, I was only able to calculate the PFOA steady state body burden for American toads and tiger salamanders (Table 1.3). Body burden varied over time for tiger salamanders and toads in the 1,000 µg/L PFOA treatment, but not for leopard frogs (Table A3.1). Steady state was reached within 5 h of exposure for leopard frogs, and within 48 h for tiger salamanders and toads. At steady state, body burdens ranged from 3,819 to 16,481 ng/g of dw and BCF ranged from 0.46 to 2.48 across all species (Table 1.3, Fig. 1.2). Survival across species and treatments was > 99%.

1.5 Discussion

I examined the uptake dynamics of PFOA and PFOS in larvae of three amphibian species. The uptake of PFAS in larval amphibians was rapid; body burdens were detected in all treatments within 5 h of exposure and steady state was reached within 48 to 96 h of exposure in most treatments, and by 144 h in all treatments, with some variation depending on treatment and

species. This rapid rate of PFAS uptake has not been previously documented in vertebrates. Steady state of PFAS in rainbow trout ranged from 10 to 43 d depending on the specific compound (Martin et al., 2009b). In bluegill (*Lepomis macrochirus*) steady state was still not reached after 62 days of exposure for PFOS (Drottar et al. 2001). While these differences in the uptake and steady state dynamics might be explained by functional differences in the skin, gills, and differences in toxicokinetics between fish and amphibians, additional comparative studies are needed to address the underlying mechanisms. Such data would allow development of more accurate amphibian toxicity reference values for use in ecological risk assessment and models.

For all species, steady state was reached within 48 h for PFOA at 1,000 µg/L, which is much faster than reported uptake rates of PFAS for other taxa elsewhere (Ulhaq et al., 2015). At steady state, I found body burdens and BCFs to be chemical and concentration dependent. Uptake of PFOA was highly variable across species and concentration whereas uptake of PFOS was relatively consistent among species and concentrations. At low concentrations (i.e. 10 µg/L), time to steady state for PFOA was delayed and body burdens were more variable compared to the other treatments. Due to this variability, I was unable to determine steady state BCF calculations for this treatment. I posit that this resulted from low bioaccumulation potential of PFOA coupled with the low concentration in the treatment. However, BCF values tended to be higher for the low concentrations of PFOA compared to the higher dose. Salamanders accumulated higher concentrations of PFOA indicating toxicokinetics differences across species (Beach et al., 2006).

Steady state was reached within 144 h regardless of PFOS concentration, but salamanders had delayed uptake compared to the anuran species. Consistent with previous investigations of PFAS in amphibians, PFOS was 1 to 2 orders of magnitude more bioaccumulative than PFOA (Hoover et al. 2017). For instance, Ankley et al. (2004) found a BCF value of 83.1 after 54 days of exposure to 10 µg/L of PFOS, which is very close to the value I found (70). However, different patterns of bioaccumulation have been found for other aquatic taxa. Bluegills reached a mean BCF of 2,796 following exposure to 86 µg/L of PFOS (Drottar et al., 2001). Additionally, BCFs in European perch ranged from 3200 to 10200 when PFOS exposure ranged from 0.059–0.137 µg/L (Ahrens et al., 2015). These data suggest that BCF can vary substantially among aquatic taxa, and potentially increase with decreased concentration exposure.

Because PFAS are highly soluble in water, can accumulate in soils and sediments, and are persistent in ecosystems, they are likely to persist in wetland environments (Anderson et al., 2016; Zareitalabad et al., 2013). Wetlands provide a number of ecosystem services (Dudgeon et al., 2006) and are significant reservoirs for biodiversity (Dudgeon et al., 2006). Although PFAS are likely deleterious to wetland communities, systematic studies addressing risk to wetland species are sparse (Ahrens and Bundschuh, 2014; Akerblom et al., 2017; Xu et al., 2014; Zhou et al., 2013). My results demonstrated that larval amphibians can rapidly bioaccumulate PFAS. Given that I examined patterns of PFAS uptake in three common and widespread amphibian species, the patterns of rapid accumulation observed are likely to be representative of amphibians across North America. Moreover, uptake rates by amphibians are notably higher compared to other aquatic taxa, which suggests that biomagnification rates could be higher than previously postulated (Ahrens et al., 2015; Martin et al., 2009a, 2009b). For instance, amphibians serve as prey for a broad diversity of aquatic and terrestrial species with varied food web positions, which could enhance biomagnification potential. Understanding biomagnification is of direct interest to humans due to consumption of game species that prey upon amphibians and other lower-trophic-order taxa that uptake PFAS. Moreover, amphibian populations across the globe are declining because of a multitude of anthropogenic stressors. While this trend is primarily due to habitat loss, anthropogenic contaminants also are significant drivers (Wake and Vredenburg, 2008). Research examining the sublethal effects of PFAS exposure on amphibians and the potential for biomagnification is necessary to determine the risk posed to this imperiled group of vertebrates and higher trophic position taxa that prey upon them.

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Table 1.1 Average (n=3) water concentrations (µg/L) detected on days 0 and 5 (prior to water change), by chemical treatment and species. Treatment groups included control (0 µg/L), 10, or 1,000 µg/L of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). The level of quantification (LOQ) was 0.55 µg/L.

Species	Treatment (µg/L)	PFOA water concentration (µg/L)		PFOS water concentration (µg/L)	
		Day 0	Day 5	Day 0	Day 5
Leopard frog	0	< LOQ	< LOQ	< LOQ	< LOQ
	10	10.92	11.22	6.35	5.84
	1,000	780.16	949.93	691.59	703.51
Tiger salamander	0	< LOQ	< LOQ	< LOQ	< LOQ
	10	12.46	16.71	3.72	3.43
	1,000	684.44	659.37	589.42	656.26
American toad	0	< LOQ	< LOQ	< LOQ	< LOQ
	10	9.26	10.96	4.28	4.54
	1,000	1,288	855.54	771.52	610.75

Table 1.2 Results of linear mixed-effects model examining the response variable body burden as a function of day for each chemical treatment, for each species. A significant p-value indicates a significant relationship between the variables body burden and day. Analyses were separated into treatment groups, of either 10 or 1,000 µg/L of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). Experimental unit was included as a random factor in the analyses.

Species	Treatment (µg/L)	PFOA			PFOS		
		t	df	*P*	*t*	df	*P*
Leopard frog	10	0.323	14	0.751	3.991	14	0.001
	1,000	-1.313	14	0.210	3.604	14	0.003
Tiger salamander	10	4.967	32	< 0.001	7.928	32	<0.001
	1,000	2.978	32	0.006	6.655	30	<0.001
American toad	10	3.869	14	0.002	4.506	14	<0.001
	1,000	2.029	14	0.062	2.724	14	0.017

Table 1.3 Body burdens (ng/g dry weight) and bioconcentration factors (BCF) calculated at steady state for each chemical treatment for each species. Treatment groups were 10 µg/L or 1,000 µg/L of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). Some PFOA steady state values could not be calculated (N/A) due to an inability to determine an asymptote for steady state.

Species	Treatment (µg/L)	PFOA		PFOS	
		Body Burden	BCF	Body Burden	BCF
Leopard frog	10	N/A	N/A	6,955	114
	1,000	3,819	0.46	489,958	70
Tiger salamander	10	6,548	N/A	9,682	230
	1,000	16,481	2.5	336,511	47
American toad	10	6,868	N/A	11,631	259
	1,000	7,713	0.87	448,125	65

N/A = data not available

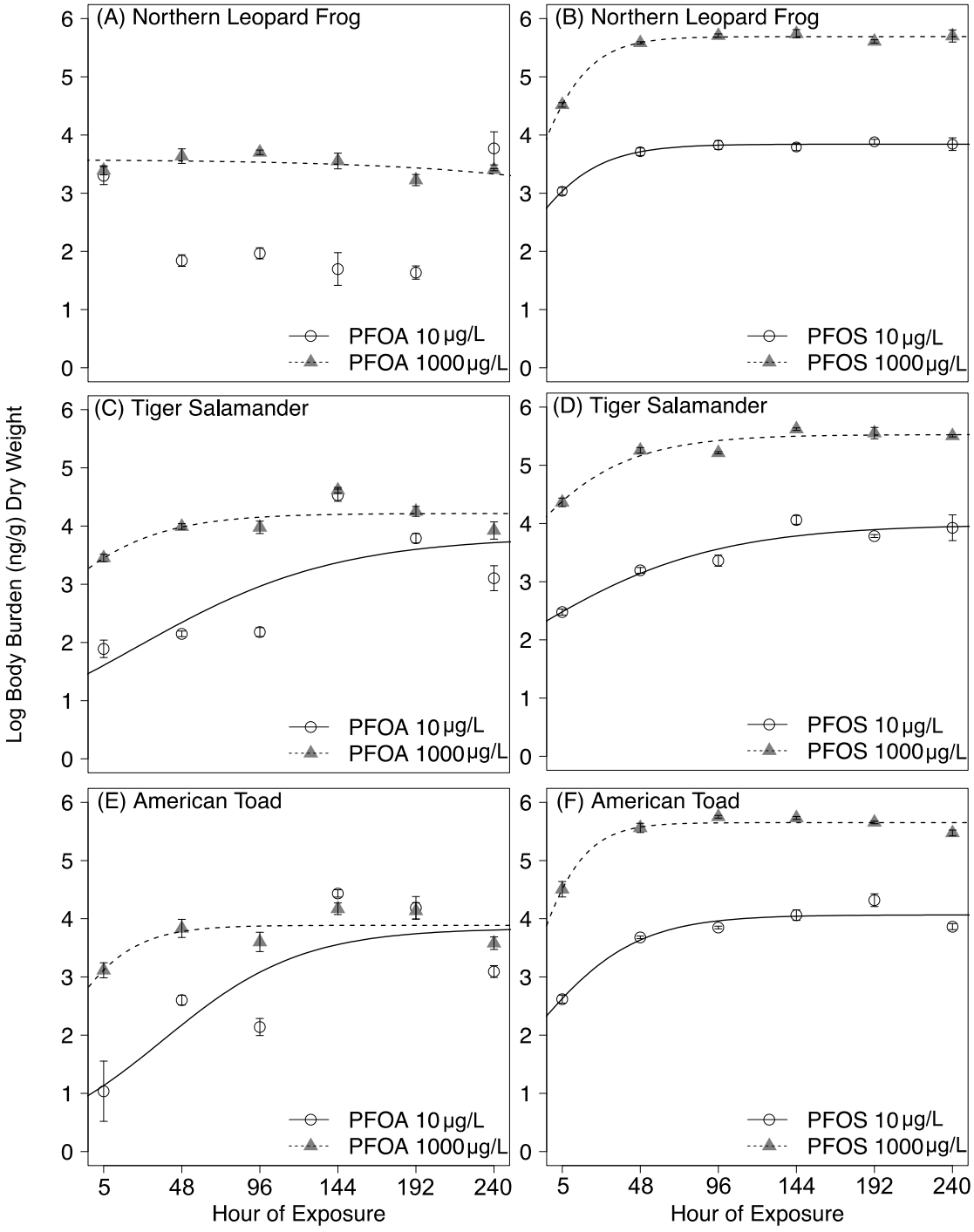

Figure 1.1 Log body burdens (mean ± 1 SE) of northern leopard frogs (A and B), eastern tiger salamanders (C and D), and American toads (E and F) after exposure to perfluorooctanoic acid (PFOA) or perfluorooctane sulfonate (PFOS) at 10 or 1,000 µg/L over time. The solid or broken line represents the calculated body burden contaminant uptake, and subsequent steady state (if applicable) for each treatment.

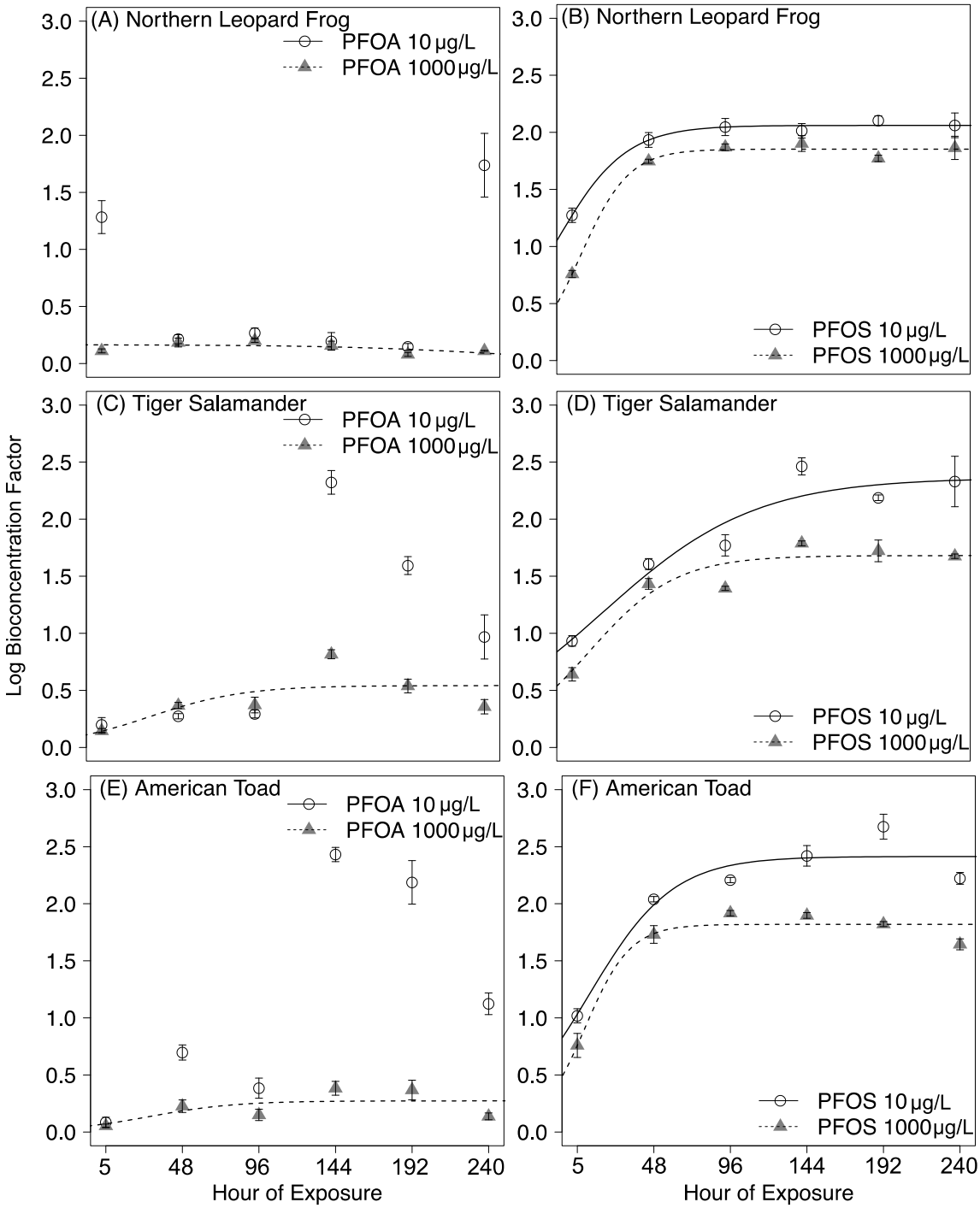

Figure 1.2 Log bioconcentration factors (mean ± 1 SE) for northern leopard frogs (A and B), eastern tiger salamanders (C and D), and American toads (E and F) after exposure to perfluorooctanoic acid (PFOA) or perfluorooctane sulfonate (PFOS) at 10 or 1,000 µg/L over time. Solid and broken lines represent the estimated contaminant bioconcentration factor, and the subsequent bioconcentration factor at steady state (if applicable) for each treatment is indicated by the line asymptote.

CHAPTER 2. THE EFFECTS OF DERMAL EXPOSURE TO POLY- AND PERFLUOROALKYL SUBSTANCES ON POST- METAMORPHIC AMPHIBIANS

2.1 Abstract

Poly- and perfluoroalkyl substances (PFAS) are distributed throughout ecosystems globally and have been targeted for regulation due to their persistence in the environment, widespread accumulation in both humans and wildlife, and potential for a variety of adverse effects. While PFAS toxicity has been examined in all vertebrate taxa, research on amphibians is limited. I examined the effects of PFAS exposure via contaminated sphagnum moss substrate on the survival and growth of juvenile American toads (*Anaxyrus americanus*), eastern tiger salamanders (*Ambystoma tigrinum*) and northern leopard frogs (*Rana pipiens*). 30 day treatments included perfluorooctanoic acid (PFOA), perfluoroocatane sulfonate (PFOS), perfluorohexane sulfonate (PFHxS), and 6:2 fluorotelomer sulfonate (6:2 FTS) at concentrations of 10, 100, and 1000 µg/L, and a control treatment. I uniquely identified individuals to assess percent change in mass and snout-vent length (SVL) over the 30-d experiment. While there was no influence of PFAS on survival or mass, I found significant effects on SVL. Salamanders exposed to PFAS generally exhibited a 10 to 37% percent increase in SVL compared to the control, while frogs and toads displayed the opposite pattern. Effects on SVL growth were strongest with exposure to 6:2 FTS and weakest with PFOA. There was limited evidence for a dose-response relationship for the PFAS. I also applied a scaled mass index (SMI) to evaluate relative body condition. In frogs, SMI was significantly greater with 6:2 FTS and PFHxS exposure compared to the control. There were limited effects on SMI in salamander and toads. While additional research is needed to determine the mechanisms underlying the contrasting effects of PFAS on salamanders and anurans, my work demonstrates that PFAS can have sublethal effects on amphibians, with effects dependent on species, chemical, and trait, but not concentration.

2.2 Introduction

Chemical contaminants including heavy metals, salts, nutrients, and pesticides are a pervasive challenge associated with human activities (Carson, 1962; Flynn et al., 2015; Lance et

al., 2012; Willson and Hopkins, 2013). Many chemical contaminants are complex compounds that are persistent in the environment, mobile, and bioaccumuative, which can adversely affect wildlife populations, communities, and ecosystems (Bickham et al., 2000; Boone et al., 2007; Stoler et al., 2017). While it is broadly recognized that chemical contaminants can have a variety of direct and indirect effects on ecological communities and ecosystems (Fleeger et al., 2003; Hua and Relyea, 2014; Lance et al., 2013; Relyea, 2005), the rapid pace of technological advances often limits out ability to assess potential environmental impacts prior to widespread usage.

A core objective of ecotoxicology is to understand the lethal and sublethal effects of chemical contaminants on wildlife (Rand, 1995). Given that most environmental exposures to chemical contaminants are expected to be below levels known to be directly toxic to wildlife, research has increasingly focused on the broad range of sublethal effects that can follow exposure (Köhler and Triebskorn, 2013). For instance, chemical exposure can reduce growth and development, alter behavior, or impair immune function (Gardner and Oberdorster, 2016; Lance et al., 2012; Svartz et al., 2015). These effects have been observed with contaminants such as pesticides, and heavy metals (Boening, 2000; Colborn et al., 1993; Grue et al., 1997; Watson et al., 2009). Moreover, this research has resulted in regulatory action to limit or prevent the negative effect of these contaminants on wildlife (Environmental Protection Agency, 1972). Despite this research, there is a paucity of information on sublethal effects for many emerging contaminants in nature. Thus, there is a need to understand the effects of emerging contaminants on wildlife so that populations can be monitored and regulatory actions implemented if proven harmful.

Poly- and perfluoroalkyl substances (PFAS) are a group of emerging contaminants of concern across the globe. Historically, PFAS were commonly found in a variety of substances including aqueous film forming foams (AFFFs), commercial household products, and manufacturing waste (U.S. Environmental Protection Agency, 2002, 2015). Although regulations have reduced PFAS production (U.S. Environmental Protection Agency, 2015), decades of use have led to their widespread distribution in the environment and associated risks for human and wildlife. Indeed, significant accumulation has been detected in both humans and wildlife raising concern over the potential adverse effects of PFAS (Beach et al., 2006; Colborn et al., 1993; U.S. Environmental Protection Agency, 2015). For instance, recent studies have found that PFAS can

function as endocrine disruptors capable of altering the hypothalamus-pituitary-thyroid (HPT) axis of vertebrates (Lau et al., 2007; Lopez-Espinosa et al., 2012). Other adverse effects linked to PFAS exposure include liver lesions, growth suppression, embryo deformities, and reduced offspring survival (Custer et al., 2014; Scheringer et al., 2014; Sonne et al., 2008; Wang et al., 2011). Despite accumulating evidence for the adverse effects of PFAS exposure, relatively few taxa have been examined.

Amphibians provide an ideal model system for examining the potential adverse effects of PFAS exposure. Amphibians are associated with both aquatic and terrestrial environments due to their biphasic life cycle. Because PFAS are water soluble and bind to sediments, the risk of PFAS exposure is high for amphibians across these environments (Ahrens et al., 2010; Higgins and Luthy, 2006; Houde et al., 2006). Additionally, in freshwater aquatic systems such as rivers and wetlands, organisms are often more sedentary, therefore they may have a higher risk of exposure to PFAS if located near a source of contamination (Boening, 1999; Custer et al., 2014; Teixeira et al., 2009). This can then lead to a more chronic exposure, and potentially increase sublethal impacts (Hontela et al., 1995). However, the effects of contaminants in the sediment surrounding these water systems are largely unknown (Ankley et al., 2004; McCarthy et al., 2017). Amphibians also have thin, permeable skin, which makes them susceptible to contaminants (Wake and Vredenburg, 2008). PFAS are known endocrine disruptors and previous studies with other endocrine disruptors contaminants have document sublethal effects on development, growth, and survivorship (Carr and Patiño, 2011; Hayes et al., 2006). Thus, it is possible that PFAS will have similar effects on amphibians.

My objectives were to compare sublethal effects of different PFAS treatments on post-metamorphic amphibians, when dermally exposed via dosed sphagnum moss substrate, which served as a pseudo-sediment, but with homogeneous properties. I addressed this objective by conducting laboratory experiments that exposed post-metamorphic individuals of three amphibian species to PFAS contaminated substrate. My focal species were northern leopard frogs (*Rana pipiens*), American toads (*Anaxyrus americanus*), and eastern tiger salamanders (*Ambystoma tigrinum*). I chose these three species because they are broadly distributed in North America. Moreover, my results should be broadly applicable to other North American amphibian species and study regions, as they are representative of three widespread and diverse amphibian families in North America (Amystomatidae, Bufonidae, and Ranidae; Lannoo, 2005; Petranka,

1998). I chose four environmentally relevant PFAS for these dermal exposures: perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorohexane sulfonate (PFHxS), and 6:2 fluorotelomer sulfonate (6:2 FTS). These chemicals are some of the most common PFAS found in the environment (U.S. Environmental Protection Agency, 2012). Additionally, these compounds vary in their chain lengths, and have either a carboxylic or sulfonate group. Because PFAS are endocrine disruptors, I hypothesized a dose-response relationship such that higher chemical concentrations will be associated with greater reductions in growth for each chemical, due to the additional stress on the organism. Additionally, I predicted stronger effects of PFOS compared to PFOA, PFHxS, and 6:2 FTS because it is more bioaccumulative (Hoover et al., 2017; Liu et al., 2018; Pi et al., 2017).

2.3 Materials and Methods

Metamorph production

In spring 2017, I collected egg masses of my three focal species from a temporary pond at the Purdue Wildlife Area, West Lafayette, IN. I collected eastern tiger salamander eggs (n = 276 masses) early March 2017; northern leopard frog eggs (n = 18 partial masses) late February to early March 2017; and America toad eggs (n = 24 masses) early April 2017. I initially housed eggs in 200-L stock tanks filled with 150 L of aged well water under common garden conditions, covered with 70% shade cloth to prevent the colonization of invertebrate predators. I fed anurans TetraMin® Tropical Flakes or rabbit chow (Country Road Rabbit Pellets) *ad libitum* with water changes approximately every 4 d. I fed salamanders zooplankton or brine shrimp (*Artemia* spp.) *ad libitum*.

When anuran tadpoles reached Gosner stage 26 (Gosner, 1960) I moved them to 40 covered mesocosms to generate metamorphs. The mesocosms were 1,200-L cattle tanks filled with 1,000 L of well water. To each tank, I added 150 g dry leaf litter (primarily *Quercus* spp.) to serve as refuges and 30 g of rabbit chow to provide an initial nutrient source. I then added 1 L of pond water containing phytoplankton and periphyton. The algal community was established for 1 week before adding 120 mL of dense zooplankton in pond water. I then stocked each mesocosm with 20 American toads and 20 northern leopard frogs. I monitored mesocosms daily for metamorphosis, and removed metamorphs and housed them in plastic husbandry tanks with wet sphagnum moss substrate, separated by species, until used in the experiments. During this

husbandry period, I fed American toads a mix of fruit flies (*Drosophila* spp.) and crickets (*Gryllodes sigillatus*) while feeding northern leopard frogs only crickets.

Once the gape size of tiger salamanders was large enough to consume tank mates, I transferred them into individual holding containers following the methods of Tornabene and Hoverman (2018) to prevent cannibalism. I fed salamanders tadpoles (*Anaxyrus americanus*) and black worms (*Lumbriculus variegatus*) *ad libitum* daily during this period. After approximately 2 months, individuals were large enough that cannibalism was no longer a concern. Thus, I moved them into covered 200-L stock tanks filled with 150 L of aged well water. I stocked the tanks at densities of ≤ 30 salamander larvae and fed them *ad libitum* daily. When noticeable gill resorption began, I relocated individuals into covered 200-L stock tanks filled with 75 L of aged well water. I angled tanks on an incline to provide dry land access and promote total gill resorption. Once gills were resorbed, I moved animals to plastic tanks with wet sphagnum moss substrate. I then fed salamanders crickets until used in the experiment.

Chemicals and stock solution preparation

I purchased PFOA (SKU-Pack Size #171468-25G, 96% pure), PFOS (SKU-Pack Size #77282-10G, ≥ 98% pure), and PFHxS (SKU-Pack Size #50929-10G-F, ≥ 98% pure) from Sigma-Aldrich (St. Louis, MO, USA) and 6:2 FTS (SKU-Pack Size #6164-3-06, 98% pure) from SynQuest Laboratories for use in the experiment. I created primary stock solutions (200 to 1000 mg/L) by dissolving the chemicals (0.2 to 1 g) into 1 L of UV-irradiated filtered well water. I mixed the solutions on a stir plate until the chemicals dissolved (2 hr for PFOA and PFHxS, and 8 h for PFOS and 6:2 FTS). For 6:2 FTS, I used vacuum-filtration (Whatman, ashless, grade 40) following mixing to remove insoluble solids that remained. I stored stock solutions in polypropylene (PP) bottles at room temperature until used in the experiments.

Experimental design and procedures

I conducted experiments in the laboratory at the Purdue Wildlife Area-Animal Care Facility. For each species, the experimental design consisted of a control and exposure to PFOA, PFOS, PFHxS, or 6:2 FTS at 10 ppb, 100 ppb, or 1000 ppb. I replicated these 13 treatments 4 times for a total of 52 experimental units per species. I used 15-L plastic bins as experimental units for toads and salamanders, and 68-L plastic bins for frogs. Larger bins were used for leopard frogs to prevent escape during feedings. I filled the units with rehydrated sphagnum

moss to a depth of 5 cm for anurans and 10 cm for salamanders. Salamander substrate depth was deeper due to their fossorial nature. For each species, I homogenized all individuals from the husbandry tanks and then randomly selected individuals for use in the experiment. I placed 12 individuals into each experimental unit and allowed them to acclimate to the housing environment and indoor conditions of approximately 22°C and a photoperiod of 12:12 L:D for one week prior to chemical dosing. At this point, I began feeding each experimental unit 24 appropriately sized crickets (average 2 per animal; 2.54 cm crickets for salamanders, 1.27 cm crickets for leopard frogs, and 0.64 cm crickets for toads) on a MWF schedule. I also randomly assigned 12 animals to a 24-h handling mortality group; survival was a 100% for all species.

To track individual patterns in growth during the experiments, I uniquely marked northern leopard frogs and American toads in the experimental units using toe clippings (Green, 2001), and uniquely marked tiger salamanders with Visible Implant Elastomer (VIE) tags (Northwest Marine Technology, Inc., Shaw Island, Washington, USA; Ferner, 2007) within the week between unit assignment and chemical exposure.

To begin the chemical exposures, I removed animals from the experimental units, placed them in temporary holding tanks, and removed the husbandry substrate. Then, I diluted the primary stock solution to the desired concentrations and added this secondary solution to pre-measured sphagnum moss to dose the experimental units. This dosed substrate was homogenized throughout the experimental unit prior to the replacement of animals, and then was not moved for the duration of the experiment for anurans, but flipped top to bottom for tiger salamanders after 5 days, to ensure homogeneity with the additional moss depth. I took surface substrate samples from 4 sites within each bin to compose one sample, at the time of dosing and at day 30 to determine initial exposure levels and possible chemical degradation, respectively. I filled and stored substrate samples in 5-mL PP centrifuge tubes at -20°C until processed.

After 30 days of exposure, I euthanized all animals with a 5.0 g/L solution of buffered MS-222 (tricaine methanesulfonate). I immediately weighed and measured the snout-vent length (SVL) of each individual (Table A2.1). I calculated the percent change ([final – initial] / initial * 100) in mass and SVL during the experiment for each individual because there was some variation across treatments at the start of the experiments (Table A2.2). This percent change in mass and percent change in SVL were used as two of my response variables. I froze whole anurans at -20°C for body burden and BCF analysis. Due to the large size of the salamanders, I

removed their livers and froze livers and carcasses separately at -20°C. I then utilized the livers for chemical analysis.

Calculation of scaled mass index

While there are several different body condition indices, I used the scaled mass index which reduces bias in estimates and has proven to be a reliable indicator of condition (Peig and Green, 2010). Following the methods of Peig and Green (2009), I conducted standardized major axis (SMA) regression of ln body mass on ln SVL to obtain the scaling exponent. This was done for each species using all individuals within the respectively datasets (northern leopard frog = 2.96, American toad = 0.95, tiger salamander = 2.92). I also calculated the mean SVL for each species (37.89 mm for northern leopard frogs, 18.21 mm for American toads, and 67.03 mm for tiger salamanders) across all individuals in the dataset, giving me a population mean. To calculate each individual's scaled mass index I used these values (Peig and Green, 2009).

Analytical procedure

I lyophilized and homogenized animal samples for approximately 48 hours before extracting PFAS analytes from the dried samples. Because leopard frogs and sediment samples were large, I homogenized them before extraction, while whole bodies could be extracted for toads. I tested tiger salamander livers for homogeneity by sectioning a liver into 11 parts and extracting these sections separately. Once homogeneity was confirmed, I used whole livers for extraction for all treatments except PFOS 1,000 µg/L treatment, where I used a liver subsample due to the high concentrations expected in the livers. . Samples were processed as described in Hoover et al. (2017), which followed a modified method from Luque et al. (2010). I then analyzed these processed samples using an automated Shimadzu (Nexera x2) ultra-HPLC (uPLC) with an AB Sciex Quadrupole Time of Flight (QTOF) 5600 mass spectrometer (MS). The mobile phase was a gradient of 0.15% acetic acid in water and 20 mM ammonium acetate in methanol. Instrument control was managed via Analyst TF1.7 software for the 5600+. I used Multiquant 3.0.1 to process this data and determine body burden (ng of PFAS /g dry weight).

Data analysis

For each species, my response variables were proportion surviving, percent change in mass, percent change in SVL, and scaled mass index. All variables met normality assumptions

for statistical analyses. To analyze proportion surviving, I used a linear model with treatment as a fixed factor, to test for treatment effects on the proportion of individuals surviving in an experimental unit. For the other response variables, I used linear mixed-effects models to examine treatment effects (*lme* in *nlme* package; R Development Core Team, 2015). I nested observations within experimental units from which individuals were sampled and included experimental unit as a random effect, to account for dependence among individuals from the same tubs (Zuur et al., 2009). If the overall test was significant, I used the linear mixed-effects model outputs to compute the least squares means (*lsmeans* in *lsmeans* package) and conducted linear contrasts to compare treatments. These contrasts addressed whether there was a dose-response relationship for each chemical and whether there were differences among the chemical treatments averaged across doses. I adjusted p-values using the Bonferroni method to control for type I error, since multiple tests were conducted on the same dataset.

2.4 Results

Survival was >99% for each species, with no significant treatment effects ($F_{12,39} \leq 1.55$, $P \geq 0.148$). Additionally, there were no significant treatment effects on percent change in mass (Table 2.1, Fig. 2.1). However, the percent change in SVL for all three species was significantly affected by PFAS exposure (Table 2.1, Fig. 2.2) but there was no evidence of dose-response relationships for the chemicals ($P \geq 0.986$). Based on these results, I pooled my concentrations within a chemical to conduct contrasts across chemicals. There were differences in percent change of SVL among the five chemical treatments, with trends varying by species (Table 2.2). In leopard frogs, percent change in SVL was 35 to 49% lower in the PFOS, PFHxS, and 6:2 FTS treatments compared to the control whereas no significant difference was observed between the control and PFOA treatment (Fig. 2.2). Percent change in SVL was 34 to 36% lower in the PFHxS and 6:2 FTS treatments compared to the PFOA treatment. There was no significant difference between the PFHxS and 6:2 FTS treatment. In American toads, percent change in SVL was 109 to 137% lower in the PFOS and 6:2 FTS treatments compared to the control. However, there were no significant differences among the control, PFHxS, and PFOA treatments. Percent change in SVL was 242 to 304% lower in the PFOS and 6:2 FTS treatments compared to the PFOA treatment. In tiger salamanders, percent change in SVL was 59% higher in the 6:2 FTS treatment compared to the control, but no significant differences among the control, PFOS,

PFOA, and PFHxS treatments. Percent change in SVL was 32 to 43% higher in the 6:2 FTS treatment compared to the PFOA and PFOS treatments. All remaining comparisons that were not mentioned specifically were not significant at α =0.95 (Fig. 2.2).

In my analysis of scaled mass index (SMI), I found significant treatment effects in northern leopard frogs and near significant effects in American toads, but no effects in tiger salamanders (Table 2.1, Fig. 2.3). Thus, I focused on leopard frogs and American toads for my linear contrasts. There was no evidence of dose-response relationships for any of the chemicals for either species (P = 1.000). Thus, I pooled the concentrations within a chemical to conduct contrasts across chemicals. Similar to percent change in SVL, there were significant differences in the SMI across the five chemical treatments, with trends varying between leopard frogs and American toads (Table 2.3, Fig. 2.3). In leopard frogs, SMI was 6 to 14% higher in the PFHxS and 6:2 FTS treatments compared to the control, PFOA, and PFOS treatments. There was no difference between the PFHxS and 6:2 FTS treatments or among the control, PFOA, and PFOS treatments. In American toads, SMI was 8 to 9% higher in the PFOS treatment compared to the PFHxS and 6:2 FTS treatments. There were no other differences detected.

2.5 Discussion

While previous studies have documented sublethal effects of PFAS on larval amphibians (Hoover et al., 2017) this is the first study to document sublethal effects on post-metamorphic amphibians. Effects were dependent on species, chemical, and trait, but not concentration. Across all three species, I found evidence that PFAS exposure influenced the percent change in SVL but not mass. These results suggest that PFAS exposure influenced resource allocation to morphology rather than resource acquisition or assimilation. However, a complicating factor in interpreting my results is the fact that opposing patterns were seen between anurans and salamanders; PFAS exposure increased percent change in SVL in salamanders while decreasing it in anurans. Given the physiological similarities among amphibian species, it is unclear why the direction of response would differ between salamanders and anurans. However, it is possible that responses could vary by order. For instance, when exposed to other contaminants such as Roundup, amphibian species responses vary by order (Egea‐Serrano et al., 2012; Relyea, 2004; Relyea and Jones, 2009). Consequently, the endocrine disruption may be impacting these

different species differently, but more comprehensive thyroid and histological studies would need to be conducted. Such studies may be able to decipher these species differences, even within taxa. Additionally, future research should explore various other anuran and salamander species across multiple genera to determine whether there are phylogenetic trends in sublethal effects to PFAS.

Despite the lack of effects on percent change in mass with PFAS exposure, I did observe significant increases in the SMI, a measure of body condition, in leopard frogs and American toads. Previous studies have shown that chemical contaminants can increase relative mass, typically measured at metamorphosis (Boone et al., 2007; Gutleb et al., 2000; Relyea, 2009; Yahnke et al., 2013). In my study, a greater SMI may be an indication of an illness (e.g., obesity) or be a mechanism of compensating for stress (Salleh, 2008). For instance, exposure to endocrine disrupting contaminants such as diethylstilbestrol, bisphenol A, phytoestrogens, phthalates, and organotins have been linked to obesity in humans and lab mice (Desvergne et al., 2009; Newbold et al., 2008; Newbold, 2010;). Endocrine disruption is known to have negative impacts on amphibians, due to the reliance on thyroid hormone during metamorphosis (Hayes et al., 2006; Legler, 2008). Polychlorinated biphenyls, an endocrine disruptor, has been shown to increase mass after both short term (10 d) and chronic exposures (end of metamorphosis) to amphibians (Gutleb et al., 2000). However, many studies that analyze endocrine disrupting chemicals in amphibians find that amphibians are typically reduced in size at metamorphosis (Rohr and McCoy, 2009). Given the endocrine disrupting potential of PFAS, but variable trends in endocrine disruption effects, future work should focus on quantifying the hormonal and physiological effects of PFAS exposure on juvenile and adult amphibians to determine the mechanisms underlying changes in body condition.

Over the course of my experiment, American toads tended to lose mass. Given their small size, this can be attributed to their inefficiency at capturing prey. Toads metamorphose at extremely small sizes, which contributed to their difficulty in capturing prey items. In addition to reductions in mass, I also found reductions in SVL for toads. However, I only observed reductions in SVL in the PFAS exposure treatments. Previous field studies have documented apparent shrinkage (i.e. reductions in length between measurements) in toads (Raney and Lachner, 1947), yet this appears to be the first evidence that such reductions can be mediated by chemical exposure.

While I observed significant variation among the PFAS tested in the experiment, the responses were not as predicted. For instance, I expected stronger negative effects with PFOS because it is known to be more bioaccumulative and toxic. However, I only found evidence of significant effects of PFOS in a single analysis case (SMI in American toads). Given the strong sorption tendencies of PFOS to sediments, uptake in my species may have been lower than anticipated (Higgins and Luthy, 2006; Zareitalabad et al., 2013). Interestingly, 6:2 FTS was often associated with strong sublethal effects, despite limited bioaccumulation potential (Hoke et al. 2015, Hoover et al. 2017). A recent study in larval tiger salamanders also found significant effects of 6:2 FTS on body condition (Hoover, 2018). Thus, 6:2 FTS should be investigated more thoroughly to determine what is driving its toxicity. I found limited evidence for sublethal effects of PFOA, which reinforces work demonstrating that PFOA is not as toxic as other compounds in this chemical group (Joung et al., 2010; Ulhaq et al., 2013; Zareitalabad et al., 2013; Zhang et al., 2013; Zheng et al., 2012). PFHxS exhibited moderate sublethal effects, but it was never the chemical with the greatest effect. This was expected because the C6 chemical is thought to be less toxic than C8 PFAS (Organization for Economic Co-operation and Development, 2013). Ultimately, variation in the sublethal effects induced by different PFAS could be mediated by their differential effects on the endocrine system. As such, future research should focus on conducting thyroid hormone assays after exposure to assess the underlying mechanisms of PFAS effects on amphibians.

In conclusion, my study demonstrates that PFAS have sublethal effects on post-metamorphic amphibians. Previous PFAS studies with larval amphibians have also documented negative effects (Ankley et al., 2004; Hoover et al., 2017; Palmer and Krueger, 2001). Collectively, this research underscores that PFAS can affect both aquatic and terrestrial life stages of amphibians. Future studies should examine exposure throughout the life cycle, from larvae through adults, to determine the long-term effects of PFAS exposure on amphibian fitness. In particular, studies that examine survival through reproduction, time of maturation, reproductive output, and maternal transfer of PFAS are critical next steps in assessing sublethal effects of PFAS on amphibians.

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Table 2.1 Results from linear mixed-effects models examining the effects of PFAS treatments on percent change in snout-vent length (SVL), percent change in mass, and scaled mass index of northern leopard frogs, American toads, and eastern tiger salamanders. Experimental unit was included as a random factor in the analyses with individuals nested within experimental units.

	% change mass			% change SVL			Scaled mass index		
	df	F-value	*P*	df	F-value	*P*	df	F-value	*P*
Northern leopard frog	12, 39	0.76	0.690	12, 39	6.41	<0.001	12, 39	5.30	<0.001
American toad	12, 39	0.63	0.800	12, 39	4.15	<0.001	12, 39	1.83	0.077
Eastern tiger salamander	12, 39	0.39	0.960	12, 39	2.49	0.016	12, 39	0.95	0.512

Table 2.2 Results from linear contrasts examining differences in percent change in snout-vent length (SVL) among chemical treatments within a species. Treatment abbreviations are: C=control, A=PFOA, S=PFOS, X=PFHxS, F=6:2 FTS. For the analysis, I pooled the three concentrations for each PFAS because there was no evidence of a dose-response relationship.

Treatment contrasts	Northern leopard frog		American toad		Tiger salamander	
	t ratio	*P*	t ratio	*P*	t ratio	*P*
C-A	2.55	0.324	1.04	1.000	-0.68	1.000
C-S	4.41	0.002	3.94	0.007	-1.27	1.000
C-X	5.98	<0.001	2.69	0.230	-1.82	1.000
C-F	6.12	<0.001	4.47	0.001	-3.69	0.015
A-S	-2.63	0.265	-4.10	0.005	0.818	1.000
A-X	-4.84	<0.001	-2.34	0.538	1.59	1.000
A-F	-5.05	<0.001	-4.86	<0.001	4.20	0.003
S-X	-2.20	0.752	1.77	1.000	0.78	1.000
S-F	-2.40	0.468	-0.75	1.000	3.42	0.033
X-F	-0.21	1.00	-2.52	0.351	2.64	0.260

Table 2.3 Results from linear contrasts examining differences in scaled mass index among chemical treatments. Treatment abbreviations are: C=control, A=PFOA, S=PFOS, X=PFHxS, F=6:2 FTS. For the analysis, I pooled the three concentrations for each PFAS because there was no evidence of a dose-response relationship. Tiger salamanders were excluded from the comparisons because the overall test of treatment effects was not significant.

Treatment contrasts	Northern leopard frog		American toad	
	t ratio	*p*	t ratio	*p*
C-A	-2.82	0.167	0.93	1.000
C-S	-2.65	0.252	-1.27	1.000
C-X	-5.58	<0.001	1.35	1.000
C-F	-5.54	<0.001	1.11	1.000
A-S	-0.23	1.000	3.10	0.079
A-X	3.90	0.008	-0.60	1.000
A-F	3.86	0.009	-0.25	1.000
S-X	4.12	0.004	-3.69	0.015
S-F	4.07	0.005	-3.35	0.040
X-F	-0.04	1.000	0.35	1.000

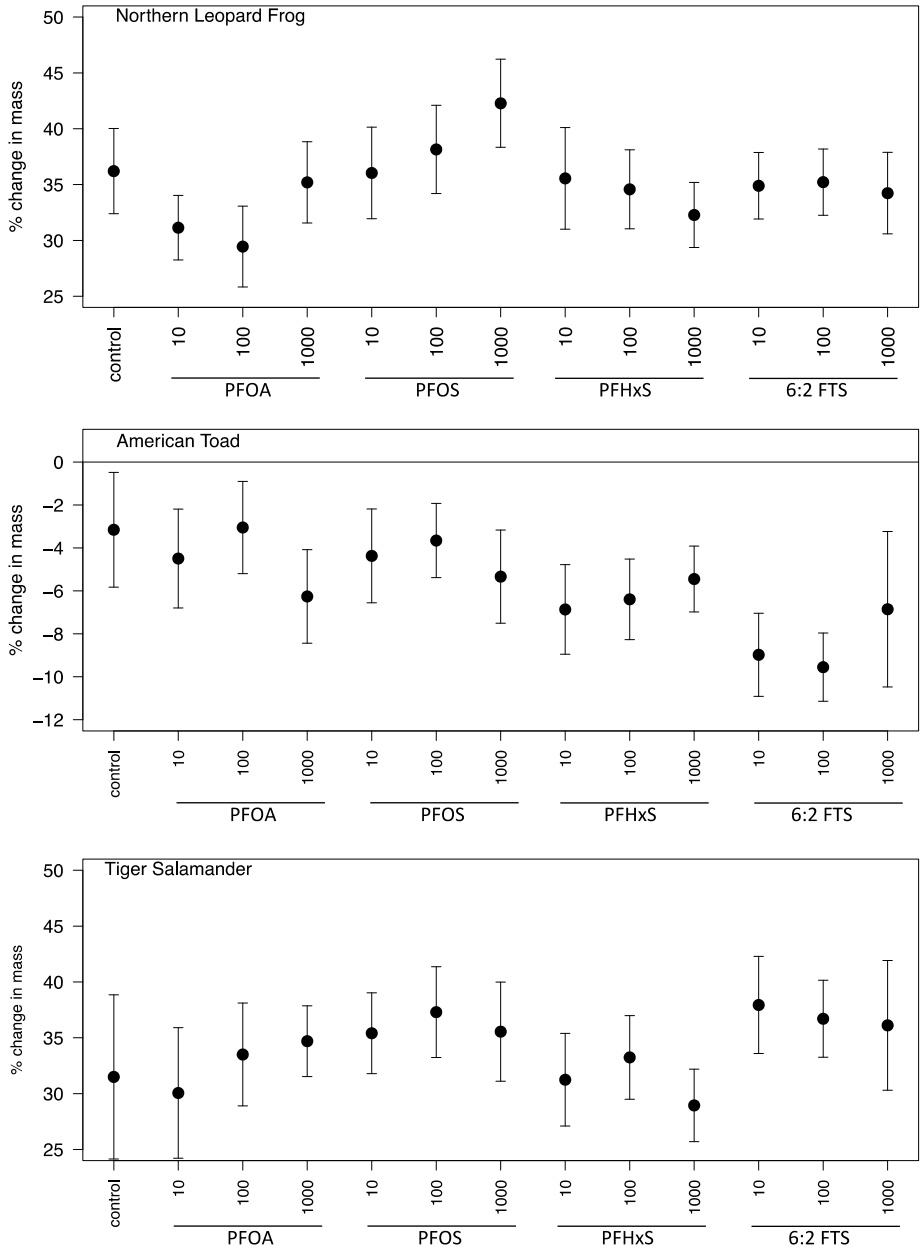

Figure 2.1 Effects of PFAS exposure via contaminated substrate on the percent change in mass of post-metamorphic northern leopard frogs, American toads, and eastern tiger salamanders. Data are means ± SE. No significant differences were observed between groups within a species.

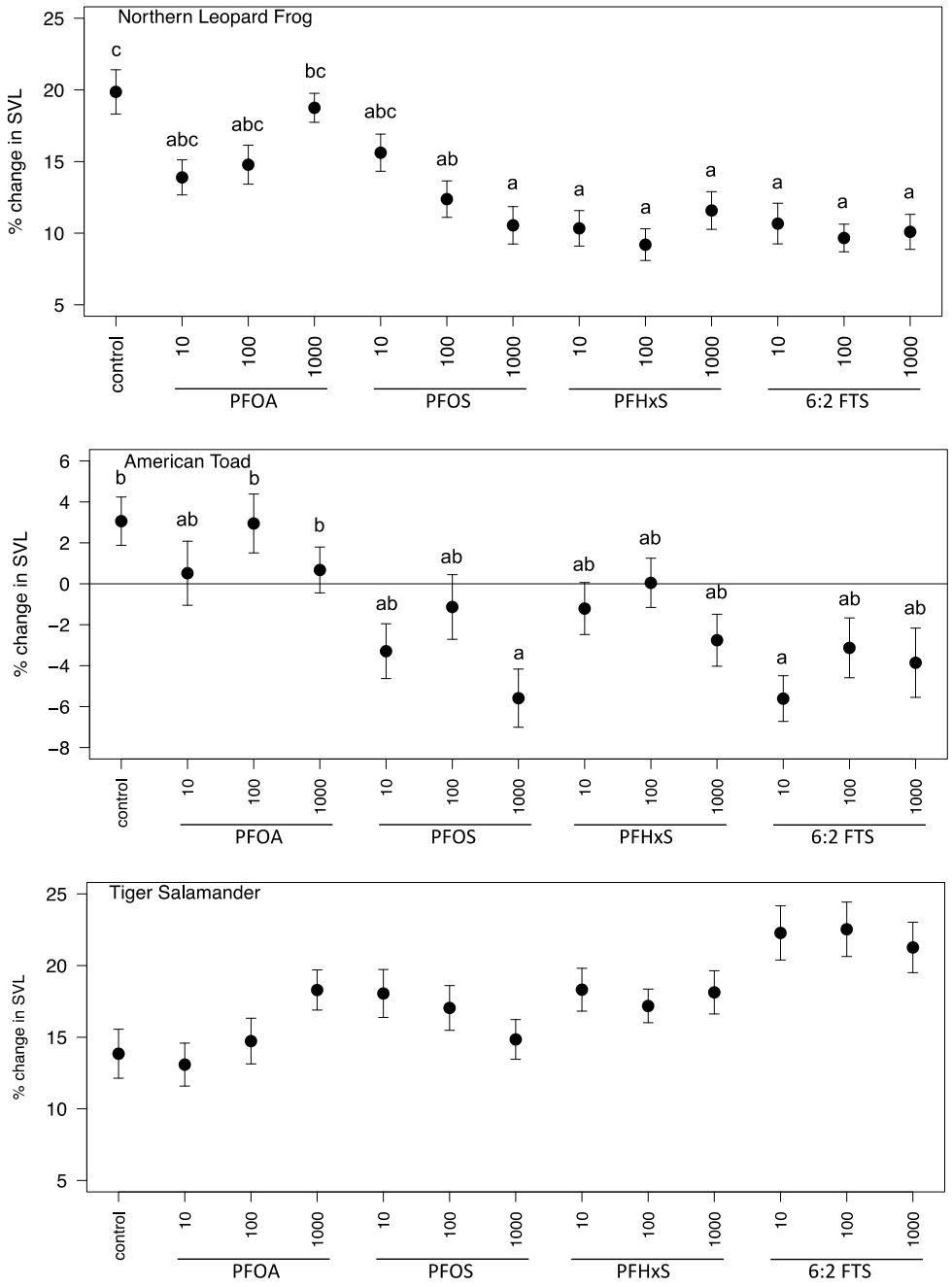

Figure 2.2 Effects of PFAS exposure via contaminated substrate on the percent change in percent change in snout-vent length (SVL) of post-metamorphic northern leopard frogs, American toads, and eastern tiger salamanders. Data are means ± SE. Letters denote results of compact letter display of pairwise comparisons examining significant differences between groups. Lack of letters indicates no significant differences observed between groups.

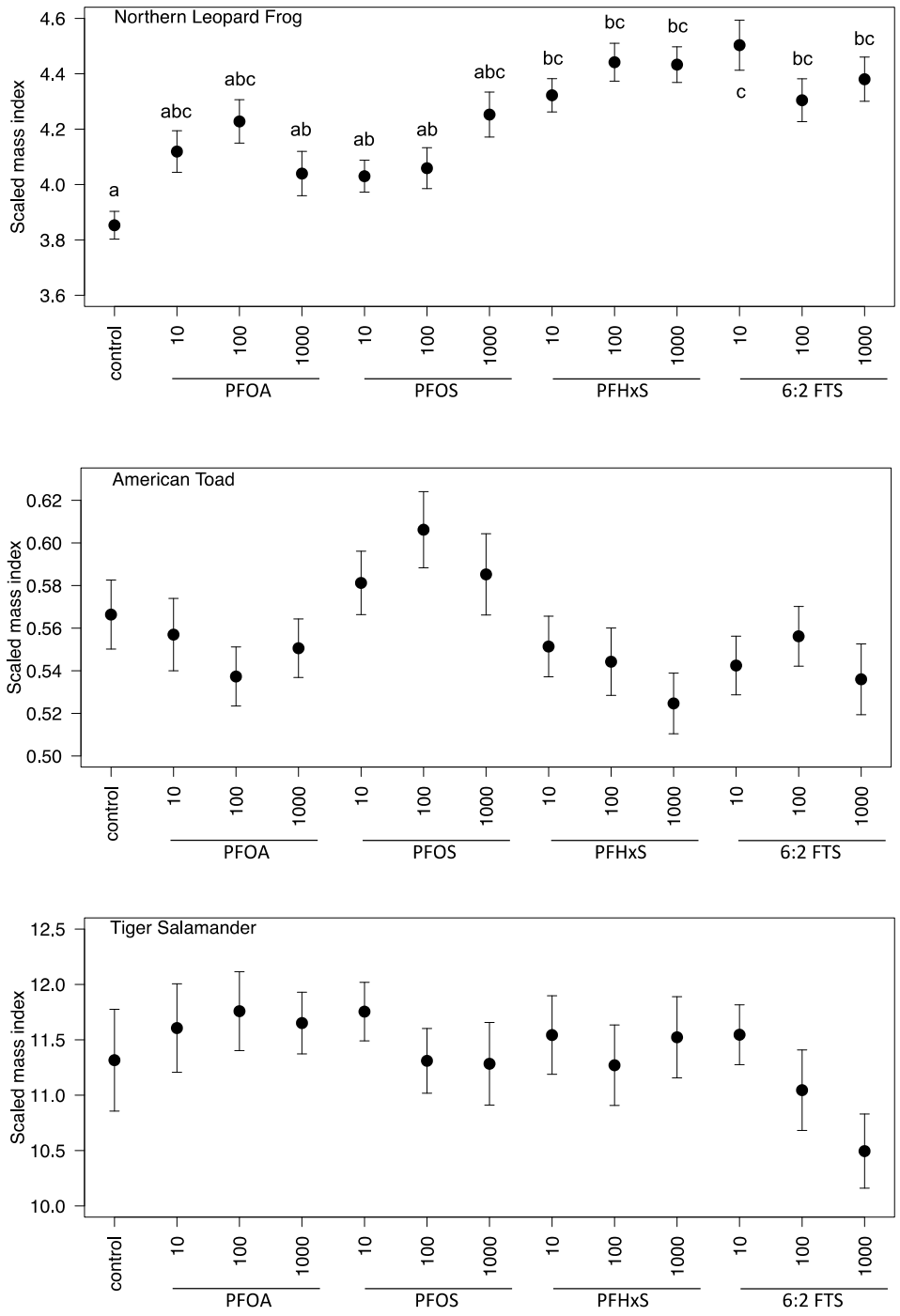

Figure 2.3 Effects of PFAS exposure via contaminated substrate on the body condition (scaled mass index) of post-metamorphic northern leopard frogs, American toads, and eastern tiger salamanders. Data are means ± SE. Letters denote results of compact letter display of pairwise comparisons examining significant differences between groups. Lack of letters indicates no significant differences observed between groups.

CHAPTER 3. CONCLUSIONS

3.1 Conclusions and future directions

Poly- and perfluoroalkyl substances (PFAS) are globally distributed throughout ecosystems, and have been targeted for regulation due to their persistence in the environment, widespread accumulation in both humans and wildlife, and potential for a variety of adverse effects. These factors support the idea that it is important to understand how these chemicals are impacting wildlife.

Relatively few studies have examined the influence of PFAS on amphibians, which are important components in freshwater ecosystems. My work demonstrated that amphibians quickly bioaccumulate PFAS and rapidly reach steady state. These data are vital to understanding the uptake kinetics of these compounds and will inform future research. Moreover, these results demonstrated that amphibian could contribute to PFAS biomagnification in food webs (Ahrens and Bundschuh, 2014). Understanding these relationships can inform mitigation efforts and the potential for PFAS biomagnification in natural food webs.

Additionally, my work on post-metamorphic amphibians found that PFAF exposure can have sublethal effects on amphibian growth and body condition. Interestingly, the direction and magnitude of effects were dependent on the species tested and the specific PFAS chemical. A critical next step in this research is to relate sublethal effects to body burdens. Some of the variation my data could be explained by differences in body burden among individuals within an experimental unit. With this knowledge, theories regarding PFOS sorption to sediment may be answered, as well as determining how bioaccumulative 6:2 FTS is with this type of dermal exposure. Additionally, there is a need for more mechanistic studies that determine the physiological processes that underlie the sublethal effects observed. Because PFAS are endocrine disruptors, there is the potential for effects on the hypothalamus-pituitary-thyroid axis.

Potential future work regarding these compounds is vast. Additional work addressing the thyroid hormone and the impacts that exposure to these PFAS have on its development would be insightful. Furthermore, addressing organ distribution of these compounds in a laboratory setting could be critical in understanding how these chemicals are distributed and therefore potentially eliminated, with species differences specifically in mind.

APPENDIX

Table A1.1 Snout-vent-length (SVL) and body weight for northern leopard frogs, eastern tiger salamander, and American toads at the conclusion of the experiments. Data are means ± 1 SE.

Species	SVL (mm)	Body weight (g)
Leopard Frog	8.18 ± 0.53	0.11 ± 0.00
Tiger Salamander	28.56 ± 0.21	0.99 ± 0.02
American Toad	6.85 ± 0.08	0.06 ± 0.00

Table A1.2 Results of two-tailed paired t-test examining differences in water concentrations between the first day of exposure and day 5 (prior to water change). Treatment groups were 10 or 1,000 µg/L of perfluorooctanoic acid (PFOA) or perfluorooctane sulfonate (PFOS).

		PFOA			PFOS		
	Treatment (µg/L)	*t*	df	*P*	*t*	df	*P*
Leopard Frog	10	-0.376	2	0.743	0.166	2	0.884
	1,000	-1.385	2	0.300	-0.169	2	0.881
Tiger Salamander	10	-1.384	2	0.301	0.394	2	0.732
	1,000	0.827	2	0.495	-0.468	2	0.686
American Toad	10	-1.974	2	0.187	-1.797	2	0.765
	1,000	3.503	2	0.073	-0.341	2	0.331

Table A1.3 Results of compact letter display of pairwise comparisons examining differences in $\log_{10}$ body burden over time within species and treatment. The linear combination of the estimated effects is displayed as lsmeans (least square means). Days sharing a group as indicated by the lower case letters are not significantly different from each other ($P > 0.05$).

			PFOA			PFOS		
Species	Treatment	Day	lsmean	SE	Group	lsmean	SE	Group
Leopard frog	10 µg/L	0	3.301	0.189	a	3.034	0.073	a
		2	1.842	0.189	b	3.713	0.073	b
		4	1.968	0.189	b	3.827	0.073	b
		6	1.697	0.189	b	3.795	0.073	b
		8	1.636	0.189	b	3.884	0.073	b
		10	3.771	0.189	a	3.842	0.073	b
	1,000 µg/L	0	3.393	0.092	ab	4.519	0.056	a
		2	3.636	0.092	ab	5.583	0.056	b
		4	3.702	0.092	b	5.707	0.056	b
		6	3.553	0.092	ab	5.740	0.056	b
		8	3.226	0.092	a	5.608	0.056	b
		10	3.404	0.092	ab	5.707	0.056	b
Tiger salamander	10 µg/L	0	1.890	0.125	a	2.478	0.108	a
		2	2.148	0.125	a	3.196	0.108	b
		4	2.179	0.125	a	3.362	0.108	bc
		6	4.530	0.125	b	4.061	0.108	d
		8	3.791	0.125	c	3.784	0.108	cd
		10	3.104	0.125	d	3.927	0.108	d
	1,000 µg/L	0	3.452	0.090	a	4.364	0.052	a
		2	3.991	0.090	b	5.256	0.056	b
		4	3.978	0.090	b	5.215	0.052	b

		6	4.615	0.090	c	5.621	0.052	c
		8	4.252	0.090	bc	5.547	0.056	c
		10	3.924	0.090	b	5.506	0.052	c
American toad	10 µg/L	0	1.037	0.241	a	2.619	0.069	a
		2	2.601	0.241	b	3.680	0.069	b
		4	2.141	0.241	ab	3.849	0.069	bc
		6	4.435	0.241	c	4.062	0.069	cd
		8	4.189	0.241	cd	4.319	0.069	d
		10	3.093	0.241	bd	3.864	0.069	bc
	1,000 µg/L	0	3.114	0.136	a	4.507	0.068	a
		2	3.833	0.136	b	5.562	0.068	b
		4	3.600	0.136	ab	5.752	0.068	b
		6	4.171	0.136	b	5.732	0.068	b
		8	4.135	0.136	b	5.655	0.068	b
		10	3.579	0.136	ab	5.474	0.068	b

Table A2.1 Mean snout-vent length (± SE) and mass (± SE) by treatment and species (leopard frog, tiger salamander and American toad) at the end of the experiment. Data were collected after the 30 d chemical exposure for each species. For snout-vent length and mass I ran a compact letter display of pairwise comparisons (cld in lsmeans package; R Development Core Team) to assess differences. Treatments sharing letters are not statistically different from each other ($P > 0.05$). Response variables without letters were not significantly different.

Treatment	(µg/L)	Snout-vent length (mm)			Mass (g)		
		Leopard Frog	Tiger Salamander	American Toad	Leopard Frog	Tiger Salamander	American Toad
Control	0	39.25±0.39^{b}	67.21±0.88^{abc}	18.83±0.24^{c}	4.31±0.14	11.46±0.58	0.59±0.02
PFOA	10	37.95±0.38^{ab}	67.08±0.94^{abc}	17.94±0.32^{abc}	4.15±0.12	11.72±0.53	0.56±0.02
	100	38.38±0.46^{ab}	65.53±0.92^{ab}	18.56±0.27^{abc}	4.44±0.16	11.62±0.45	0.55±0.02
	1000	38.22±0.41^{ab}	65.68±0.73^{ab}	18.61±0.20^{bc}	4.17±0.14	11.46±0.32	0.56±0.02
PFOS	10	38.55±0.43^{ab}	66.06±0.75^{abc}	18.19±0.26^{abc}	4.27±0.14	11.65±0.34	0.58±0.02
	100	38.15±0.45^{ab}	64.89±0.73^{a}	18.05±0.29^{abc}	4.17±0.14	11.05±0.38	0.61±0.03
	1000	348.34±0.28^{ab}	64.32±0.80^{a}	17.38±0.26^{ab}	4.41±0.11	10.98±0.46	0.56±0.02
PFHxS	10	36.98±0.44^{a}	67.55±0.94^{abc}	18.30±0.26^{abc}	4.06±0.14	11.73±0.48	0.56±0.02
	100	37.92±0.37^{ab}	65.79±0.80^{abc}	18.66±0.26^{bc}	4.46±0.11	11.19±0.47	0.56±0.02
	1000	37.08±0.46^{a}	68.45±0.99^{abc}	18.55±0.24^{abc}	4.22±0.16	11.84±0.49	0.54±0.02
6:2 FTS	10	37.32±0.40^{ab}	69.97±0.88^{bc}	18.41±0.22^{abc}	4.31±0.12	12.06±0.38	0.55±0.02
	100	37.34±0.44^{ab}	68.69±0.92^{abc}	18.01±0.21^{abc}	4.15±0.13	11.38±0.47	0.55±0.02
	1000	37.14±0.44^{a}	70.46±1.14^{c}	17.30±0.34^{a}	4.15±0.14	11.08±0.47	0.52±0.02

Table A2.2 Mean snout-vent length (± SE) and mass (± SE) by treatment and species (leopard frog, tiger salamander and American toad) at the start of the experiment. Data were collected three days prior to chemical exposure for each species. For snout-vent length and mass, I ran a compact letter display of pairwise comparisons (cld in lsmeans package; R Development Core Team) to assess differences. Treatments sharing letters are not statistically different from each other ($P > 0.05$). Response variables without letters were not significantly different.

		Snout-vent length (mm)			Mass (g)		
Treatment	(µg/L)	Leopard Frog	Tiger Salamander	American Toad	Leopard Frog	Tiger Salamander	American Toad
Control	0	32.85±0.38^{ab}	59.29±0.89	18.30±0.23^{ab}	3.22±0.11	9.02±0.36	0.61±0.02
PFOA	10	33.38±0.33^{abc}	59.52±1.00	17.86±0.20^{a}	3.18±0.08	9.21±0.38	0.58±0.02
	100	33.52±0.42^{abc}	57.30±0.83	18.07±0.24^{a}	3.46±0.12	8.81±0.32	0.57±0.02
	1000	32.23±0.38^{a}	55.62±0.59	18.52±0.21^{ab}	3.11±0.10	8.56±0.21	0.61±0.02
PFOS	10	33.45±0.47^{abc}	56.19±0.78	18.84±0.20^{ab}	3.22±0.13	8.71±0.28	0.61±0.02
	100	34.02±0.40^{abc}	55.59±0.58	18.32±0.30^{ab}	3.05±0.10	8.15±0.26	0.63±0.03
	1000	34.80±0.43^{bc}	56.13±0.72	18.45±0.23^{ab}	3.16±0.11	8.12±0.26	0.60±0.02
PFHxS	10	33.56±0.35^{abc}	57.22±0.78	18.58±0.29^{ab}	3.05±0.11	9.04±0.37	0.60±0.02
	100	34.82±0.44^{c}	56.28±0.82	18.69±0.26^{ab}	3.40±0.13	8.47±0.35	0.60±0.02
	1000	33.29±0.39^{abc}	58.11±0.89	19.12±0.25^{ab}	3.19±0.11	9.17±0.29	0.57±0.02
6:2 FTS	10	33.80±0.35^{abc}	57.49±0.86	19.55±0.23^{b}	3.21±0.08	8.90±0.32	0.61±0.02
	100	34.08±0.36^{abc}	56.33±0.92	18.70±0.31^{ab}	3.10±0.11	8.37±0.33	0.62±0.02
	1000	33.79±0.39^{abc}	58.14±0.62	18.08±0.36^{ab}	3.12±0.10	8.28±0.28	0.56±0.02