

**FINAL REPORT**

# Development of Amphibian PFAS Toxicity Reference Values for Use in Ecological Risk Assessment at AFFF Sites

**A Critical Review of the Purdue Amphibian PFAS Ecotoxicity Research Studies (2017-2022): Identification of Screening Levels in Water and Other Useful Resources for Site-specific Ecological Risk Assessments**

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**May 2023**

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**REPORT DOCUMENTATION PAGE**

Form Approved  
OMB No. 0704-0188

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<b>1. REPORT DATE (DD-MM-YYYY)</b> 30/05/2023		<b>2. REPORT TYPE</b> SERDP Final Report		<b>3. DATES COVERED (From - To)</b> 3/30/2022 - 3/30/2023	
<b>4. TITLE AND SUBTITLE</b>  Development of Amphibian PFAS Toxicity Reference Values for Use in Ecological Risk Assessment at AFFF Sites  A Critical Review of the Purdue Amphibian PFAS Ecotoxicity Research Studies (2017-2022): Identification of Screening Levels in Water and Other Useful Resources for Site-specific Ecological Risk Assessments				<b>5a. CONTRACT NUMBER</b> 22-P-0020	
				<b>5b. GRANT NUMBER</b>	
				<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b>  Maria Sepúlveda, Jason Hoverman, Linda Lee, Tyler Hoskins, Youn Choi and Elizabeth Allmon Purdue University  Jason Conder and Zacharias Pandelides Geosyntec Consultants				<b>5d. PROJECT NUMBER</b> ER-2626	
				<b>5e. TASK NUMBER</b>	
				<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Purdue University 155 South Grant Street West Lafayette, IN 47907				<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>  ER-2626	
<b>9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> Strategic Environmental Research and Development Program (SERDP) 4800 Mark Center Drive, Suite 16F16 Alexandria, VA 22350-3605				<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b> SERDP	
				<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b> ER-2626	
<b>12. DISTRIBUTION/AVAILABILITY STATEMENT</b> DISTRIBUTION STATEMENT A. Approved for public release: distribution unlimited.					
<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> With the goals of aiding risk assessors conducting site-specific ecological risk assessments at PFAS-contaminated sites, this critical review synthesizes information on the ecotoxicity of PFAS to amphibians produced by the Purdue University research group over 6 years, in 9 amphibian species, and across 14 peer-reviewed publications. The Purdue studies focused upon in this review consisted of spiked-PFAS chronic toxicity experiments with PFOS, PFOA, PFHxS, and 6:2 FTS that evaluated apical endpoints typical of ecological risk-based decision making (survival, growth, and development). Body mass was the most sensitive endpoint, showing clear and biologically meaningful population level adverse effect sizes (≥ 20% adverse effects). From these results, we recommended chronic no observed effect concentration (NOEC) screening levels of 120 µg/L for PFOS and 130 µg/L for PFOA. At or above recommended chronic LOEC screening levels of 1400 µg/L PFOS and 1400 µg/L PFOA, there is an ecologically significant chance of adverse chronic effects. Biologically relevant adverse effects were not observed for PFHxS and 6:2 FTS, so unbounded NOEC screening values or 1300 µg/L PFHxS and 1800 µg/L 6:2 FTS are recommended. Screening levels for the evaluation of potential risks to amphibians are also provided for concentration of PFAS in amphibian diet, amphibian tissue, and moss substrate. Additionally, we recommend bioconcentration factors that can be useful to predict concentrations of PFAS in amphibians using concentrations in water; these values are useful for food web modeling to understand risks to vertebrate wildlife that prey on amphibians. Overall, this paper provides a guide to the wealth of ecotoxicological research on PFAS conducted by this research group, and also highlights the need for additional work that that would improve the understanding of chemical risks to amphibians.					
<b>15. SUBJECT TERMS</b> Risk assessment, Perfluoroalkyl substances, PFAS, Amphibian, Chronic toxicity					
<b>16. SECURITY CLASSIFICATION OF:</b>			<b>17. LIMITATION OF ABSTRACT</b>  UNCLASS	<b>18. NUMBER OF PAGES</b>  51	<b>19a. NAME OF RESPONSIBLE PERSON</b> Maria Sepulveda
<b>a. REPORT</b>  UNCLASS	<b>b. ABSTRACT</b> UNCLASS	<b>c. THIS PAGE</b> UNCLASS			<b>19b. TELEPHONE NUMBER (Include area code)</b> 765-496-3428

**FINAL REPORT**  
Project: ER-2626

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## ACKNOWLEDGEMENTS

The authors declare no conflicts of interest. This review effort was funded by the Strategic Environmental Research and Development Program (SERDP), project number ER2626. The work reviewed in this effort was funded by SERDP, project number ER2626. The authors would like to thank Dr. Axel Berky, who assisted with data compilation and many other students and laboratory technicians that assisted during this work.

**Data Availability statement:** All raw data used in the critical risk assessment are archived through the PURR - Purdue University Research Repository using the following link:

<https://doi.org/10.4231/T1KN-3T16>

**Key words:** Risk assessment, Perfluoroalkyl substances, PFAS, Amphibian, Chronic toxicity

## ABSTRACT

With the goals of aiding risk assessors conducting site-specific ecological risk assessments at PFAS-contaminated sites, this critical review synthesizes information on the ecotoxicity of PFAS to amphibians produced by the Purdue University research group over 6 years, in 9 amphibian species, and across 14 peer-reviewed publications. The Purdue studies focused upon in this review consisted of spiked-PFAS chronic toxicity experiments with PFOS, PFOA, PFHxS, and 6:2 FTS that evaluated apical endpoints typical of ecological risk-based decision making (survival, growth, and development). Body mass was the most sensitive endpoint, showing clear and biologically meaningful population level adverse effect sizes ( $\geq 20\%$  adverse effects). From these results, we recommended chronic no observed effect concentration (NOEC) screening levels of 120  $\mu\text{g/L}$  for PFOS and 130  $\mu\text{g/L}$  for PFOA. At or above recommended chronic LOEC screening levels of 1400  $\mu\text{g/L}$  PFOS and 1400  $\mu\text{g/L}$  PFOA, there is an ecologically significant chance of adverse chronic effects. Biologically relevant adverse effects were not observed for PFHxS and 6:2 FTS, so unbounded NOEC screening values or 1300  $\mu\text{g/L}$  PFHxS and 1800  $\mu\text{g/L}$  6:2 FTS are recommended. Screening levels for the evaluation of potential risks to amphibians are also provided for concentration of PFAS in amphibian diet, amphibian tissue, and moss substrate. Additionally, we recommend bioconcentration factors that can be useful to predict concentrations of PFAS in amphibians using concentrations in water; these values are useful for food web modeling to understand risks to vertebrate wildlife that prey on amphibians. Overall, this paper provides a guide to the wealth of ecotoxicological research on PFAS conducted by this research group, and also highlights the need for additional work that that would improve the understanding of chemical risks to amphibians.

## 1.0 INTRODUCTION

Due to the ubiquity of per- and polyfluoroalkyl substances (PFAS) in aquatic ecosystems, there is a need to understand the potential risks to aquatic life. A key part of the site-specific ecological risk assessment process for aquatic life is the comparison of measured (modeled) concentrations in samples of abiotic and biotic media to quantitative risk-based screening levels. The availability of screening levels for aquatic life is limited, and the few values that are available from the literature or from regulatory agencies are limited to screening levels for perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in freshwater (Conder et al., 2021; Zodrow et al., 2021). These screening levels are primarily derived from laboratory studies with fish and invertebrates exposed to water spiked with PFOA and PFOS.

In 2022, the United States Environmental Protection Agency (USEPA) released a review of the available literature on the aquatic ecotoxicology of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), and this review was used to generate draft aquatic life ambient water quality criteria for PFOA and PFOS in freshwater and salt water (USEPA, 2022a; USEPA, 2022b). As of 2022, these reviews are comprehensive and up-to-date compilations of PFAS ecotoxicology data, and include information from a wide variety of species. Information on amphibians, which are often included in site-specific aquatic ecological risk assessments based on the perception that they are environmental sentinels (Johnson et al., 2017), was included in the USEPA reviews. Importantly, from this analysis, amphibians were among the more sensitive taxa to PFAS, and were used directly in the calculation of the USEPA's PFOA chronic criterion and the PFOS acute criterion.

Much of the available data on PFAS effects in amphibians that was reviewed by USEPA originated from an extensive 6+ year research program conducted at Purdue University under the direction of Dr. Maria Sepúlveda, which was funded by the Strategic Environmental Research and Development Program (SERDP ER2626), began in 2016 and examined the ecotoxicology of PFAS in amphibians. To date, this research effort has evaluated the ecotoxicity of PFOS, PFOS, perfluorohexane sulfonate (PFHxS), and 6:2 fluorotelomer sulfonate (6:2 FTS) in 9 amphibian species using different experimental venues (e.g., laboratory tests, outdoor mesocosms) and exposure routes (e.g., water, sediment, moss, diet), as detailed in 14 peer-reviewed scientific articles. Some of these studies were not available to USEPA at the time of their review. Also, the USEPA's review of these studies was focused on generation of screening levels for PFOS and PFOA in water. The Purdue amphibian PFAS research also features information on bioaccumulation evaluations and exposure scenarios involving media aside from water, results that may be useful in understanding alternate exposure routes to amphibians and exposures of vertebrate wildlife that consume amphibians. Lastly, several studies included here were conducted in outdoor mesocosms, incorporating abiotic variability and community context, which will be useful in determining if and how lab-derived results translate to systems more reflective of the field.

The purpose of this paper was to conduct a critical review of the Purdue amphibian research, synthesizing and highlighting key results from the 14 studies. The primary audience for this review is ecological risk assessors evaluating site-specific risks to amphibians or site-specific risks to vertebrate wildlife that may consume amphibians. Specific goals included the identification of

recommended PFAS screening levels in water, amphibian tissue, and amphibian diet items that could be directly used in site-specific ecological risk assessments evaluating the toxicity of PFAS to amphibian populations. Additionally, we identify recommended bioaccumulation factors to predict the bioaccumulation of PFAS in amphibian tissues. These values could be used to predict PFAS daily dietary doses for wildlife that may prey upon amphibians inhabiting aquatic systems in which PFAS are present.

## **2.0 METHODOLOGY**

### **2.1 GENERAL REVIEW APPROACH**

Controlled laboratory and outdoor spiked-PFAS toxicity studies with amphibians published in 14 peer-reviewed articles by Purdue University from the years 2017 to 2022 were considered in this review (hereafter, “Purdue studies”). The primary goal of this review was to identify recommended risk-based screening levels for PFAS in water that would be protective of long-term, chronic adverse effects on amphibians. Overall, 9 of the 14 Purdue studies were identified for this purpose (Table S1, Supplemental Information) based on the review approach and considerations described in this section, and detailed discussions of all 14 studies is presented in the SI. Among these 9 key chronic studies (Abercrombie et al., 2019; Brown et al., 2021; Flynn et al., 2019; Flynn et al., 2021a; Flynn et al., 2022; Foguth et al., 2020; Hoover et al., 2017; Hoskins et al., 2022; Lech et al., 2022), PFOS was tested the most frequently (all 9 studies), followed by PFOA (5 studies), PFHxS (4 studies), and 6:2 FTS (2 studies). The most common species exposed was the northern leopard frog (*Rana pipiens*; 7 studies), while the other three species (eastern tiger salamanders, *Ambystoma tigrinum*; American toads, *Anaxyrus americanus*; and American bullfrogs, *Rana (Lithobates) catesbeiana*) were exposed in two studies. Additional detailed information on the biological effects, exposure approaches, and adverse effects considered in derivation of PFAS screening criteria are provided in the subsections below.

The body of work reviewed represents high quality, peer-reviewed scientific findings. Regardless, some of the conclusions and interpretations of data and statistical and/or biological significance of the results presented in this review may be different than those of the original authors in the Purdue studies. Generally, these differences are due to the overall goal of this critical review, which is to distill the research findings for use in regulatory-driven ecological risk assessments at contaminated sites. This highlights the different goals and burdens of proof for academic researchers exploring open-ended questions in the field of ecotoxicology versus those of risk assessors balancing hypothetical chemical risks, stakeholder concerns, and the ecological health of sites being assessed in a regulatory-driven decision-making process.

### **2.2 BIOLOGICAL EFFECTS CONSIDERED IN SCREENING LEVEL IDENTIFICATION**

A variety of biological effects were evaluated in the Purdue studies; however, this review focused on apical sub-acute, sub-chronic, or chronic responses on survival, growth, and development that are ecologically relevant at the population level. These endpoints are typically used for regulatory screening levels and decisionmaking purposes at contaminated sites (Conder et al., 2020; ECCC, 2018; EPAV, 2016; RIVM, 2010; Suter, 2018; USEPA, 2022a, 2022b). As there were no statistically significant effects of PFAS exposure on survival in the 9 key chronic Purdue studies, only sublethal endpoints were used for chronic screening level identification. Growth and development were measured using different approaches, and for the purpose of this review, growth and development effects considered body mass, snout-vent length (SVL), Gosner Stage (GS; (Gosner, 1960)) that was reached at the end of the exposure (noted as “GS” in our review), and the time (number of days) it took amphibians to reach a certain terminal key stage of development (most often, time to metamorphosis (GS 42) or time to tail resorption (GS 46)).

Studies evaluating any (or all) of these apical endpoints were selected for this focused review. Of the growth parameters, SVL was measured in all 9 studies, mass was measured in 8 studies, GS attained at termination of exposure was reported in 8 studies, and the time to GS 46 was reported in 3 studies. Foguth et al. (2019) was not considered in screening level identification, as that study did not examine apical endpoints (see SI discussion of Foguth et al., (2019)).

Scaled Mass Index (SMI) is a measure of body condition and is calculated on a population basis using body mass and SVL (Peig and Green, 2009). Although several of the studies report changes in SMI after PFAS exposure, it was not considered as an endpoint for identification of screening levels mostly because there is no clear understanding on the relationship between SMI and population fitness in amphibians. Additional ecological studies on relationships between SMI and the health of individual animals are needed to better understand the use of amphibian SMI values in site-specific ecological risk assessment. Ongoing studies at Purdue University are exploring the relationship between SMI and other potential measures of health (e.g., body lipid content and composition) and additional research should shed more insights into the use of SMI as an indicator of health and potential for use as an apical endpoint that can be considered in environmental site decision making.

### **2.3 PFAS EXPOSURES CONSIDERED IN SCREENING LEVEL IDENTIFICATION**

PFAS exposures were considered if the exposure to PFAS was for a continuous duration of at least 10 days. Study length for the 9 key chronic studies varied from 10 to 120 days, with the median study duration being 40 days (Table S1). We note that in some cases the minimum number of days may not be sufficient to detect growth effects. Most acute studies in amphibians are generally considered a 96 h or less (OECD, 2009), and acute studies conducted by Purdue (Flynn et al., 2019; Hoover et al., 2019; Tornabene et al., 2021) focused primarily on survival endpoints, and reported effect concentrations that were orders of magnitude higher than the thresholds for more sensitive endpoints evaluated in the chronic exposures. Acute effect data from these three studies were not considered quantitatively in identification of screening levels, but are discussed in the SI.

Most of the Purdue studies focused on waterborne PFAS exposures with larval amphibians. Chronic waterborne exposure doses of PFAS ranged from 0.5 to 1800 µg/L. These exposure concentrations are orders of magnitude below the calculated lethal concentration for 10% of the population (LC<sub>10</sub>) values generated for amphibians by Tornabene et al. (2021), as LC<sub>10</sub> values ranged from 10,000 to 909,000 µg/L. Seven of the 9 studies evaluated for identification of screening levels in water measured PFAS in water (Table S1). Water samples were not filtered prior to analysis. As the water in these tests was comprised of pure filtered water relatively free of turbidity, organic carbon, and other solids that could reduce PFAS availability, it is likely that the majority of the PFAS present in the water was dissolved and freely available. Thus, concentrations of PFAS in water as reported in the Purdue studies are likely comparable to either filtered water samples from the field or relatively clean unfiltered or centrifuged water samples collected from natural waters in the field.

Concentrations in water from the tests (Table S1) are represented by average concentrations of PFAS in samples obtained at the beginning and ending of the exposure period. In one study, amphibians were exposed to PFAS-spiked sediment and overlying water. Exposure in this study was based on measurements of PFAS in the overlying water, and these values were considered in the data review for identification of screening levels for water (see SI discussion (Flynn et al., 2021a)).

In Abercrombie et al., (2021) and Flynn et al., (2021b), PFAS exposures were facilitated by spiking PFAS in moss substrate and food, respectively; these studies were not considered for screening levels in water, but were used for identification of other screening levels, as noted below.

## 2.4 ADVERSE EFFECTS QUANTIFICATION FOR IN SCREENING LEVEL IDENTIFICATION

Identification of screening levels in water in each study was based on the most sensitive survival, growth, or development effect endpoints. For studies that reported effects at multiple timepoints during the exposure, effects at the termination of PFAS exposure were considered for derivation of adverse effect thresholds. For each study, the percent magnitude of the effects in each exposure concentration were normalized to the control response by dividing the mean biological measurement of the PFAS exposed animals by using the following equation  $\frac{(V2-V1)}{V1} \times 100\%$ , with V1 being the average biological measurement in the control, and V2 being the biological measurement in the tank replicate. After the percent magnitude of effects was calculated for individual replicates, they are displayed as the arithmetic mean  $\pm$  standard deviation. Thus, negative values indicate adverse performance relative to the controls (e.g., a -15% change in body mass would mean the treated amphibians weighed 15% less than controls), and positive values indicating better performance than the controls. An exception to this was the time to GS 46 measurement, as noted in the results. Biologically measured values were obtained from raw data provided by the authors for each key chronic study; with exception of SMI data for Lech et al (2022) which was not provided and values derived to 2 significant figures using analysis of study figures (e.g., bar charts provided in the peer-reviewed publications) using ImageJ image processing software (<https://imagej.nih.gov/ij/>).

In this review, adverse effects were considered ecologically significant at the population level if the results from PFAS-exposed animals were statistically different from control animals ( $p \leq 0.05$ ) and the magnitude of difference was -20% or less (i.e., a 20% or greater level of adverse effect relative to the controls). We also present benchmark values for a magnitude of difference of -10%. The lowest PFAS exposure meeting those criteria was identified as the Lowest Observed Effect Concentration (LOECs), and the highest exposure less than the LOEC was identified as the No Observed Effect Concentration (NOEC). Calculation of effective concentration ( $EC_x$ ) estimates using dose response modeling was not attempted due to the relatively low number of dose levels in most of the experiments (e.g.,  $\leq 3$  PFAS doses), lack of clear dose-dependent adverse effects, lack of adverse effects at the 20% or greater effect size, and data variability.

In the United States, an adverse 20% effect level has generally been considered the threshold for ecological significance for aquatic life at the population level; effect sizes less than this can occasionally be detectable as statistically different from control responses in laboratory toxicity tests, but they are considered to be not severe enough to result in chronic effects at the population level and are unlikely to be statistically detectable in wild populations (Suter, 2000; USEPA, 2016). It is acknowledged, however, that some environmental regulatory agencies (Canadian Council of Ministers of the Environment, 2007; ECHA, 2008; Australian Government Department of Agriculture and Water Resources, 2018) and stakeholders involved in site-specific decision making consider exposures associated with the 10% level of effect (e.g., Effective Concentrations causing 10% effect,  $EC_{10}$  values) as the upper bound value for exposures that do not result in

ecologically significant effects, such that exceedance of this effect size could be potentially unacceptable and require additional investigation and/or management. Recently, in a draft document released for public comment, the USEPA (2022a; 2022b) proposed the use of EC<sub>10</sub> values for derivation of aquatic life values for PFOS and PFOA. For the purposes of our discussion, we incorporated the consideration that the 10% level of effect may be considered as a potential benchmark, despite the potential uncertainties noted above and the fact that most effect sizes of less than 20% in this review were not statistically detectable from control responses.

## **2.5 ADDITIONAL AMPHIBIAN ECOLOGICAL RISK ASSESSMENT RESOURCES CONSIDERED IN THE REVIEW**

A secondary product of our review includes the identification of recommended screening values for PFAS in amphibian diet items (i.e., invertebrates), amphibian tissue, and moss substrate. These screening levels allow the evaluation of site-specific risk to amphibians via the measurement (or model prediction) of concentrations of PFAS in amphibian diet items, amphibian tissue, or moss (or similar vegetation) upon which terrestrial amphibians may contact dermally. The review of data and identification of these screening levels in these media followed the above approaches used for water-based screening levels. However, the available data on these measures of dose were limited, and only unbounded NOEC screening levels for amphibian diet items, tissue, and moss substrate could be identified (see SI detail on the Flynn et al. (2021b), Hoover et al., (2017), and Abercrombie et al. (2021) studies, respectively). In cases in which site-specific concentrations are below these unbounded NOECs, there is relatively high confidence that population level adverse effects in amphibians would not be expected; however, exceedance of these NOECs only implies uncertain levels of risk since no LOECs were identified (i.e., the likelihood of adverse effects are unknown).

Additionally, this review highlighted a PFAS bioaccumulation study (Hoover et al., 2017) in northern leopard frogs (see SI detail). Using data from this study, we calculated average bioconcentration factor (BCF) values that could be used to predict the concentration of PFOS, PFOA, PFHxA, and 6:2 FTS in larval northern leopard frogs (or other larval amphibians) using site-specific concentrations in water. The predicted values for PFAS in amphibian tissue could, in turn, be used with wildlife dietary exposure models for PFAS (Larson et al., 2018; Conder et al., 2020; Conder et al., 2021) to estimate site-specific dietary exposure for a vertebrate wildlife species that preys upon amphibians.

## 3.0 RESULTS AND DISCUSSION

### 3.1 EFFECTS OF PFAS ON BODY MASS

As shown in Figure 1, the review of the 9 key aquatic chronic PFAS-spiked toxicity tests with amphibians (Table S1) provided dose-response data for evaluating the effects of PFOS and PFOA on body mass for 4 key species (American bullfrog, American toad, eastern tiger salamander, and northern leopard frog) and the effects of PFHxS and 6:2 FTS on body mass for 3 key species (American toad, eastern tiger salamander, and northern leopard frog). Compared to the other chronic endpoints evaluated in this review (SVL in Figure 2, GS in Figure 3, time to GS in Figure 4), body mass (Figure 1) was the most sensitive endpoint, causing dose-dependent effects at an approximate 20% adverse effects size (relative to controls) at concentrations of approximately 1400 µg/L for PFOS and 1400 µg/L for PFOA. A slight (mean effect 14% relative to controls) on SVL in American bullfrogs was observed at 300 µg/L; this could be a potential LOEC for PFOA, if a 10% adverse effect size is considered biologically relevant, but there is high uncertainty since only two doses were evaluated in this study.

Results at the 20% adverse effect size were statistically significantly different from controls for PFOA and PFOS, as indicated by the filled in symbols shown in Figure 1a and Figure 1b. For example, there was a statistically significant reduction in mass of northern leopard frogs (-16% difference relative to controls) exposed to 1400 µg/L PFOS (Flynn et al., 2022), as indicated by the filled in red triangle shown in Figure 1a. All other exposures of PFOS to northern leopard frog (empty red triangles, values ranging from 0.06 to 700 µg/L) were not statistically different from controls.

The variance of the mass results among replicates in these studies was large (SD values often ranged ± 10% to 20%). This resulted in a difficulty to statistically detect adverse effects sizes 20%, and this is likely a function of natural variability given the levels of replication used in these tests are reasonable for typical toxicity tests (i.e., 3-4 replicates per dose).

Effects of PFHxS and 6:2 FTS on amphibian growth were less clear. In American toads, although no dose-response was observed, larvae exposed to 600-800 µg/L were significantly heavier (15-25%). There is no evidence to suggest an increase in body mass of this magnitude would cause a reduction in population fitness, so they were not considered evidence of adverse effects.

LOEC values for PFOS for body mass were identified using the data highlighted in Figure 1. Of the nine studies conducted, PFOS statistically significantly decreased the mass of northern leopard frogs by a mean (SD) of 16% ± 20% in one exposure concentration (1400 µg/L, Flynn et al., 2022). The 16% adverse effect for PFOS at 1400 µg/L was considered approximate to the 20% level of biologically significant effects (there were no other higher doses tested (Figure 1a)). A statistically significant effect of PFOS on body mass was also detected in American bullfrogs (Flynn et al., 2019). In this study, 144 µg/L PFOS (nominal) resulted in a statistically significant mean (SD) decrease in mass (19% ± 6%), although the higher dose of 288 µg/L (nominal) resulted in a lower level of effect that was not statistically significant (15% ± 7%). High variation and identification of a potential outlier result in the control data complicates interpretation of this study. A second study (Lech et al., 2022) evaluated two lower doses (2 and 6 µg/L), and found increases in mass (25-30%) relative to controls that were not statistically significant.

Due to the wide variation in individual body mass of this species, the high variability in the control data, and the lack of a clear dose response, the effects of PFOS on American bullfrog mass were only considered qualitatively in this review, and the potential adverse effect noted at 144 µg/L was not considered a LOEC. Exposing American bullfrogs to a wider range of concentrations and more concentrations than was used in the two Purdue studies reviewed would be helpful to address this uncertainty.

A PFOS NOEC of 120 µg/L was identified from the Flynn et al. (2022) study. This result was the bounded NOEC that was the dose less than the 1400 µg/L PFOS LOEC identified above. As shown in Figure 1a and discussed in the SI, Abercrombie et al. (2019) noted an absence of statistically significant results in northern leopard frogs in concentrations as high as 700 µg/L. This study was conducted over a shorter time period (10 days) compared to the Flynn et al. (2022) study (30 days), so we conservatively selected the Flynn et al. (2022) value of 120 µg/L as the NOEC. The 120 µg/L NOEC value is also lower than the (higher uncertainty) 144 µg/L PFOS dose that significantly reduced American bullfrog mass in the Flynn et al. (2019) study.

LOEC and NOEC values for PFOA for body mass were identified using the data highlighted in Figure 1. In the study by Flynn et al. (2022), exposure to 1400 µg/L PFOA caused a statistically significant dose dependent decrease in mass (mean (SD) of 19% ± 14%), and this concentration was used as the LOEC for PFOA. The next lowest concentration tested in the Flynn et al. (2022) study (130 µg/L) was identified as the NOEC for PFOA. As shown in Figure 1b and discussed in the SI, Abercrombie et al. (2019) noted an absence of statistically significant results in northern leopard frog in concentrations as high as 870 µg/L. This result was not identified as a NOEC for PFOA since it was a shorter duration than the Flynn et al. (2022) study.

No adverse effects above the approximately 20% threshold were identified for mass (as well as the other two endpoints) for PFHxS and 6:2 FTS. Given this, unbounded NOEC values of 1300 µg/L PFHxS and 1800 µg/L 6:2 FTD were identified from Figure 1c and Figure 1d, respectively.

### **3.2 EFFECTS OF PFAS ON SVL**

As shown in Figure 2, the review of the 9 key chronic PFAS-spiked toxicity tests with amphibians (Table S1) provided dose-response data for evaluating the effects of PFOS and PFOA on SVL for 4 key species (American bullfrog, American toad, eastern tiger salamander, and northern leopard frog) and the effects of PFHxS and 6:2 FTS on SVL for 3 key species (American toad, eastern tiger salamander, and northern leopard frog). Statistically significant adverse effects of approximately 20% or more were not detected for SVL. The only significant adverse outcome of PFAS exposure on SVL was found in American bullfrogs exposed to PFOA (Figure 2b). There was a dose dependent decrease in SVL which was statistically significantly reduced by a mean (SD) of 14% ± 6% in amphibians exposed to 288 µg/L PFOA (nominal) by Flynn et al. (2019). This adverse effect size (14%) was less than the adverse 20% effect size considered to be biologically relevant, so this concentration was not considered as a LOEC for growth effects in this review. Additionally, there were statistically significant increases in SVL for eastern tiger salamanders and American toads exposed to PFOS, PFHxS and 6:2 FTS; however, the effect size was small (3-7% increase), and this minor difference was not considered an adverse effect.

### 3.3 EFFECTS OF PFAS ON GS AND TIME TO GS

As shown in Figure 3, the review of the 9 key chronic PFAS-spiked toxicity tests with amphibians (Table S1) provided dose-response data for evaluating the effects of PFOS and PFOA on GS reached at the end of the PFAS exposure period for 3 key species (American bullfrog, American toad, and northern leopard frog) and the effects of PFHxS and 6:2 FTS on SVL for 1 key species (northern leopard frog). Figure 4 depicts the available PFOS, PFHxS, (northern leopard frog) and PFOS, PFOA, PFHxS, and 6:2 FTS (American bullfrog) dose response data derived from the number of days required to reach terminal GS 46. Statistically significant adverse effects of approximately 20% or more were not detected for GS (as measured at the end of the exposure) or the time required to reach GS 46.

Exposures to doses of PFOS as low as 0.06 µg/L (water concentration) caused a statistically significant reduction in GS (mean (SD) of 4% ± 2%, a reduction of 1.5 GS compared to controls) in northern leopard frogs exposed to PFOS for 30 days (Flynn et al., 2021a). The latter experiment was conducted outdoors in mesocosms and animals exposed through spiked sediment, so uptake occurred through multiple routes. Effects on GS detected in the Flynn et al. (2021a) study were not dose dependent, as exposure to 0.06 to 15.6 µg/L PFOS (water concentrations) resulted in a similar 3-4% reduction in GS. In a separate study exposing northern leopard frogs for 30 days to 9.6 to 1376 µg/L PFOS indoors in aquaria, this effect was not observed (Flynn et al. 2022). This study found that 9.6 and 125 µg/L PFOS caused no statistically significant effect on GS and 1376 µg/L PFOS statistically significantly reduced GS by a mean (SD) of 3.6% ± 3.8%. Exposure to PFOA, PFHxS or 6:2FTS also either caused no statistically significant, or small (< 5%) changes to GS. Overall, the effects of PFAS on GS tended to be small (< 5%), typically resulting in animals being 1-2 GS lower than controls when statistically significantly different.

Although effects on development were consistent (delayed) across multiple species, chemicals, and exposure regimes, the ecological relevance of these developmental delay at the levels reported by the Purdue group are unclear. Given the importance of phenotypic plasticity to larval performance, some authors argue that any chemical-induced change in timing represents an adverse effect, that the timing of metamorphosis in response to chemicals is highly variable, and that this variability should not be interpreted as inconsistencies across studies (Rohr and McCoy, 2010). Rohr and McCoy (2010) further suggest that the size at metamorphosis is expected to decrease with increasing chemical dose, thus, studies that evaluate development should consider a measure of size (i.e., body mass and length) in addition to developmental stage. The ecological relevance of these minor levels of effect size for this endpoint in the decision-making process are considered low in the present review, but amphibian risk assessments would benefit greatly from more consensus around how to treat altered developmental rates in effects assessments.

A few studies measured the effects of PFAS on the period of time required to complete metamorphosis (i.e., the time required for animals to develop from GS 25 to GS 46), as shown in Figure 4. Two studies did not detect statistically significant effects of PFAS in the time required to reach GS 46 (Foguth et al., 2020; Hoskins et al., 2022). Flynn et al. (2022) did not detect any statistically significant effect of PFOS, PFHxS or 6:2 FTS on the time required for American toads to reach GS 46. In this same study, exposure to 10 and 94 µg/L PFOA also did not statistically significantly affect time to reach GS 46; however, exposure to 873 µg/L PFOA did cause a statistically significant increase in time to GS 46 (mean (SD) of 5% ± 4%) with the controls taking 35 days on average to reach GS 46 and the high dose PFOA American toads taking 36.7 days.

Despite this statistically significant result, the effect is small, and a 1.7-day delay to reach full development is unlikely to be biologically relevant to the success of most populations.

Developmental rate in amphibians is influenced by environmental factors such as temperature and can result in variation across experiments. For example, Goldstein et al. (2017) measured time to metamorphosis for GS 25 relict leopard frogs (*Rana [lithobates] onca*) raised at 20, 25, and 30°C, and noted that frogs required 264, 67, and 98 days, respectively, to complete metamorphosis. Similarly, in the study by Flynn et al. (2021a), GS 25 control northern leopard frogs reached GS 40 in 30 days, while in another study by Flynn et al. (2022), GS 25 control northern leopard frogs reached GS 34 after 30 days. One factor that certainly influenced the difference in growth patterns between these two studies is that the 2021a study was conducted in an outdoor mesocosm, where temperatures were much higher, densities were lower, and their diet was more diverse than in the 2022 study conducted in the laboratory. However, there is still substantial variation in development schedules between lab studies. For example, Hoover et al. (2017) GS 26 control northern leopard frogs only reached GS 30 in a 40-day laboratory experiment. Standardized test conditions could help with comparisons across studies, but plasticity in developmental rates and associated changes in growth rates are a basic life history feature of amphibians that will complicate risk assessment in the absence of a strategy to assess repeatability across an inherently variable set of endpoints.

### **3.4 RECOMMENDED AMPHIBIAN SCREENING LEVELS FOR PFAS IN WATER**

As noted above, the most robust toxicity data from the 9 key chronic studies indicated that northern leopard frogs were the most sensitive to chronic PFAS exposure of the four species tested (northern leopard frogs, eastern tiger salamanders, American toads, and American bullfrogs). Based on body mass responses from the most comprehensive study (Flynn et al., 2022), as detailed in the above discussion, we summarize the recommended chronic NOEC and LOEC screening levels for waterborne exposure to PFOS, PFOA, PFHxS and 6:2 FTS (Table 1). These NOECs and LOECs are based on statistically significant and biologically relevant effects to mass, and are protective of the other growth, survival, and developmental endpoints measured in the Purdue studies, as shown in Figures 2-4.

Aside from the Purdue studies, there are few chronic sublethal PFAS toxicity studies that report apical endpoint measurements. Ankley et al., (2004) found an approximate 20% reduction in length (relative to controls, statistically significant) in northern leopard frogs exposed to a measured concentration of 3420 µg/L PFOS (LOEC) and no effect in the next-lowest exposure of 957 µg/L PFOS (NOEC). Fort et al., (2019) noted statistically significant reductions of 5% and 14% (relative to controls) in length and mass in the western clawed frog (*Xenopus tropicalis*) at the highest concentration tested (measured), 1050 µg/L PFOS; no significantly different effects were noted at the next-highest concentration (590 µg/L). The NOEC and LOEC values for PFOS identified from the review of the Purdue studies, 120 and 1400 µg/L, respectively, are consistent with the results from the Ankley et al. (2004) and Fort et al. (2019) studies.

The geometric mean of the three concentrations with effects < 20% among Ankley et al. (2004), Fort et al. (2019), and Flynn et al. (2022) studies (i.e., 957, 1050, and 120 µg/L) would indicate a NOEC of approximately 500 µg/L. The geometric mean of studies with demonstrated chronic sublethal effects with > 20% adverse effects (Flynn et al., 2022; Ankley et al., 2004) would indicate a LOEC of 1200 µg/L. These values could also be considered as alternate NOEC and LOEC screening values to those presented in Table 1.

In general, the amphibian NOEC and LOEC values for PFOA and PFOS discussed in this review are greater than or similar to thresholds observed for other aquatic life that may be present in PFAS-contaminated aquatic systems in which amphibians are found. For example, toxic thresholds for PFOS in some other aquatic species, as identified by the USEPA (2022a; 2022b) conducted in support of derivation of PFOS ambient water quality criteria, were two orders of magnitude lower than the NOEC values recommended in this review (i.e., 120 or 500  $\mu\text{g/L}$  PFOS). The USEPA's draft Criterion Continuous Concentration (CCC) value for PFOS was 8.3  $\mu\text{g/L}$ , indicating amphibian species will not likely influence risk-based decision-making for PFOS at aquatic sites. In contrast, amphibians were among the more sensitive aquatic species for PFOA (USEPA, 2022b), and the draft CCC for PFOA was 94  $\mu\text{g/L}$ , very similar to the amphibian NOEC value of 130  $\mu\text{g/L}$  recommended in this review. Thus, amphibians may be among the more sensitive aquatic species to PFOA.

The NOEC and LOEC screening values shown in Table 1 can be used by decision makers evaluating the potential site-specific risks of PFAS in water to amphibian populations inhabiting aquatic systems where PFAS presence is suspected. A typical use of these screening levels would be to serve as points of comparison when measured concentrations of PFAS are available in site-specific water samples. For example, in cases in which a concentration in surface water is below the NOEC screening levels, there is a high probability that PFAS waterborne exposures will not affect amphibian populations. In cases in which a concentration in surface water is equal to or exceeds the LOEC screening levels for PFOS and/or PFOA, there is a possibility of an ecologically meaningful effect. Exceedance of these LOECs should not be interpreted to be evidence of an adverse effect at any given site, but only that additional site-specific evaluations (additional measurements, toxicity testing, ecological study, etc.) may be needed to complete an assessment of risks to amphibians. Concentrations of PFOS and PFOA that are between the NOEC and LOEC screening levels represent potential uncertainty such that there may be some minor level of adverse effects, especially for values that approach the LOEC screening values. Exceedances of the unbounded NOEC screening values for PFHxS and 6:2 FTS also represent uncertain conditions with regards to the likelihood of ecologically significant population-level effects, but they should not be interpreted to indicate the presence of risk.

Most importantly, the recommended NOECs and LOECs (Table 1) should not be considered default cleanup or management goals. Risk management options should take site-specific considerations into account prior to action, as well as follow the general recommendation that any "cure" one might consider to mitigate potential chemical risks should not result in conditions that represent a net loss in ecological health to amphibians or other valued natural resources.

The application of the Table 1 screening levels recommended in this paper should be on a single-PFAS basis such that the potential for mixture interactions between PFAS are not accounted for. This is a typical approach for screening criteria for most chemicals until robust quantitative approaches for evaluating mixture toxicity in aquatic animals (i.e., the calculation of polycyclic aromatic hydrocarbons (PAH) toxic units when multiple PAHs are detected in aquatic environmental media) are available. These approaches are not yet available for PFAS. Mixture interactions of PFAS were assessed by some the Purdue studies (Flynn et al., 2019; Foguth et al., 2020; Hoover et al., 2019; Hoskins et al., 2022; Lech et al., 2022). Although the studies generally suggested the PFAS tested interacted on an additive basis, additional work is needed to develop an algorithm capable of incorporating concentrations of multiple PFAS in water to produce an estimate of combined risk.

### **3.5 OTHER USEFUL DATA RESOURCES FOR PFAS ECOLOGICAL RISK ASSESSMENTS INVOLVING AMPHIBIANS**

Aside from data that can be used to identify concentrations in water associated with effects (and a lack of significant effects), the Purdue studies also provide additional data that may be helpful to site-specific PFAS risk assessments that focus on the risks of adverse effects to amphibians. As detailed in the SI, concentrations of PFAS were measured in northern leopard frogs in the study by Hoover et al. (2017), and these were used to derive unbounded NOECs for PFOS and PFOA of 100000 and 940 ng/g wet weight (ww), respectively. These values could be useful in interpreting site-specific measurements of PFOS and PFOA in amphibians.

Flynn et al. (2021b) conducted a chronic dietary exposure with PFOS, PFHxS, PFOA, 6:2 FTS with post-metamorphic salamanders, and we used this data to derive unbounded NOECs in amphibian diet of 1900, 1300, 4800, and 2200 ng/g ww for PFOS, PFOA, PFHxS, and 6:2 FTS, respectively (see SI section on Flynn et al. (2021b)). These values could be useful in interpreting site-specific measurements of PFAS in samples of insects and other biota of amphibians.

Lastly, Abercrombie et al. (2021) exposed post-metamorphosis American toads, eastern tiger salamanders, and northern leopard frogs dermally to moss substrate spiked with PFOS, PFOA, PFHxS, and 6:2 FTS, and we used these data to identify unbounded NOECs in moss substrate ranging from 2800 to 7100 ng/g dry weight (dw; see SI section on (Abercrombie et al., (2021))). These values could be useful in interpreting site-specific measurements of PFAS in samples of moss or other substrates (e.g., leaf litter, vegetation) to which terrestrial forms of amphibians may be exposed via dermal contact.

In addition, several of the Purdue studies evaluate the bioaccumulation of PFAS (Abercrombie et al., 2019; Flynn et al., 2021a; Foguth et al., 2020; Hoover et al., 2017; Hoskins et al., 2022), and this information can be useful for predicting the concentrations of PFAS in amphibians. For example, as detailed in the SI, we used data from Hoover et al. (2017) to calculate bioconcentration factors (BCFs) that can be used to predict the concentrations of PFOS, PFOA, PFHxS, and 6:2 FTS in northern leopard frogs (whole body) using site-specific values for concentrations in water. The predicted values for PFAS in amphibian tissue could, in turn, be used with wildlife dietary exposure models for PFAS (Larson et al., 2018; Conder et al., 2020; Conder et al., 2021) to estimate site-specific dietary exposure for a vertebrate wildlife species that preys upon amphibians.

### **3.6 RECOMMENDATIONS FOR FUTURE RESEARCH**

The research produced by the Purdue group since 2017 represents a sizable and critical contribution to our understanding of PFAS toxicity to amphibians. There are still some data gaps and areas for future studies for understanding PFAS amphibian ecotoxicology. One endpoint that has not been explored very well in amphibians is reproductive effects. Reproductive effects in zebrafish have been shown to be a sensitive endpoint that is affected at levels of PFOS lower than those that affect growth or survival (Wang et al., 2011). Female fish have been demonstrated to reduce their body concentration of PFOS through maternal deposition to their eggs, and it would be important to know if this also occurs in other species (Sharpe et al., 2010; Wang et al., 2011).

Another area that could be explored further is examining effects of early life stage (larval) exposure on amphibians post-metamorphosis. As amphibians bioaccumulate PFAS during their aquatic stage, their likely primary mechanism for PFAS depuration (gills) is lost, such that terrestrial depuration may be slow. Understanding terrestrial depuration rates will be critical in understanding if and how amphibians could export aquatically sourced PFAS to terrestrial food webs. Further, terrestrial life stages have a disproportionately large influence on population dynamics relative to larval stages (Biek et al., 2002; Vonesh and De la Cruz, 2002).

There is large individual variance in the growth and development of animals in the Purdue results, making it difficult to detect statistically significant changes in growth and replicate results across experiments. This could be due to phenotypic plasticity or other sources of experiment-to-experiment variation typical of toxicity testing with organisms. Future studies interested in elucidating growth effects should adhere to robust standard practices typically used in the Purdue studies, such as measuring the animals at the start of the experiment to ensure there is no significant difference in body mass and SVL between the initial groups of animals, adjusting the feeding ration based on the density and size of the animals, whenever possible, including a positive control toxicant for growth effects, measuring the size of the animals when they reach metamorphosis (to capture monotonic growth effects), and/or state what the biologically relevant effect size for the growth endpoints are and use a sample size that is able to detect this effect. Future avenues of research include studies that measure the effects of PFAS exposure on amphibian reproduction, incorporate terrestrial life stages, quantify mixture effects, and assess the interaction between PFAS exposure and other stressors. Together, these steps will lead to better, evidence-based decision making.

As noted in the results section and in the SI, American bullfrogs were exposed to PFAS in only two of the nine studies. In the Flynn et al. (2019) American bullfrog study indicated possible effects between 150 to 300  $\mu\text{g/L}$  PFOS and PFOA, although results were uncertain due to the small dose range and a lack of dose response for PFOS. Future studies could elucidate these effects by conducting a larger dose response range within the NOEC and LOEC range for PFOS and PFOA reported in this review (120-1400  $\mu\text{g/L}$ ), with an increase in the number of doses to (ideally 4 to 5 or more).

GS is an endpoint that was recorded in many studies as a measure of amphibian development. This endpoint tended to have relatively small variance between replicates, allowing for relatively small changes (3% to 5%) to be detected as statistically significantly different from controls. The biological significance of the effects of PFAS on GS are challenging to interpret because these small changes generally indicated that PFAS-exposed amphibians were approximately 1 stage behind controls at the end of many of the exposure periods tested. However, when similar studies were conducted, these significant effects on GS were not observed, indicating significant study-to-study variation. For example, in the study by Flynn et al. (2021a), there was a statistically significant reduction in GS by  $3\% \pm 2\%$  ( $\sim 1$  GS) in northern leopard frogs exposed to 0.96  $\mu\text{g/L}$  PFOS for 30 days. In contrast, in the 2022 study by Hoskins et al. (2022), neither exposure to 0.56 or 0.93  $\mu\text{g/L}$  PFOS statistically significantly impacted GS ( $-0.2\% \pm 2\%$  or  $+1\% \pm 3\%$  change, respectively) in northern leopard frogs by day 30. It should be noted that GS is a qualitative measurement where animals are visually assessed. A best practice worth considering in future studies would be imaging the amphibians when they are sampled. This would allow all (or a portion of) results to be staged by independent blinded observers, an additional quality control/quality assurance step that could potentially improving reproducibility and confidence, in the results.

## 4.0 CONCLUSIONS

Amphibians are generally underrepresented in environmental risk assessment and ecotoxicology (Johnson et al., 2017). To our knowledge, this is one of the most comprehensive risk assessments for a chemical class to amphibians, aside from atrazine (Hanson et al., 2019; Rohr and McCoy, 2010). The Purdue research group has contributed substantially to the understanding of the toxicity of PFAS to amphibian species. Across the 14 peer-reviewed studies, there is a strong weight of evidence for relatively high PFAS tolerance of the four amphibian species that were evaluated. Among the statistically significant adverse effects that were detected, effects tended to be minor (< 10%), not dose dependent, and not necessarily reproducible by laboratory studies conducted under similar conditions.

Based on the nine key chronic studies identified in this review, we identified chronic screening levels (NOEC and LOEC, respectively) in water that can be used to evaluate concentrations of PFOS (120 and 1400  $\mu\text{g/L}$ ) and PFOA (130 and 1400  $\mu\text{g/L}$ ) in water measured at sites. This evaluation will be helpful for site-specific risk assessments that focus on amphibian population level health. For PFHxS and 6:2 FTS, only unbounded NOEC screening levels were able to be identified ( $\geq 1300$  and  $\geq 1800$   $\mu\text{g/L}$ , respectively). While other aquatic biota might be more sensitive for PFOS, consideration of PFOA levels to amphibians is advisable, as amphibians can be among the most sensitive aquatic vertebrate species. Additional screening levels are also provided for evaluating the risks of amphibians using site-specific measurements of PFAS in amphibian tissue, amphibian diet items, and terrestrial substrates. Information on bioaccumulation in amphibians is also presented, and values are available for use in food web bioaccumulation modeling for evaluation of dietary risks of vertebrate wildlife that consume amphibians.

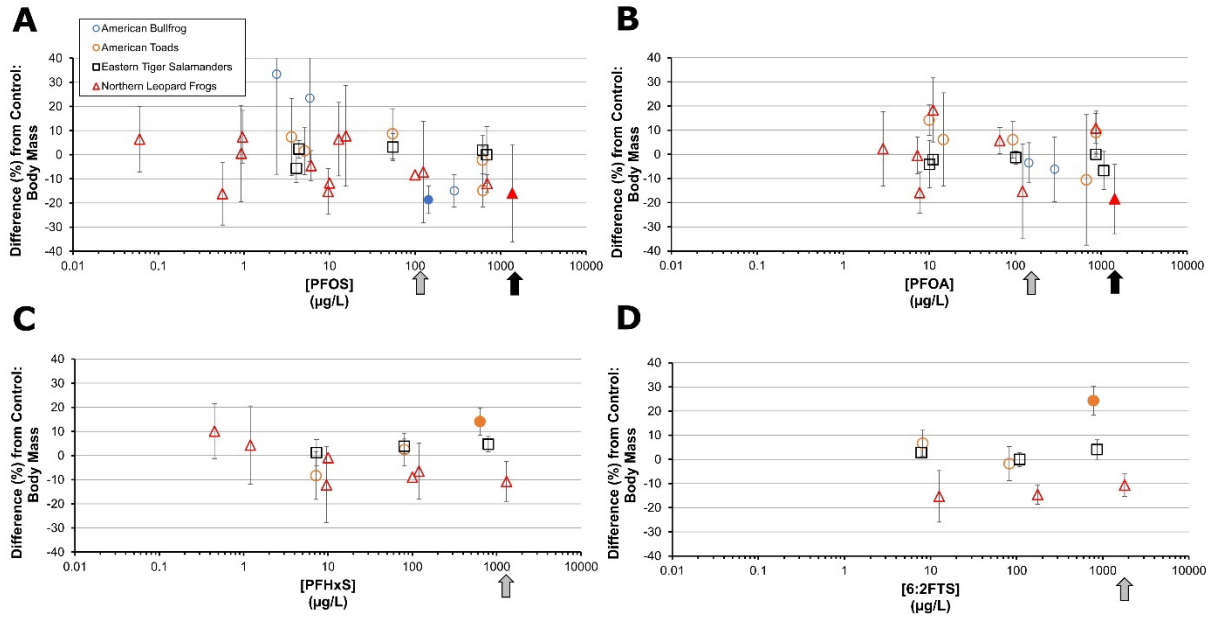
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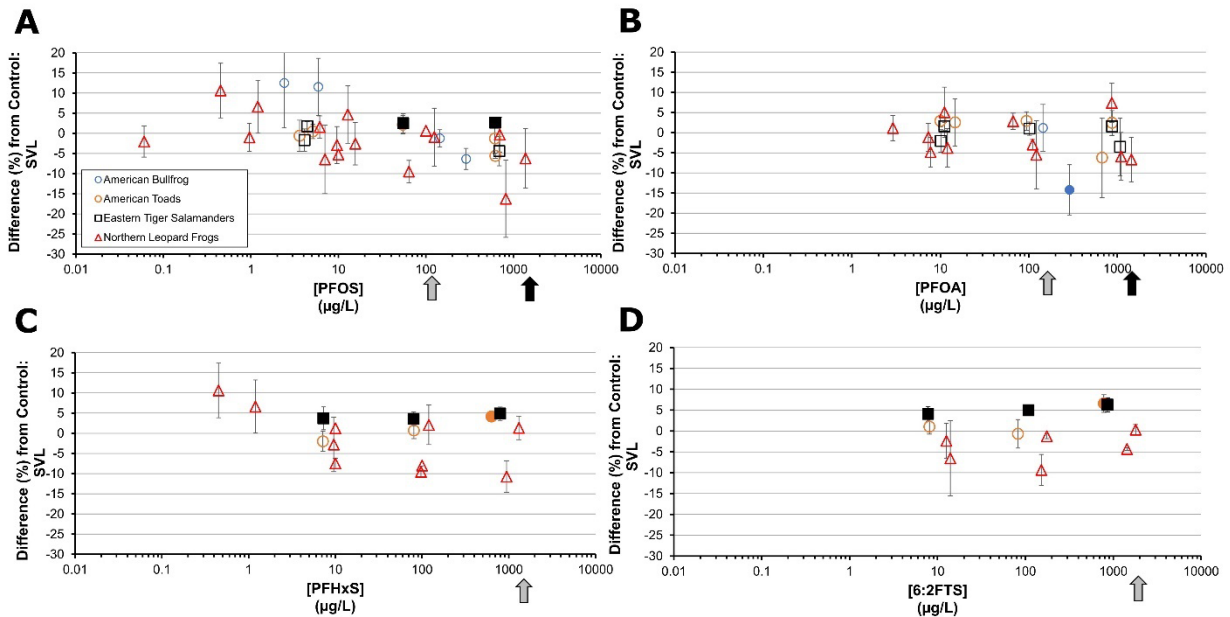
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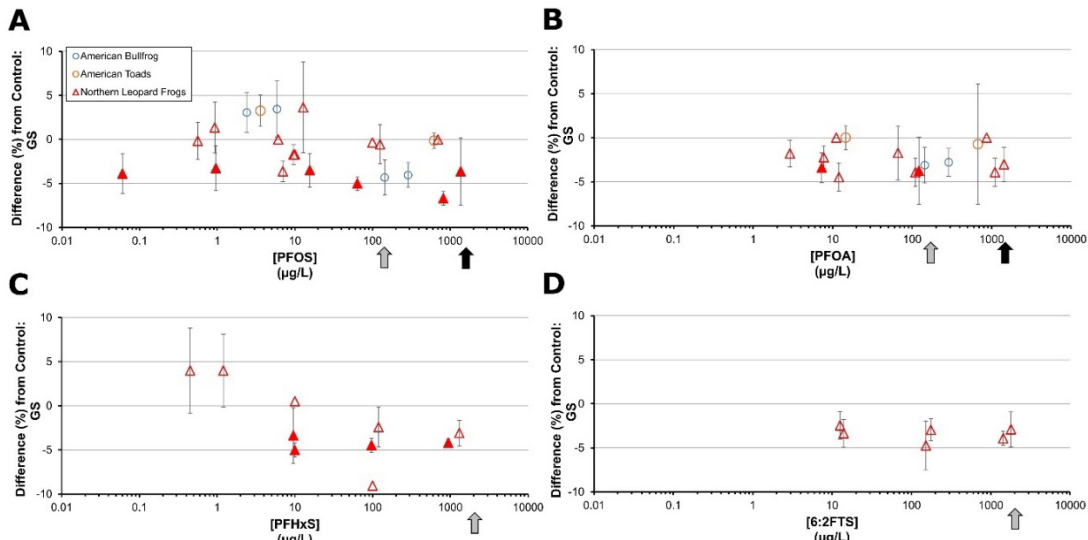
**Figure 1. Effects of PFAS Exposure (Log Scale,  $\mu\text{g/L}$ ) on Amphibian Body Mass.**

Data are presented as the percent change from controls (mean  $\pm$  SD). Symbols that are filled in represent statistically significant differences from controls for each PFAS. Grey and black arrows represent the overall recommended NOEC and LOEC values, respectively, identified from a review of all growth and development endpoints (Table 1).



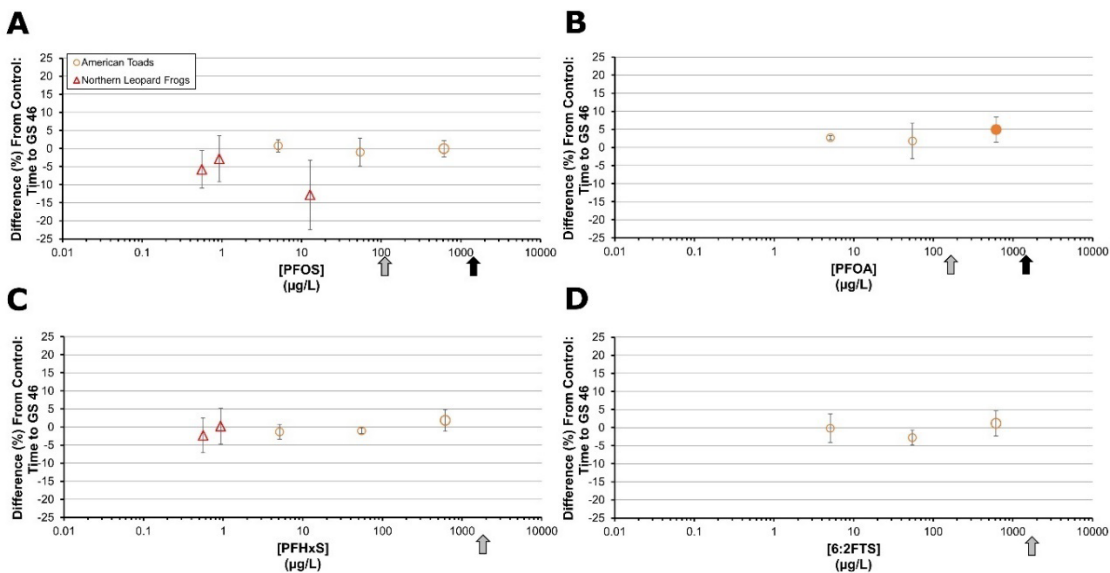
**Figure 2. Effects of PFAS exposure (log scale,  $\mu\text{g/L}$ ) on SVL.**

Data are presented as the percent change from controls (mean  $\pm$  SD). Symbols that are filled in represent statistically significant differences from the controls for the each PFAS. Grey and black arrows represent the overall recommended NOEC and LOEC values, respectively, identified from a review of all growth and development endpoints (Table 1).



**Figure 3. Effects of PFAS exposure (log scale, µg/L) on Gosner Stage (GS).**

Data are presented as the percent change from controls (mean ± SD). Symbols that are filled in represent statistically significant differences from the controls for each PFAS. Grey and black arrows represent the overall recommended NOEC and LOEC values, respectively, identified from a review of all growth and development endpoints (Table 1).



**Figure 4. Effects of PFAS exposure (log scale, µg/L) on time to complete metamorphosis (time to reach GS 46).**

Data are presented as the percent change from controls (mean ± SD). Symbols that are filled in represent statistically significant differences from controls for each PFAS. Grey and black arrows represent the overall recommended NOEC and LOEC values, respectively, identified from a review of all growth and development endpoints (Table 1). Please note that unlike the other figures (Figures 1-3), a negative percent difference relative to controls indicates a faster time to metamorphosis (not necessarily an adverse outcome), while a higher percent relative to controls represents a longer (delayed) metamorphosis (a potential adverse outcome). For example, the only statistically significant effect on time to GS 46 for any PFAS was for an exposure to 873 µg/L PFOA which caused a 5% ± 4% increase in the time it took to complete metamorphosis (1.7 days longer than control).

**Table 1. Recommended Amphibian Chronic No-effect (NOEC) and Low-effect (LOEC) Screening Levels for PFAS in Water.**

<b>PFAS</b>	<b>NOEC <sup>[1]</sup> (µg/L)</b>	<b>LOEC <sup>[1,2]</sup> (µg/L)</b>	<b>Average (SD) Adverse Growth Effect Associated with LOEC <sup>[3]</sup></b>
PFOS	120	1400	-16% (20%)
PFOA	130	1400 <sup>[5]</sup>	-19% (14%)
PFHxS	≥ 1300	NA <sup>[4]</sup>	NA <sup>[4]</sup>
6:2 FTS	≥ 1800	NA <sup>[4]</sup>	NA <sup>[4]</sup>

**Notes**

[1] NOEC and LOEC screening levels are based on growth (body mass) measurements, the most sensitive ecologically relevant endpoint with relatively clear dose-response relationships.

[2] LOECs are exposure concentrations at which an effect was statistically significantly different from control ( $p \leq .05$ ) and an effect size  $\geq 10\%$ -20%.

[3] Effects  $< 20\%$  may not be ecologically significant or experimentally reproducible, but in these cases, the LOECs identified were from the highest dose available in the study, so doses associated with effect sizes  $> 20\%$  are unavailable.

[4] No statistical differences between doses and control responses in any of the PFHxS or 6:2 FTS toxicity tests were identified, thus LOECs are not available.

[5] A slight (mean effect 14% relative to controls) on SVL in American bullfrogs was observed at 300 µg/L; this could be a potential LOEC for PFOA, but there is high uncertainty (see discussion).

## SUPPLEMENTAL INFORMATION

### Summary Table S1

A summary and interpretation of the 9 key chronic toxicity study results is provided in Table S1 and as an Excel file attached to this PDF file. The following information is provided in Table S1 for each study:

- Life Stage (at beginning of exposure);
- Species (common name);
- Exposure duration;
- Number of dose levels (including control);
- Replicate tanks per dose;
- Information about the experimental design and PFAS doses, how (or if) the concentrations of PFAS in the exposure waters/media were measured, the number of replicate measurements per dose, and the dose values (measured values were provided if available);
- Endpoints measured;
- The most sensitive endpoint, which was determined as the endpoint that indicated a statistically significant difference (adverse effect) from the control at the lowest PFAS dose; in cases in which two endpoints both indicated a statistically significant effect at the same (low) dose, the endpoint with the largest adverse effect size was selected as the most sensitive endpoint;
- The statistical NOEC, which was defined as the highest dose level at which the study authors could not detect a statistically significant difference from the control;
- The statistical LOEC, which was defined as the next dose greater than the statistical NOEC (at which a statistical difference from controls was detected); the magnitude of the effect at this dose is noted;
- NOECs, assuming either 10% or 20% is considered ecologically significant – these are evaluated as the doses less than the dose with a statistically significant difference from the control that is at least 10% or 20% different, respectively, from the control;
- LOECs, assuming either 10% or 20% is considered ecologically significant – these are evaluated as the doses with a statistically significant difference from the control that is at least 10% or 20% different, respectively, from the control;
- Consideration of the utility of the study for setting aquatic benchmarks,, particularly focusing on the number of doses and replication, and if doses were nominal (not measured) or confirmed with measurement;
  - Note that many studies were designed with goals other than deriving aquatic benchmarks. Comments here refer to utility of the data for the present effort and are not appraisals of the utility of the original approaches.
- Unless otherwise noted data are presented as mean  $\pm$  standard deviation; and
- Additional notes as needed.

## **Individual Study Summaries**

This section of the Supporting Information contains detailed reviews of the 14 Purdue amphibian PFAS Studies, and includes the following subsections (arranged alphabetically by author in each subsection):

- Key Chronic Studies (Presented in Table S1)
  - Abercrombie et al. (2019)
  - Brown et al. (2021)
  - Flynn et al. (2019) – Chronic data
  - Flynn et al. (2021a)
  - Flynn et al. (2022)
  - Foguth et al. (2020)
  - Hoover et al. (2017)
  - Hoskins et al. (2022)
  - Lech et al. (2022)
- Other Chronic Studies
  - Abercrombie et al. (2021)
  - Flynn et al. (2021b)
  - Foguth et al. (2019)
- Acute Studies
  - Flynn et al. (2019) – Acute data
  - Hoover et al. (2019)
  - Tornabene et al. (2021)

## **Key Chronic Studies (Presented in Table S1)**

### **Abercrombie et al. (2019): Larval amphibians rapidly bioaccumulate poly- and perfluoroalkyl Substances**

This study by Abercrombie et al. (2019) assessed the uptake of PFOS and PFOA in three species of amphibians, northern leopard frogs (*Rana pipiens*), American toads (*Anaxyrus americanus*), and eastern tiger salamanders (*Ambystoma tigrinum*). The study was 10 days long and was not designed to look at growth effects. Nevertheless, animals were measured (mass, SVL, GS) and survival quantified. Exposure was static renewal waterborne. Amphibians were exposed to 0, 10 and 1000 µg/L (nominal) PFOS or PFOA, in three replicate, 15-L plastic bins (22 tadpoles / replicate, northern leopard frogs and toads) or individual 300-mL plastic cups (42 experimental units, eastern tiger salamanders). Concentrations of PFOS and PFOA were measured in the water (0 h and 120 h, as well as in the animals at 5 h and every 48 h) until termination of the exposure. Measured concentrations of PFOS or PFOA in water are summarized in the table below:

Species (common name)	PFAS	PFAS Doses ( $\mu\text{g/L}$ )
American toads	PFOS	0.08, 3.6, 623
	PFOA	0.08, 14.6, 672
Eastern tiger salamanders	PFOS	0.09, 4.1, 691
	PFOA	0.09, 10.1, 1072
Northern leopard frogs	PFOS	0.03, 6.1, 698
	PFOA	0.03, 11.1, 865

Water exposures were renewed every 120 h until the end of exposure at day 10. The animals were GS 26-28 (variance not provided) or HS 46 for salamanders at the onset of exposure, acclimated for 2 days prior to exposure, and maintained under standard laboratory conditions: 21°C and a 12-h:12-h light:dark photoperiod.

Exposure to PFOS or PFOA for 10 days did not significantly affect survival, SVL, or mass in any of the three species tested. GS was measured in northern leopard frogs and American toads, but the information about the statistical analysis is not present in the paper. Based on data provided by the Purdue researchers, American toad GS differed from -1 to +3% from controls following PFOS or PFOA treatment over the 10-day exposure, a level of difference that is not biologically meaningful. There was no difference in GS for northern leopard frogs after 10 days of exposure to PFAS (they were all still GS 26). There was a statistically significant difference between the mass of northern leopard frogs in the PFOA 10- $\mu\text{g/L}$  treatment and the two PFOS treatments, but no differences in PFOS- or PFOA-exposed animals from control are stated. Since PFOS or PFOA did not significantly impact growth or survival, the following unbounded NOECs are reported for the 10-day exposure:

Species (common name)	PFAS	NOEC ( $\mu\text{g/L}$ )
American toads	PFOS	$\geq 623$
	PFOA	$\geq 672$
Eastern tiger salamanders	PFOS	$\geq 691$
	PFOA	$\geq 1072$
Northern leopard frogs	PFOS	$\geq 698$
	PFOA	$\geq 865$

Concentrations of PFOA and PFOS in the animals measured at the end of the 10-d exposure ranged from 3819 to 16481 ng/g dry weight PFOA and 6955 to 489958 ng/g dry weight PFOS (these were converted to ww in Supplemental Table 1). PFAS steady state occurred rapidly in the larval amphibians particularly for PFOS.

Concentrations in of PFOS and PFOA measured in American toad (whole body) in the highest exposure in this study was 112000 and 1900 ng/g, ww, respectively. Concentrations in of PFOS and PFOA measured in eastern tiger salamander (whole body) in the highest exposure in this study was 84000 and 4100 ng/g, ww, respectively. Concentrations in of PFOS and PFOA measured in northern leopard frog (whole body) in the highest exposure in this study was 122000 and 960 ng/g,

ww, respectively. These values, from animals at which no adverse effects were observed, were similar to the tissue NOECs derived for PFOS and PFOA from the Hoover et al. (2017) study (100000 and 940 ng/g ww, respectively, see below), with the exception of the concentration of PFOA in tiger salamander whole body (4100 ng/g ww), which was approximately 4X higher than the northern leopard frog NOEC for PFOA. This confirms the unbounded NOECs developed from the Hoover et al. (2017) in this review are reasonably applicable to other amphibians, but could suggest tiger salamanders are less sensitive to PFOA than northern leopard frogs.

### **Brown et al. (2021): Perfluoroalkyl Substances Increase Susceptibility of Northern Leopard Frog Tadpoles to Trematode Infection**

The study by Brown et al. (2021) evaluated the impacts of a chronic PFOS and PFHxS waterborne exposure on infection risk by frogs. Animals were measured (mass, SVL, GS) and survival examined. Frogs were exposed to PFOS and PFHxS solutions of 10 and 100 µg/L, in one replicate 15-L tank/treatment (n = 20 tadpoles/replicate) for 10 days in a static renewal system. Initial tadpoles were evaluated with a median GS of 26.5 and median weight of 0.109 g, and were acclimated for 1-day prior to treatment. Water was changed on day 5 of PFAS exposure. Concentrations of PFAS in water were not measured, so nominal values were used in our review. After 10 days of PFAS exposure, tadpoles were transferred to clean water in the same 15-L bins. Tadpoles were held in clean water for 7 days before parasite (*Echinoparyphium*) exposure to examine tadpole susceptibility to parasitic infection after PFOS exposure. PFAS-exposed tadpoles were assigned to individual 1-L cups (clean water), and each tadpole was exposed to 50 trematode cercariae for six hours. After trematode cercaria exposure, frogs were transferred to clean water, held for 48 hours, euthanized, measured for body mass, length, and GS, then dissected to evaluate parasite infection. The entire experiment was conducted at 22°C with a 14-h:10-h day:night light cycle.

Survival was 100% over the course of the study, with no statistically significant effect of PFOS or PFHxS exposure. There was no statistically significant effect of 10-day exposure to PFOS or PFHxS on mass, GS, or SVL. We did not use the data reported on number of cysts found in relation to PFAS exposure for deriving NOEC and LOEC values because of a lack of clear dose response and it is not a typical apical endpoint for risk assessment. In conclusion, unbounded NOECs of 100 µg/L are reported for PFOS and 100 µg/L for PFHxS for the lack of effects on growth and survival.

### **Flynn et al. (2019): Acute and chronic effects of perfluoroalkyl substance mixtures on larval American bullfrogs (*Rana catesbeiana*)**

This study evaluated the chronic effects of PFOS and PFOA waterborne exposure on survival and growth (mass, SVL, GS) of the American bullfrog (*Lithobates catesbeiana*) during a 72-day unmeasured, static renewal study. American bull frogs were exposed to nominal test concentrations of 0 (control), 144, and 288 µg/L of PFOS or PFOA in 4 replicate tubs (n = 10 tadpoles/replicate). Concentrations of PFAS in water were not measured, so nominal values were used in our review. A factorial combination of the three PFOS treatments and the three PFOA concentrations was used, resulting in nine total exposure groups. Initial tadpoles were GS 25 (variance in initial size not specified), acclimated for 1-day prior to exposure, and maintained under standard laboratory conditions: 21°C and a 12-h:12-h light:dark photoperiod. Complete water changes were performed every 3-4 days, at which point chemical exposure was renewed. Frogs were fed at a constant rate based on the control biomass throughout the experiment.

There was no effect of PFOS exposure on survival. The growth of the animals was measured at six timepoints over the 72-days. After 72-days exposure to PFOS, there was no statistically significant effect on SVL. There were inconsistencies between the significant differences reported in the text and in the figures of this study for GS and body mass, so for this study, the raw data were obtained and reanalyzed for statistical confirmation. Exposure to 144 µg/L or 288 µg/L PFOS did not statistically significantly affect GS (one-factor ANOVA on GS values,  $p > 0.05$ ). Mass was the most sensitive endpoint for PFOS exposure to 144 µg/L reducing the weight by an average (SD) of  $19\% \pm 6\%$  relative to control (ANOVA on mass data with Dunnett's post hoc comparison,  $p = 0.043$ ). Exposure to 288 µg/L did not significantly affect weight (reduced by average (SD) of  $15\% \pm 7\%$ ). Thus, the effects of PFOS did not follow a dose response for any endpoint measured with statistically significant effects detected at the low but not the high concentrations. Using a sensitivity analysis, one of the four control replicates was determined to be an outlier (per Grubb's test) and dropped from the analyses. The result of this analysis indicated that neither the 144 µg/L nor the 288 µg/L mass was statistically significantly different from the controls.

Due to the lack of dose response, the use of only 2 PFOS doses in the study, and the potential high mass outlier in the control group that could influence study interpretation by inflating the average mass of the control group, the results of this study are considered to be inconclusive, and a NOEC and LOEC is not able to be derived from this study.

There was no effect of PFOA exposure on survival. Exposure to 0, 144 or 288 µg/L PFOA did not significantly affect mass or GS, but did cause a significant reduction of SVL at 288 µg/L (mean (SD) of  $14\% \pm 6\%$ , ANOVA on ranks,  $p < 0.001$ ) but not at 144 µg/L ( $1.2\% \pm 5\%$  reduction). Thus, the statistical NOEC for PFOA exposure is 144 µg/L and the statistical LOEC is 288 µg/L. However, when considering both statistical significance and the lower biological relevance of the statistical (i.e., effect size  $< 20\%$ ), we consider the NOEC for PFOA as 288 µg/L.

### **Flynn et al. (2021a): Chronic Per-/Polyfluoroalkyl Substance Exposure Under Environmentally Relevant Conditions Delays Development in Northern Leopard Frog (*Rana pipiens*) Larvae**

Flynn et al. (2021a) evaluated the chronic effects of PFOS and PFOA on survival and growth (mass, SVL, GS) on northern leopard frogs, *Lithobates pipiens* (formerly, *Rana pipiens*), via a 30-day, sediment-spiked, static mesocosm study. Frogs were exposed to sediment spiked with PFAS (0, 10, 100, 1000 ng/g dw sediment) in four or five replicate mesocosms (180-L plastic wading pools, 25 larvae/pool). Concentrations of PFAS in sediment were measured before and at the end of the 30-day exposure; average concentrations were: 6.9, 67.3, and 597.6 ng/g PFOS in the PFOS sediment; and 13.3, 28.2, 281.4 ng/g PFOA in the PFOA sediment. One control sediment was used for both experiments, and the average concentration of the sum of PFOA and PFOS was 0.09 ng/g. Concentrations were also measured in the overlying water. The concentration of PFOS in overlying water in the dosed PFOS mesocosms was 0.06, 0.96 and 15.6 µg/L PFOS, and the concentration of PFOA in the overlying water of the PFOA mesocosms was 2.9, 7.3 or 66.2 µg/L PFOA. There was no PFOS or PFOA detected in the water overlying the control sediment. The water was then inoculated with algae and zooplankton from local pond water and allowed to establish for five days, after which the Gosner stage 25 frog larvae (initial variance not specified) were added to each mesocosm.

There was no statistically significant effect of 30-day exposure to PFOS on survival, mass, or SVL. There was a statistically significant reduction of GS in frogs exposed to all concentrations of PFOS (0.06, 0.96, or 15.6 µg/L water, 6.9, 67.3, 597.6 ng/g sediment), and GS was reduced from controls by a mean (SD) of 4% ± 2%, 3% ± 2.5%, or 3% ± 2%, respectively, a difference of approximately 1 Gosner stage at the end of the 30-day exposure. Given the magnitude of the adverse effect decreased slightly with increasing PFOS dose, a clear dose response was not evident. Due to the effect size (3-4%) being less than a biologically relevant effect size (10-20%), a NOEC of 30-day exposure of northern leopard frogs to PFOS of ≥ 16 µg/L water (≥ 598 ng/g sediment) is reported.

There was no statistically significant effect of 30-day exposure to PFOA on survival, mass, or SVL. There was a statistically significant reduction of GS in frogs exposed to the middle concentration of PFOA (7.3 µg/L water, 28.2 ng/g sediment) of an average (SD) of 3% ± 2% (~1 GS), but not the high or the low concentration (2.9 and 66.2 µg/L water, 13.3 and 281.4 ng/g sediment). Like PFOS, the effects of PFOA did not follow a dose response. Due to the effect size (3%) being less than a biologically relevant effect size (10-20%) a NOEC of 30-day exposure of northern leopard frogs to PFOA of ≥ 66 µg/L water (281 ng/g sediment) is reported.

The use of concentrations in the surface water from these experiments as potential aquatic life screening values in water does is not without uncertainty. The frogs remained on the surface of the PFAS-spiked sediment and did not burrow in the sediment (personal communication from the authors), and thus, they were primarily exposed to PFAS via the water and potentially exposed via diet (algae and incidental ingestion of sediment). Given that the amphibians were also dermally exposed to sediment porewater (through body surfaces in contact with the sediment), this could have resulted in a higher degree of exposure. Thus, use of the concentration in surface water (e.g., 66 µg/L in the PFAS experiment) to represent the exposure concentration to which frogs were exposed was likely conservative.

The minor (3-4%) effects on GS merit further discussion, as the amphibians were measured at multiple time points. By 5 days exposure, there was already a reduction of 0.5 GS across all PFAS treatments (a 2.5% change). After 5 days in the mesocosms, 55% of the controls had increased 1 GS from GS 25 to GS 26, while the other 45% of the controls increased 2-3 GS, from GS 25 to GS 27 or 28. In contrast, by the first 5 days of exposure, 24% of PFOA-exposed and 16% of PFOS-exposed frogs were still GS 25 (despite increasing in mass), 58% and 64% of PFOA- and PFOS-exposed frogs were GS 26, and 5% and 0% were GS 27 or 28. Overall, the slight adverse effects on GS, appeared to occur rapidly (within the first 5 days exposure), and were maintained until the end of the experiment, when an approximate 1 Gosner stage difference between the controls and PFAS-dosed frogs was observed. Thus, overall, this study is only useful for identifying unbounded NOEC values.

### **Flynn et al. (2022): Comparative Toxicity of Aquatic Per- and Polyfluoroalkyl Substance Exposure in Three Species of Amphibians**

This study by Flynn et al. (2022) determined the chronic effects of PFOS, PFOA, PFHxS, and 6:2FTS exposure on survival and growth (mass, SVL, Scaled Mass Index (SMI)) on northern leopard frogs, American toads, and eastern tiger salamanders in a measured static renewal waterborne exposure. Animals were exposed for 30 days (frogs and salamanders) or until metamorphosis (toads) to 0, 10, 100, or 1000 µg/L PFAS. Groups of 20 larvae per replicate tank

frogs & toads (GS 25, initial variance not specified) or ten salamanders (1 per cup, Harrison Stage (HS) 46 (no variance as they were all at the same stage) were exposed in 4 replicates. Frogs and toads acclimated to laboratory conditions in 15-L polypropylene tubs and salamanders were acclimated in 1-L cups ( $20 \pm 2$  °C, 12-h:12-h light:dark cycle) for 1 week prior to the start of the experiments. Measured exposure concentrations are listed below:

Species (common name)	PFAS	PFAS Doses ( $\mu\text{g/L}$ )
Northern leopard frogs	PFOS	0.1, 7.74, 122, 1437
	PFOA	0.12, 9.6, 125, 1376
	PFHxS	0.16, 9.6, 120, 1307
	6:2FTS	0.1, 12.6, 175, 1795
American toads	PFOS	0, 5.1, 55, 616
	PFOA	0.4, 10, 94, 873
	PFHxS	0, 7.2, 81, 634
	6:2FTS	0, 8.1, 82, 783
Eastern tiger salamanders	PFOS	0, 4.4, 55, 621
	PFOA	0.05, 11, 102, 868
	PFHxS	0.01, 7.3, 80, 797
	6:2FTS	0, 7.8, 109, 859

Exposure to PFOS, PFOA, PFHxS, and 6:2FTS did not statistically significantly affect the survival or SVL of northern leopard frogs. Exposure to 1437  $\mu\text{g/L}$  PFOS or 1376  $\mu\text{g/L}$  PFOA caused a statistically significant reduction in mass (average (SD) of  $16\% \pm 20\%$  and  $19\% \pm 14\%$ , respectively). The 16% effect for PFOS at 1437  $\mu\text{g/L}$  was considered to approximate to the 20% level of biological significant effects, thus, we considered it a LOEC for PFOS in northern leopard frog, with the NOEC being 122  $\mu\text{g/L}$ . The 19% effect for PFOA at 1376  $\mu\text{g/L}$  was considered approximate to the 20% level of a biologically significant effect, and was considered a LOEC for PFOA in northern leopard frog, with the NOEC being 125  $\mu\text{g/L}$ .

There were no statistically detectable differences in mass for 6:2 FTS; however, exposure to the low dose (9.6  $\mu\text{g/L}$ ) of PFHxS caused a statistically significant reduction in GS by a mean (SD) of  $3.4\% \pm 3.2\%$  (~1 GS). Gosner stages were statistically significantly reduced in the middle dose of PFOA (125  $\mu\text{g/L}$ , reduction in GS of  $3.8\% \pm 3.8\%$ , ~1 GS), and the high dose of PFOS (1437  $\mu\text{g/L}$ , reduction in GS of  $3.6\% \pm 3.8\%$ , ~1 GS). These differences are slight (< 4%) and do not provide strong evidence of a dose-dependent effect on GS, with the possible exception of PFOS (effect at the highest dose). Given this, we did not consider the GS effects to be biologically significant and evidence of a PFAS-dependent effect. As there were no clear adverse effects of PFHxS, so an unbounded NOEC of 1307  $\mu\text{g/L}$  is reported for northern leopard frog. There were no statistically detectable differences in mass or GS for 6:2 FTS, so an unbounded NOEC of 1795  $\mu\text{g/L}$  is reported for northern leopard frog for 6:2 FTS.

Despite the relatively small changes in other measures of northern leopard frog growth in this study, SMI was significantly reduced by 4-13% following exposure to almost all PFAS concentrations (except for the high dose of PFOA, 1376  $\mu\text{g/L}$ ). The lack of effect for the high dose

of PFOA to SMI does not correspond to other biological measurements, since, at this concentration, there was also a statistically significant decrease in mass (19%), a biologically meaningful adverse effect size in an apical endpoint typically considered in ecological risk assessments for aquatic life. These results indicate SMI, as currently calculated in this study, is not as useful an endpoint as mass or SVL for identifying adverse effects in northern leopard frogs in this study. Additional discussion on the uncertain utility of SMI as an endpoint is noted in our detailed discussions of the Flynn et al. (2022) and Hoskins et al. (2022) studies.

Exposure to PFOS, PFOA, PFHxS, and 6:2FTS did not statistically significantly affect the survival of American toads. Exposure to all concentrations of PFOS did not significantly affect mass, SVL or time to GS 46, so an unbounded NOEC of 616  $\mu\text{g/L}$  is reported for American toads for PFOS. Time to GS 46 is a measure of the time it takes an amphibian to complete metamorphosis. The time it takes an amphibian to complete metamorphosis is more ecologically relevant than measuring the GS after an arbitrary number of days of exposure since it represents the time it takes the animal to reach the stage of maturity where it leaves the aquatic environment. Time to a terminal GS is thus a more holistic endpoint that considers the entire developmental process. When both GS at specific time points and time to GS 46 were measured by an experiment, the time to GS 46 was used for risk assessment since it is ecologically relevant rather than a time-specific measurement of GS at the end of exposure. Exposure to all doses of PFOA did not statistically significantly affect mass or SVL; however, exposure to the highest dose (873  $\mu\text{g/L}$ ) did significantly increase the time it took the toads to reach GS 46 by an average (SD) of  $4.9\% \pm 3.5\%$  (~1 day). Given this level of effect is below the level of 20% biological significance, an unbounded NOEC of 873  $\mu\text{g/L}$  is reported for American toads for PFOA. Exposure to all concentrations of PFHxS did not statistically significantly affect time to GS 46, but exposure to the high dose (1307  $\mu\text{g/L}$ ) did cause a significant increase in mass (mean (SD) of  $14.1\% \pm 6\%$ ) and SVL ( $4.1\% \pm 1\%$ ); however, increases in growth are not considered to be an adverse effect. Given the lack of detectable adverse effects, an unbounded NOEC of 634  $\mu\text{g/L}$  is reported for American toads for PFHxS. Detectable adverse effects were not identified for 6:2 FTS, so an unbounded NOEC of 783  $\mu\text{g/L}$  is reported for American toads for 6:2 FTS. The few detectable increases in mass and SVL did not result in a statistically significant effect of any PFAS to SMI in American toads.

Exposure to PFOS, PFOA, PFHxS, and 6:2FTS for 30-days did not statistically significantly affect the survival or mass of eastern tiger salamanders. Exposure to all three concentrations of PFOA did not statistically significantly affect SVL, so an unbounded NOEC of 868  $\mu\text{g/L}$  is reported for PFOA for eastern tiger salamander. Exposure to 55 and 621  $\mu\text{g/L}$  PFOS caused a slight statistically significant increase in SVL (average (SD) of  $2.5\% \pm 2.3\%$  and  $2.6\% \pm 0.5\%$ , respectively). Exposure to all three doses of PFHxS (7.3, 80.2, and 797  $\mu\text{g/L}$ ) statistically significantly increased SVL by a mean (SD) of  $3.7\% \pm 2.8\%$ ,  $3.5\% \pm 1.8\%$ , and  $4.9\% \pm 1.7\%$ , respectively. Exposure to all three doses of 6:2 FTS (7.8, 108.6, and 859  $\mu\text{g/L}$ ) also significantly increased SVL by a mean (SD) of  $4.1\% \pm 1.8\%$ ,  $5\% \pm 1\%$ , or  $6.3\% \pm 2\%$  respectively. Given a lack of detectable adverse effects in survival, mass, and SVL, unbounded NOECs of 621, 797, and 859  $\mu\text{g/L}$  are reported for PFOS, PFHxS, and 6:2 FTS, respectively, for eastern tiger salamander.

Compared to other endpoints, SMI was not a clear metric for identifying adverse effects in eastern tiger salamander. Exposure to 7.3  $\mu\text{g/L}$  PFHxS significantly reduced SMI by 6% and the high dose 797  $\mu\text{g/L}$  (reduced SMI 4%), but there were no statistically detectable reductions in SMI in the middle dose (80.2), indicating a lack of dose response. Exposure to all three doses of 6:2 FTS

reduced SMI by an average of 5 to 9%. There was no statistically significant effect of PFOS on SMI. SMI in PFOA was only reduced in the lowest dose, indicating a complete lack of dose response. Overall, the tiger salamander results from this study indicate PFAS-exposed animals were longer compared to control animals (statistically significant increases in SVL for many doses) and heavier (or similar) mass compared to control animal (although not statistically different from controls). This pattern would seem to indicate healthy animals (i.e., longer and heavier/similar animals), yet, the SMI values generally indicated lower SMI values with a lack of dose response (PFHxS and PFOA), no changes in SMI values (PFOS), or a slight decrease in SMI with increasing dose (6:2 FTS). More work is needed to understand the relationship between SMI to an individual amphibian's health and between SMI and the health of a population before it can be used as a metric for identifying ecologically significant effects on populations.

LOECs and NOECs for PFAS exposure are summarized in the inset table below for all three species:

Species (common name)	PFAS	PFAS NOEC (µg/L)	PFAS LOEC (µg/L)
Northern leopard frogs	PFOS	122	1437
	PFOA	125	1376
	PFHxS	≥ 1307	> 1307
	6:2FTS	≥ 1795	> 1795
American toads	PFOS	≥ 616.2	> 616.2
	PFOA	≥ 873	> 873
	PFHxS	≥ 634.4	> 634.4
	6:2FTS	≥ 782.7	> 782.7
Eastern tiger salamanders	PFOS	≥ 621	> 621
	PFOA	≥ 868	> 868
	PFHxS	≥ 797	> 797
	6:2FTS	≥ 859	> 859

**Foguth et al. (2020): Single and mixture per- and polyfluoroalkyl substances accumulate in developing Northern leopard frog brains and produce complex neurotransmission alterations**

This study evaluated the chronic effects of PFOS (singly) and a PFAS mixture, mimicking AFFF waterborne exposure on survival and growth (mass, snout vent length (SVL), Gosner Stage (GS)), of northern leopard frogs via a 116-day measured, static, outdoor mesocosm study. Frog larvae were exposed to 0 (control), 10 µg/L (12.8 measured PFOS) and a PFAS mixture totaling 10 µg/L containing 4 µg/L PFOS, 3 µg/L PFHxS, 1.25 µg/L PFOA, 1.25 µg/L PFHxA, and 0.5 µg/L PFPeA nominal (5.83, 1.87, 3.87, 1.86, 0.87 µg/L, as measured), in four replicate 150-L mesocosms (25 tadpoles/replicate). Mesocosms were prepared in 150-L cattle tanks inoculated with periphyton and phytoplankton (i.e., algal food resources for leopard frog larvae), as well as zooplankton from the wetland. After setting for twelve days, four control mesocosms and four treatment mesocosms were spiked the PFAS doses. After 7 days the experiment was started by adding 25 tadpoles at GS 25 (initial variance not specified) to each mesocosm (4 reps/treatment). After 30 days, eight

tadpoles were removed from each mesocosm, sacrificed and measured for SVL, mass, and GS. Organisms that reached GS 46 were sacrificed and measured for the same endpoints, as well as time to reach GS 46, and the hearts were collected and tested for neurotransmitters. The water quality conditions during the test were reported as pH of 7.41 to 8.54, DO of 2.1 to 9.4 mg/L and temperature of 13.1 to 29.8°C.

Exposure to PFOS (singly) or the PFAS mixture did not significantly affect survival. There was no effect of PFOS or the PFAS mixture on mass, SVL, or GS at day 30 or by the end of experiment (116 days). Due to the lack of biological relevance of effect size or statistical significance of effects a NOEC of  $\geq 12.8 \mu\text{g/L}$  PFOS ( $\geq 14.3 \mu\text{g/L}$  sum of PFAS in the mixture study) is reported for up to 116-days of exposure.

**Hoover et al. (2017): Uptake and Depuration of Four Per/Polyfluoroalkyl Substances (PFASS) in Northern Leopard Frog *Rana pipiens* Tadpoles**

Hoover et al. (2017) evaluated the chronic toxicity of PFOS, PFHxS, PFOA, and 6:2 FTS (waterborne exposure) on survival and growth (mass, SVL, GS) in northern leopard frog tadpoles in a 40-day measured static renewal study. Frogs were exposed through water spiked with PFOS, PFHxS, PFOA, or 6:2 FTS (nominally 0, 10, 100, 1000  $\mu\text{g/L}$ ) solutions, in two duplicate 15-L plastic aquaria per concentration (35 tadpoles/tank). Water concentrations were renewed every four days and exposure concentrations were measured prior to and after each water change. Treatments consisting of control and exposure to PFOS at three concentrations were conducted in duplicate ( $n = 2$ ) with each tank containing 35 tadpoles. Average concentrations of PFAS in water measured over the test duration are shown in the inset table below:

Species (common name)	PFAS	PFAS Doses ( $\mu\text{g/L}$ )
Northern leopard frogs	PFOS	< 0.22, 7, 64, 820
	PFOA	< 0.22, 12, 110, 1101
	PFHxS	< 0.22, 10, 97, 943
	6:2 FTS	< 0.22, 14, 152, 1431

Tadpoles were exposed for 40 days; every 10 days six animals were randomly sampled from each aquarium. The animals were euthanized, and growth (SVL and GS) was measured (total length at 10 days, SVL otherwise). After 40 days, the depuration phase was initiated by removing animals, cleaning each aquarium with a methanol-soaked sponge, and rinsing to remove adsorbed compound. Aquaria were refilled with clean water; animals were returned to the same aquarium and monitored. Two tadpoles were sampled every 10 days for an additional 30 days. Animals were maintained at  $20 \pm 2 \text{ }^\circ\text{C}$  with a 12-h:12-h light/dark photoperiod for 10 days to acclimate to indoor conditions and were fed a Tetramin slurry *ad libitum*.

Exposure to PFOS for 40 days did not statistically significantly affect survival, and the survival for all PFAS treatments was above 90%. There also was no statistically significant effect of PFOS exposure to SVL. Exposure to 64 or 820  $\mu\text{g/L}$  PFOS caused a statistically significant mean (SD) reduction of GS relative to the control of  $5\% \pm 1\%$  and  $7\% \pm 1\%$ , respectively, (a

decrease of 1.5 to 2 Gosner stages relative to control) by 40 days of exposure. However, due to the small effect size (5-7%) being less than a biologically relevant effect size (20%), an unbounded NOEC of 40-day exposure of northern leopard frogs to PFOS of 820 µg/L is reported.

Exposure to PFOA for 40 days did not statistically significantly affect survival. There also was no statistically significant effect of PFOA exposure to SVL or GS. Thus, due to the lack of biological relevance or statistical significance an unbounded NOEC of 40-day exposure of northern leopard frogs to PFOA of 1101 µg/L is reported.

Exposure to PFHxS for 40 days did not statistically significantly affect survival, and the survival for all PFAS treatments was above 90%. There also was no statistically significant effect of PFHxS exposure to SVL. Exposure to all concentrations of PFHxS (10, 97, or 943 µg/L) caused a mean (SD) statistically significant reduction of GS relative to the control of 5% ± 1%, 4.5% ± 1%, or 4% ± 0.5%, respectively, (a decrease of 1 to 1.5 Gosner stages relative to the control) by 40 days of exposure. However, due to the effect size (4-5%) being less than a biologically relevant effect size (20%), an unbounded NOEC of 40-day exposure of northern leopard frogs to PFHxS of 943 µg/L is reported.

Exposure to 6:2FTS for 40 days did not statistically significantly affect survival. There also was no statistically significant effect of 6:2FTS exposure to SVL or GS. Due to the lack of biological relevance or statistical significance a NOEC of 40-day exposure of northern leopard frogs to 6:2FTS, an unbounded NOEC of 1431 µg/L is reported. A summary of the unbounded NOECs for survival and growth of all four PFAS compounds can be found in the inset table below:

Species (common name)	PFAS	PFAS NOECs (µg/L)
Northern leopard frogs	PFOS	≥ 820
	PFOA	≥ 1101
	PFHxS	≥ 943
	6:2 FTS	≥ 1431

Body mass was not measured during this study. It should be noted that the northern leopard frogs grew in a different manner than those in the Flynn et al. (2021a) study. For example, the Hoover et al. (2017) frogs started exposures at GS 26 and did not change in GS after 10 days (not one animal had changed GS across the controls and three treatment groups for the 4 compounds). In contrast, 5 days following the start of the Flynn et al. (2021a) experiment, controls increased in GS from 25 to GS 26, 27, or 28, which is similar in development of frogs after 20 days of development in the Hoover et al. (2017) study. It is not apparent why the developmental progress of the control frogs in these two studies responded so differently (i.e., no progression in Gosner stage during a 10-day period in in one study compared to a progression of 1 to 3 Gosner stages over a 5-day period in another study). This comparison highlights the likely high natural biological variation in this response metric, and reinforces our position that minor differences in GS (i.e., 5%, 10%, etc.) are likely within the range of natural variation, are not ecologically meaningful, and are not useful in identifying threshold dose levels.

All four compounds were shown to be able to bioaccumulate into the frogs by day 10, with PFOS having the highest bioconcentration factor (BCF) and the other compounds having BCFs below 10. PFOS levels stayed relatively stable in the frogs while the other three compounds decreased over time with all the BCFs being below 1 by day 30. The BCFs appear to be concentration independent for PFOS. For the other three PFAS compounds BCFs were highest in the low dose and generally below 1 at the middle and high dose. Additionally, the depuration half-life in northern leopard frog tadpoles via a 40-day aqueous exposure was similar for the four PFAS compounds ranging from 1.2 to 3.3 days. The following inset table below depicts the calculation of average BCF values (L/kg ww) from this study data:

PFAS	[Water] µg/L	[Whole Body] ng/g ww	BCF (L/kg ww)	Average BCF (L/kg ww)	SD BCF (L/kg ww)
PFOS	7.0	2678	383	243	132
	64	14411	225		
	820	99196	121		
PFOA	12	10	0.8	0.83	0.02
	110	90	0.8		
	1101	941	0.9		
PFHXS	10.0	18	1.8	1.7	0.35
	97	191	2.0		
	943	1226	1.3		
6:2FTS	14	31	2.2	2.1	0.19
	152	330	2.2		
	1431	2666	1.9		

Note: Concentrations assume a 75% moisture content of amphibian tissue.

As all four PFAS did not appear to adversely affect frogs, and concentrations in the tissues of the frogs were measured at the end of the exposure (i.e., 40 days, raw data from Table 1 of Hoover et al. (2017) provided to authors), we also evaluated NOECs in tissue. Mean concentrations of PFOS, PFOA, PFHxS, and 6:2 FTS in frog tissue (above inset table) were approximately 400000, 3800, 4900, and 11000 ng/g dw, respectively. Assuming a 75% moisture content of amphibian tissue, this would correspond to concentrations of PFOS, PFOA, PFHxS, and 6:2 FTS in frog tissue of 100000, 940, 1200, and 2700 ng/g ww, respectively. These concentrations represent unbounded NOEC whole body tissue values for northern leopard frogs.

### **Hoskins et al. (2022): An Environmentally Relevant Mixture of Perfluorooctanesulfonic Acid and Perfluorohexanesulfonic Acid Does Not Conform to Additivity in Northern Leopard Frogs Exposed Through Metamorphosis**

This study by Hoskins et al. (2022) determined the chronic effects of PFOS and PFHxS exposure on survival and growth (mass, SVL, SMI, GS, time to GS 42, time to GS 46) on northern leopard frogs in a measured static renewal waterborne exposure. Animals were exposed until metamorphosis (or 120-days, whichever was longer) to 0, 0.5, and 1 µg/L PFAS. Groups of 20 larvae per replicate tank (GS 25, initial GS variance not specified, mean ± SD snout–vent length

and body mass of  $5.0 \pm 0.4$  mm and  $23 \pm 6$  mg, respectively) were exposed in 4 replicate tanks per exposure concentration. Frogs were acclimated to laboratory conditions in 15-L polypropylene tubs and salamanders were acclimated in 1-L cups ( $20 \pm 0.1$  °C, 12-h:12-h light:dark cycle) for 3 days prior to the start of the experiments. Measured exposure concentrations in PFOS-spiked water was 0.56 and 0.93  $\mu\text{g/L}$ , and measured concentrations in PFHxS-spiked water as 0.45 and 1.2  $\mu\text{g/L}$ . Neither PFOS nor PFHxS was detected in control water. Growth was measured at day 31 (when most control animals were at GS 29), and two additional time points when animals reached GS 42 and GS 46. Concentrations of PFAS in whole frogs was measured at day 31 and when animals reached GS 46 (concentrations at both time points were similar, indicating steady state had been reached within 31 days of exposure). Mean concentrations in tissue of frogs (assuming a 75% moisture content) exposed to 0.56 and 0.93  $\mu\text{g/L}$  were 81 and 189 ng/g ww for PFOS, respectively, while exposure to 0.45 and 1.2 PFHxS resulted in tissue concentrations of 1 and 1.2 ng/g ww for PFHxS, respectively. Mean concentrations measured at day 31 and GS 46 in control frogs was 5 ng/g ww for sum PFAS.

There was no statistically significant effect of exposure to any PFAS on survival. There also was no statistically significant effect of PFAS exposure on length, or SVL at day 31, GS 42, or GS 46.

Hoskins et al. (2022) reported a statistically significant difference between controls and PFHxS exposed frogs for length time required for the metamorphosis from GS 42 to GS 46. Control frogs required approximately 8 days on average. Frogs exposed to 1.2  $\mu\text{g/L}$  PFHxS required an additional 1.1 days than controls, on average, but frogs exposed to 0.45  $\mu\text{g/L}$  PFHxS required approximately 0.5 days less, on average (results from both PFHxS exposures were reported as statistically significantly different from controls). A statistically significant difference was also noted in the 0.56  $\mu\text{g/L}$  (but not 0.93  $\mu\text{g/L}$ ) PFOS exposure group, with the lower PFOS exposed animals requiring an additional  $\sim 0.25$  days to metamorphose from GS 42 to GS 46.

The differences in developmental results for particular portions of the metamorphosis noted by Hoskins et al. (2022) were not quantitatively considered for identifying NOECs or LOECs in this review. We took the approach of examining the entire development process, as opposed to calculating differences in the length of metamorphosis as an adverse effects endpoint. While lengthened metamorphosis is predicted to increase aquatic predation rates (reviewed by Werner 1980, see Touchon et al., 2013 for experimental evidence), total time to metamorphosis or completion of metamorphosis are chronic measures and are routinely used as field-validated, fitness-related endpoints (Semlitsch et al., 1988).

Therefore, we re-examined the Hoskins et al. (2022) data for delays in time required for metamorphosis over the maximum time evaluated in their experiment (i.e., GS 25 to GS 46). As noted above, the most significant difference in the GS 42 to GS 46 transition (noted by Hoskins et al. (2022)) indicated an extra 1.1 days was needed for the animals exposed to 1.2  $\mu\text{g/L}$  PFHxS. The total length of time required for the control animals and 1.2  $\mu\text{g/L}$  PFHxS exposed animals to metamorphose from GS 25 to GS 46 was 100.5 vs 100.7 days, respectively, a difference of 0.2%. Given this result and that there were only two doses tested, we concluded that there are no clear dose dependent effects of PFOS or PFHxS on development in this study.

As in the other Purdue amphibian studies, SMI was uncertain metric of health in PFAS-exposed frogs. There was no statistically significant effect of PFOS on SMI at either time points (day 31

and GS 42) or PFHxS at GS 42. Earlier in the experiment, at day 31, there was a statistically significant reduction in SMI of frogs exposed to 0.45 µg/L PFHxS (but not 1.2 µg/L), from an average (SD) of  $1.1 \pm 0.1$  in the control to  $0.9 \pm 0.1$  in the exposed group (a difference of 19%). This is an uncertain result given the lack of dose response and the use of only 2 PFHxS doses. However, it should be noted that while not statistically significantly different, the 0.45-µg/L PFHxS frogs weighed generally greater (mass (g):  $1.3 \pm 0.1$  exposed,  $1.2 \pm 0.2$  control), and were longer (SVL (mm):  $20 \pm 1.2$  exposed,  $19 \pm 1.2$  control). According to measures of growth (mass and length) typically used as endpoints in toxicity testing, it is clear there is no difference in growth between the control frogs and the 0.45-µg/L PFHxS frogs at day 31, yet, the SMI calculation indicates a difference in body shape. This is potentially a mathematical artifact of the calculation process, as small increases in length resulted in a large effect on the calculated SMI; thus, it is difficult to interpret the biological relevance of SMI. SMI is mathematically determined from measurements on the control animals at a specific point in time or stage (i.e., day 30, or GS 42 (day 54-112), and is not relative over the course of the experiment. Ongoing studies at Purdue are exploring the relationship between SMI and body lipid content and composition, which should shed more insights into the use of SMI as an indicator of health.

Our evaluation determined that there was no statistically significant effects of PFOS or PFHxS on survival or growth (GS, mass, SVL, time to GS 46). We report unbounded NOECs of 0.93 µg/L for PFOS and 1.2 µg/L for PFHxS.

#### **Lech et al. (2022): Effects of Per- and Polyfluoroalkyl Substance Mixtures on the Susceptibility of Larval American Bullfrogs to Parasites.**

Lech et al. (2022) determined the chronic effects of PFOS exposure on survival and growth (mass, SVL, GS, and SMI) on American bullfrogs in a measured static renewal waterborne exposure. Animals were exposed for 62 days to 0, 6, or 10 µg/L PFOS. Groups of 10 larvae per replicate tank (GS 25, initial variance not specified) were exposed in 10 replicate tanks per concentration. Frogs were exposed to in 15-L polypropylene tubs (23°C, 12-h:12-h light:dark cycle). Measured exposure concentrations of PFOS in water was 2.4 and 5.9 µg/L, several-fold different in concentration compared to the nominal concentrations of 6 and 10 µg/L. PFOS was not detected in the control water (i.e., < 0.22 µg/L); however, PFPeA and PFHxA was present in both the PFOS-spiked and control water at concentration of 1.2 and 1.8 µg/L on average.

There were no statistically significant adverse effects of the 62-day PFOS exposure on survival or growth (mass, SVL, GS) of American bullfrogs. There was a trend (not statistically significant) of increased mass and GS following exposure to both concentrations of PFOS. There also was a statistically significant increase in SVL (not dose dependent) from ~10.5 mm (control) to 11.5 mm in the 2.4 or 5.9 µg/L PFOS exposures. Due to the lack of statistically significant adverse outcomes an unbounded NOEC of 5.9 µg/L PFOS is reported.

#### **Other Chronic Studies**

This section details three additional chronic studies that were published by Purdue; however, they were not included in Table S1 and were not included in derivation of NOEC and LOEC values for PFAS in water for reasons identified in detail below.

**Abercrombie et al. (2021): Sublethal Effects of Dermal Exposure to Poly- and Perfluoroalkyl Substances on Postmetamorphic Amphibians**

This study evaluated the chronic effects of PFOS, PFOA, PFHxS, and 6:2 FTS on survival and growth (mass, SVL, SMI) of American toads, eastern tiger salamanders, and northern leopard frogs during a 30-day study. This study differs from the other studies since it used juvenile amphibians (non-larval) that had transitioned to the terrestrial life stage. The exposure system only contained moist moss. Amphibians were exposed dermally to PFAS-spiked moss/substrate (nominal concentrations of 0, 80, 800, 8000 ng/g dw in moss) in four replicate 15-L plastic bins (salamanders & frogs) or 68-L bins (toads), with 12 juveniles/bin. PFAS was quantified in the exposure substrate (moss) at time 0 and day 30. A summary of measured concentrations of PFAS from the exposure substrate (average of measurement taken at day 0 and day 30) is below:

<b>Species (common name)</b>	<b>PFAS</b>	<b>Concentrations of PFAS in Moss Substrate (ng/g dw)</b>
American toads	PFOS	27, 556, 603, 5680
	PFOA	27, 58, 479, 5978
	PFHxS	27, 52, 621, 7055
	6:2FTS	27, 79, 349, 3981
Eastern tiger salamanders	PFOS	7.1, 78, 684, 4340
	PFOA	7.1, 98, 634, 5780
	PFHxS	7.1, 48, 530, 5184
	6:2FTS	7.1, 29, 254, 4001
Northern leopard frogs	PFOS	20, 1544, 1250, 7091
	PFOA	20, 83, 542, 5704
	PFHxS	20, 37, 411, 5446
	6:2FTS	20, 31, 344, 2797

The concentration of PFAS reported in the control moss (inset table above) was reported as the sum PFAS (PFOS + PFOA + PFHxS + 6:2FTS). There were some inconsistencies in dosing the moss, with the time 0 PFOS concentrations for the low dose (80 ng/g dw PFOS) being much higher than the target concentration for American toads and northern leopard frogs (e.g. average PFOS was ~1100 ng/g dw instead of 80 ng/g dw at time 0 for American toads).

No additional manual mixing occurred for the duration of the anuran experiments. For the tiger salamanders, the moss was flipped from top to bottom after 5 days and 24 days to ensure homogeneity given the additional moss depth.

The initial GS for the toads and frogs were GS 26-28 (variance not provided), and HS 46 for salamanders. Animals were acclimated for 2 days prior to exposure, and maintained under standard laboratory conditions: 22 °C (range = 21.4 – 25.6 °C) and a 12-h:12-h light:dark photoperiod.

Because these animals were exposed via moss substrate, not water, we did not include this study in Table S1, which focuses on summarizing studies that could be used to derive NOEC and LOEC

values in water. However, as site-specific ecological risk assessments may have access to measured or model-predicted concentrations of PFAS in vegetative or moss substrates on which amphibians may be living, we reviewed the Abercrombie et al. (2021) study to identify potential criteria that could apply. This review is presented below.

There was no effect of exposure to any of the four PFAS compounds on survival of the three species. There was no dose response of any compound in the three species for mass, SVL, or SMI, with one exception – SMI was significantly higher (~13%) in American toads exposed to moss dosed with 3981 ng/g dw 6:2 FTS. As an increase in SMI was not considered to be an adverse outcome, especially considering the uncertain biological relevance of the SMI metric relative to PFAS exposure (noted previously). Due to a lack of biological relevance of effects and no statistical difference between exposed and controls the following unbounded NOECs are reported for 30-day exposure to moss/substrate dosed with PFAS.

Species (common name)	PFAS	NOEC, PFAS in Moss Substrate (ng/g dw)
American toads	PFOS	≥ 5680
	PFOA	≥ 5978
	PFHxS	≥ 7055
	6:2FTS	≥ 3981
Eastern tiger salamanders	PFOS	≥ 4340
	PFOA	≥ 5780
	PFHxS	≥ 5184
	6:2FTS	≥ 4001
Northern leopard frogs	PFOS	≥ 7091
	PFOA	≥ 5704
	PFHxS	≥ 5446
	6:2FTS	≥ 2797

Please note that the authors of the study pooled biological results from all three PFAS-doses treatment groups together and compared them to the control results, despite the two order of magnitude differences in the concentration of PFAS in the moss substrates among the three different spiking levels. When pooled, some minor changes were detected in growth between the controls and the pooled data from the three PFAS-dosed treatment levels; however, this is not a valid toxicological comparison, and the results cannot be used to identify NOEC or LOEC values.

This study also measured concentrations of PFAS in whole body (northern leopard frogs and American toads) and liver (eastern tiger salamander) in biota samples obtained at the termination of exposures. In the highest dose levels, concentrations of PFOS, PFOA, PFHxS, and 6:2 FTS in leopard frog tissue (whole body, Table S3 of Abercrombie et al. (2012)) were 1406, 33, 142, and 0 (non detect) ng/g dw, respectively, which would be approximately 350, 8.3, 36, and 0 ng/g ww, assuming a 75% moisture content of tissue. These values were two to three orders of magnitude lower than the unbounded tissue NOECs derived from the Hoover et al. (2017) study (100000, 940, 1200 and 2700 ng/g ww, respectively). Concentrations of PFOS, PFOA, PFHxS, and 6:2 FTS in American toad (whole body) in the highest dose level were 3752, 98, 155, and 28 ng/g dw,

respectively, which is approximately 940, 25, 39, and 7 ng/g ww (assuming a 75% moisture content), and were also similarly much lower than the northern leopard frog tissue NOECs. Concentrations of PFOS, PFOA, PFHxS, and 6:2 FTS in tiger salamander liver from the highest dose level were 1229, 46, 260, and 0.13 ng/g dw, respectively, which is approximately 310, 12, 65, and 0.033 ng/g ww (assuming a 75% moisture content), and were also lower than the northern leopard frog NOECs. Overall, the animals in these studies accumulated concentrations of PFOS, PFOA, PFHxS, and 6:2 FTS in tissues that were much lower than the unbounded tissue NOECs we derived from the Hoover et al. (2017) study. This suggests lower uptake from dermal exposure to dosed substrate (moss) in this study than waterborne exposure (Hoover et al., 2017).

**Flynn et al. (2021): Dietary exposure and accumulation of per- and polyfluoroalkyl substances alters growth and reduces body condition of post-metamorphic salamanders**

Flynn et al. (2021) conducted a chronic dietary exposure of PFOS, PFHxS, PFOA, 6:2 FTS to evaluate effects on survival and growth (mass, SVL, SMI) to post-metamorphic salamanders during a 30-day study. The salamander diet consisted of crickets exposed to three different levels of PFAS-spiked food and water, which resulted in concentrations of PFAS in the crickets ranging from ~20-5000 ng/g ww. Each salamander was fed 9 crickets per week, regardless of the salamander’s size, Salamanders were housed in 15-L polypropylene tubs with one salamander per tub, each treatment was conducted with 10 replicates of 1 salamander per treatment. Animals were housed under standard laboratory conditions 22 °C (mean = 22.2 °C, range = 19.5 - 23.6°C) on a 12-h:12-h light:dark cycle.

Concentrations of PFAS in salamander livers of 0.15-2000 ng/g ww PFAS were noted, as measured at the end of the 30-day dietary exposure. These data, along with measured concentrations in the crickets, were used to calculate food-to-salamander liver biomagnification factors (BMFs). A summary of the concentrations for the controls and the 3 different levels of PFAS in crickets is shown in the inset table below:

Species (common name)	PFAS	Concentration in Food (ng/g ww)	Concentration in Liver (ng/g ww)
Eastern tiger salamanders	PFOS	1.3, 62, 463, 1990	1.4, 189, 623, 2000
	PFOA	0.29, 35, 242, 1280	0.16, 2.6, 5.3, 6.3
	PFHxS	0.69, 45, 407, 4840	0.32, 1.9, 11, 59
	6:2 FTS	0.023, 17, 158, 2240	0.013, 0.19, 0.22, 0.15

Because these animals were exposed via the diet, not water, we did not include this study in Table S1, which focuses on summarizing studies that could be used to derive NOEC and LOEC values in water. However, as site-specific ecological risk assessments may have access to measured or model-predicted concentrations of PFAS in amphibian diet items, we reviewed the Flynn et al. (2021) study to identify NOECs and LOECs for PFAS in the diet. This review is presented in the subsequent paragraphs in this section.

The salamanders used in this experiment were measured on day 0 and day 30 of exposure. Unfortunately, treatment groups of the adult animals in some exposure groups were statistically

significantly different from controls on day 0 (start of exposure). For example, the average animals exposed to concentrations of PFOS in the diet of 463 ng/g, ww were 12% lower than controls, and the difference was statistically significant. Flynn et al. (2021) present the growth data as a difference in growth from day 0 to day 30 (e.g., control animals gained 3 grams between day 0 and day 30, so a result of 3 grams is reported and statistically evaluated in their paper).

Typically, in risk assessment the size of the animals at the end of exposure is compared. We re-examined the Flynn et al. (2021) data by comparing the PFAS-exposed animals to control animals at the end of exposure, and determined if their relative size differences changed due to exposure. When we examined the differences this way the largest effect of PFOS dietary exposure was the two highest doses of PFOS (463 or 1990 ng/g food) which caused a statistically significant decrease in mass of 10.6 or 8.7%, respectively, compared to controls, which we do not consider to be a biologically significant effect (i.e., effects are < 20%). For PFOA exposure the low dose (35 ng/g food) caused a statistically significant decrease in mass of 13.9% but no significant effects at the two higher doses (242 and 1280 ng/g food), which we do not consider to be biologically relevant. There was no statistically significant effect on mass for PFHxS or 6:2FTS exposure.

The growth data are summarized in the tables below. There were some statistically significant, response dependent differences between controls and PFOS-exposed salamanders for mass at the end of the experiment, but the adverse effect sizes were approximately 10%, which is below the 20% threshold for ecologically significant effects in this review.

PFAS	Conc.in Food (ng/g ww)	Conc. in Liver (ng/g ww)	Average Mass g (SD) Day 30		% Control Mass Day 30
Control	2.3	1.9	18.9	(1.1)	100.0
PFOS	62	189	18.7	(1.3)	98.7
PFOS	463	623	<b><u>17.0</u></b>	<b><u>(1.1)</u></b>	<b><u>89.6</u></b>
PFOS	1990	2000	<b><u>17.3</u></b>	<b><u>(2.1)</u></b>	<b><u>91.3</u></b>
PFOA	35	2.6	<b><u>16.3</u></b>	<b><u>(3.3)</u></b>	<b><u>86.1</u></b>
PFOA	242	5.3	18.6	(1.7)	98.5
PFOA	1280	6.3	18.5	(1.6)	97.7
PFHxS	45	1.9	17.9	(1.4)	94.4
PFHxS	407	11	18.9	(1.3)	99.6
PFHxS	4840	59	17.9	(1.6)	94.4
6:2FTS	17	0.19	18.4	(2.0)	97.4
6:2FTS	158	0.22	19.0	(1.7)	100.5
6:2FTS	2240	0.15	17.7	(1.6)	93.8

Notes:

**Bold and underlined** values for mass are statistically significantly different than the control (ANOVA or ANOVA on ranks, Dunnett/Dunn's posthoc test).

Control concentrations are sum PFAS, individual PFAS in food and are 1.3, 0.29, 0.69, 0.023 (PFOS, PFOA, PFHxS, 6:2 FTS) ng/g ww, and in the liver are 1.4, 0.16, 0.32, 0.013 (PFOS, PFOA, PFHxS, 6:2 FTS) ng/g ww.

SVL data for this study are presented in the inset table below. No statistically significant adverse differences in SVL were noted between PFAS-exposed animals and control animals at the end of

the exposure (day 30). Some animals in the PFAS-exposed groups were longer than control animals at the beginning of exposure, and these animals were generally longer (by a similar percentage) than animals at day 30.

PFAS	Conc.in Food (ng/g ww)	Conc. in Liver (ng/g ww)	Average SVL mm (SD)		% Control Mass Day 30
Control	2.3	1.9	81.7	(2.4)	100.0
PFOS	62	189	<b><u>85.3</u></b>	<b><u>(2.2)</u></b>	<b><u>104.3</u></b>
PFOS	463	623	82.0	(2.8)	100.3
PFOS	1990	2000	<b><u>84.9</u></b>	<b><u>(2.4)</u></b>	<b><u>103.9</u></b>
PFOA	35	2.6	81.5	(6.1)	99.7
PFOA	242	5.3	84.3	(6.6)	103.1
PFOA	1280	6.3	83.7	(2.1)	102.4
PFHxS	45	1.9	84.1	(2.9)	102.8
PFHxS	407	11	<b><u>86.3</u></b>	<b><u>(2.0)</u></b>	<b><u>105.6</u></b>
PFHxS	4840	59	80.2	(3.2)	98.2
6:2FTS	17	0.19	79.6	(3.6)	97.3
6:2FTS	158	0.22	81.9	(2.1)	100.2
6:2FTS	2240	0.15	79.6	(1.8)	97.4

Notes:

**Bold and underlined** values for SVL are statistically significantly different than the control (ANOVA or ANOVA on ranks, Dunnett/Dunn's posthoc test).

Control concentrations are sum PFAS, individual PFAS in food are 1.3, 0.29, 0.69, 0.023 (PFOS, PFOA, PFHxS, 6:2 FTS) ng/g ww, and in the liver are 1.4, 0.16, 0.32, 0.013 (PFOS, PFOA, PFHxS, 6:2 FTS) ng/g ww.

There was no statistically significant effect of exposure to PFOS on survival compared to controls. Based on the low levels of effects on growth (body mass, in the highest exposures ~ 10%) an unbounded NOEC of 1900 ng/g ww (diet) is reported for PFOS. If a 10% effect size is considered biologically significant, a NOEC and LOEC of 62 and 463 ng/g ww (diet), respectively, could be identified from this study.

There was no statistically significant effect of dietary exposure to PFOA on survival compared to controls. Based on the lack of biological relevance of effects and no dose response, an unbounded NOEC of 1300 ng/g ww (diet) is reported for PFOA.

There was no statistically significant effect of the 30-day dietary exposure to PFHxS on survival or mass. Based on the lack of biological relevance of effects or dose response between exposed and controls an unbounded NOEC of 4800 ng/g ww (diet) is reported for PFHxS.

There was no statistically significant effect of 30-day exposure to 6:2 FTS on survival. Exposure to 6:2 FTS also did not statistically significantly affect weight or SVL. Based on the lack of biological relevance of effects and no statistical difference between exposed and controls an unbounded NOEC of 2200 ng/g ww (diet) is reported for 6:2 FTS.

Note that PFOS, PFHxS, PFOA, 6:2 FTS was detected in the control livers (0.013 – 1.4 ng/g ww (liver)). It is unclear why salamanders were not split up into groups of similar size prior to the initiation of the experiment. SMI was included in the published paper, but was not included in the supplemental data, so we were unable to assess it. Effects of the PFAS on SMI was variable, but PFOA, PFOS and PFHxS reduced SMI at the lower two doses (and the highest dose for PFOS).

It is also worth noting that based on the initial SVL reported animals ranged in length relative to the control from -3% to +8% with two significant differences that were due to randomization of the animals and not due to chemical exposure. Additionally, for mass the initial animals ranged in size from -12% to +3% with two significant differences -12 and -11% also due to random chance and not due to exposure. This suggests that the natural variance in amphibian animal size could be as high as 10%.

### **Foguth et al. (2019): Developmental exposure to perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) selectively decreases brain dopamine levels in Northern leopard frogs**

This is a companion paper to the Flynn et al. (2021a) study. It had the same experiment design and exposure conditions. While the Flynn et al, (2021a) study focused more on growth effects, this study focused on the effects of PFAS on neurotransmission. Exposure for 30 days to spiked sediment resulting in 0.06-15.6 or 2.9-66.2 µg/L water PFOS or PFOA respectively, did not affect glutamate, GABA, Ach, HVA, NE, 5-HT, or DOPAC levels. Exposure to PFOA and PFOS caused a significant dose dependent decrease in DA which was significant at 0.96 and 15.6 µg/L PFOS (34.3-41.4%) or 66.2 µg/L PFOA (48.6%) and a significant dose dependent increase in DA turnover at the highest dose of PFOS (233%) and PFOA (317%). This study is not included in Table S1 because these endpoints are not typical apical endpoints used in the derivation of NOEC and LOEC screening criteria.

### **Acute Studies**

This section details acute data that were published by Purdue; however, this data were not included in Table S1 and were not included in derivation of NOEC and LOEC values for PFAS in water because they focused on acute endpoints that were less sensitive than chronic endpoints.

### **Flynn et al. (2019): Acute and chronic effects of perfluoroalkyl substance mixtures on larval American bullfrogs (*Rana catesbeiana*)**

In addition to the chronic effects (noted previously and shown in Table S1), this study evaluated the acute effects of PFOS and PFOA on the American bullfrog (*Lithobates catesbeiana*) during a

96-hour unmeasured, static study. American bullfrogs were exposed to nominal test concentrations of 0 (control), 10,000, 25,000, 50,000, 75,000, 100,000, 150,000, 300,000 and 500,000 µg/L PFOS or 0, 10,000, 100,000, 250,000, 500,000, 750,000, 1,000,000, 1,250,000, 1,500,000, 1,750,000, 2,000,000 and 2,500,000 µg/L PFOA. Each treatment contained 10 replicates with 1 Gosner Stage 25 tadpole in a 0.25-L plastic tub maintained at 21°C and a 12-h:12-h light:dark photoperiod. This experimental design resulted in median lethal concentration (LC<sub>50</sub>) curves with the majority of concentrations tested causing either 0 or 100% mortality, with only two points in the exponential phase, creating uncertainty in the model LC<sub>50</sub> estimates. Additionally, the two points in the exponential phase of the PFOS treatment were non-monotonic. Despite these limitations, LC<sub>50</sub> were generated. PFOS was found to be 10x more toxic than PFOA, with an LC<sub>50</sub> of 144,000 µg/L (95% confidence interval: 101,000 – 187,000 µg/L) for PFOS compared to 1,004,000 µg/L (95% confidence interval: 910,000 – 1,099,000 µg/L) for PFOA. Following the single compound toxicity tests, binary mixtures were tested using a toxic unit approach. This second experiment showed that PFOS and PFOA generally follow additive mixture toxicity.

### **Hoover et al. (2019): In vitro and in silico modeling of perfluoroalkyl substances mixture toxicity in an amphibian fibroblast cell line**

The study by Hoover et al. (2019) determined the acute toxicity of PFOS, PFOA, PFHxS, and PFHxA to a *Xenopus tropicalis* cell line, with a static 48h nominal exposure. First acute toxicity tests were done exposing the cells to a range of concentrations, and the calculated median inhibitory concentrations (IC<sub>50</sub>) (95% CI) in µg/L were as follows: PFOS 212000 (192000-231000), PFOA 501000 (461000-542000), PFHxS 499000 (418000-580000), PFHxA 2217000 (2014000-2419000). Binary mixtures were conducted and most PFAS combinations followed strict additivity. However, one notable exception is the PFAS + PFOA mixture which demonstrated weak synergism

### **Tornabene et al. (2021): Relative acute toxicity of three per- and polyfluoroalkyl substances on nine species of larval amphibians**

The study by Tornabene et al. (2021) established 96-h acute toxicity values for 9 amphibian species exposed to PFOS and PFOA, as well as 2 species to PFHxS. Like Flynn et al. (2019) bullfrog study, PFOS was 8X more toxic than PFOA for all species. There was little difference among species in LC<sub>50</sub> values for PFOS, but American bullfrogs tended to be a bit less resistant to PFOS than the other species. Toxicity of PFOA was split between the species, with one group (small-mouthed salamanders, tiger salamanders, American toads, grey frogs and leopard frogs) being more sensitive to PFOA, while the other species, including Jefferson salamanders, American bullfrogs, green frogs and wood frogs, were less sensitive. Of the two species were exposed to PFHxS, survival was significantly lower for green frogs than for American bullfrogs. Toxicity of PFAS also varied between developmental stages of larvae. Gray tree frogs were more sensitive at later developmental stages, and small-mouthed salamanders were more sensitive at earlier developmental stages.

Overall, the most sensitive toxicity metric estimated in the Tornabene et al. (2021) study was a 96-h 10% lethal concentration (LC<sub>10</sub>) value of 10000 µg/L for PFOS in small-mouthed salamander. This value is one to two orders of magnitude higher than the sublethal NOEC and LOEC values

from growth that is highlighted in our review, indicating that acute lethality thresholds for PFAS in amphibians are less sensitive than those for sublethal effects.

**Additional references cited in the supplemental**

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