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Toxicity of PFAAs to freshwater mussels

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Partial life-cycle and acute toxicity of perfluoralkyl acids to freshwater mussels

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Abstract

Freshwater mussels are among the most sensitive aquatic organisms to many contaminants and have complex life-cycles that include several distinct life stages with unique contaminant exposure pathways. Standard acute (24 to 96-h) and chronic (28-d) toxicity tests with free larva (glochidia) and juvenile mussels are effective at generating data on contaminant effects at two discrete life-stages, but do not incorporate effects on brooded glochidia. We developed a novel partial life-cycle assay that incorporates exposures to brooding adult female mussels and used this method in combination with acute toxicity tests to assess adverse effects of perfluoroctanesulfonic acid (PFOS) and perfluoroctanoic acid, (PFOA) on freshwater mussels. Fatmucket (*Lampsilis siliquoidea*) were exposed to PFOS at two life stages: brooding glochidia (in marsupia) for 36 d and free glochidia in water for 24 h. In standard acute tests with glochidia (24- 48 h exposures) and juveniles (48-96 h exposures) of fatmucket and black sandshell (*Ligumia recta*) glochidia were 8 to 25 times more sensitive than juveniles. PFOS significantly reduced duration of glochidia viability and reduced probability of metamorphosis at concentrations 3,000 times lower than the most sensitive acute endpoint (24-h-EC50). The partial life-cycle test is adaptable to a variety of endpoints and research objectives, and is useful for identifying adverse effects at contaminant concentrations below those required for an acute lethal response.

Keywords: Perfluoralklyl acids, PFAAs, Perfluorinated compounds, AIC, Unionidae

INTRODUCTION

Freshwater mussels (Order Unionoida) are among the most imperiled taxanomic groups worldwide [1]. In North America, as many as 72% of the native fauna are listed as endangered, threatened, or of special concern, and more than 7% are likely extinct [1]. Understanding the effects of contaminants on freshwater mussels is critical to conservation efforts and environmental risk assessment as mussels are often among the most sensitive species to aquatic contaminants [2-4]. Freshwater mussels have a complex life history that includes internal fertilization of gametes, gestational brooding of larvae (glochidia) within the adult female for one to many months and obligate parasitic metamorphosis on the gills or fin epithelial tissue of host fish, followed by excystment from the host and growth through a juvenile stage to adulthood [5]. Each discrete life-stage is critically important to the recruitment of breeding adults in a population [6] and represents a unique exposure period to contaminants [3].

Internationally approved standard acute and chronic toxicity test guidelines have been developed for larval and juvenile freshwater mussels [7]. Acute tests consist of aqueous contaminant exposures to glochidia for 24 and 48 h, and to juveniles for 24, 48, and 96 h and are efficient methods for determining median effective (EC50) and lethal concentrations (LC50) [7]. Chronic toxicity tests (28 d) of water-only contaminant exposures and aqueous exposures in the presence of sediment, have also been effective at determining lethal and sublethal (e.g., growth effects) contaminant concentrations on juvenile mussels [7-9]. However, because these approaches focus on only two distinct life-stages of freshwater mussels, the relevance to environmental exposures is limited and may differ among toxicants [3]. Further development of partial life-cycle assays is warranted to determine the most-sensitive life-stages to environmental contaminants, and to improve predictive power of risk assessments.

Perfluoralkyl acids (PFAAs) uniquely repel both water and oil, which explains their primary use in surface protection of carpets, upholstery, paper, food containers, and fabric as well as in fire suppressants [10, 11]. PFAAs are fully fluorinated organic compounds with terminal carboxylate or sulfonate groups. Fluorine-carbon bonds have greater polarity and thus are much stronger than carbon-hydrogen bonds, resulting in environmental persistence of many chemicals in this class [10]. The PFAAs with eight or more fluorocarbons (e.g., perfluoroctanesulfonic acid [PFOS] and perfluoroctanoic acid [PFOA]) exhibit moderate tendencies to bind to blood serum proteins, can accumulate in hepatic tissue, and demonstrate biomagnification in aquatic food webs [10, 12-14]. Concentrations of PFAAs in the aquatic environment are typically in the ng/L range [13], but concentrations have been measured near 1 μg/L in areas of long-term pollution [11, 15], and in excess of 2,200 μg/L in locations of accidental spills of fire-fighting foams [16, 17]. Several studies have reported PFAA bioconcentration in estuarine bivalves [12, 14], but the effects of PFAAs on native freshwater mussels have not been published to date. Persistent exposure to PFAAs may result in decreased reproduction of aquatic species [13, 18]; thus, the effect of PFAAs on freshwater bivalves needs to be addressed.

In the present study, we combined use of a novel partial life-cycle approach with standard acute toxicity test methodology to assess the effects of PFAAs on freshwater mussels. In the partial life-cycle study, mussels were exposed to PFOS at one or both of two discrete life-stages: (1) in marsupia exposure: glochidia brooding within the adult female, and (2) free glochidia exposure: glochidia in water. This approach facilitated identification of the most sensitive exposure period for larval mussels and presents a unique approach to complement current acute and chronic toxicity tests for freshwater mussels [7].

METHODS & MATERIALS

Test organsims

Fatmucket *Lampsilis siliquoidea* (Barnes) and black sandshell *Ligumia recta* (Lamarck) were used in acute toxicity tests in the present study; however, only fatmucket were used in the partial life cycle tests and juvenile toxicity tests. Brooding female fatmucket were collected from the Silver Fork of Perche Creek in Boone County, Missouri and brooding female black sandshell mussels were collected from the Meramec River in Jefferson County, Missouri. Both rivers support healthy, stable mussel communities. Juvenile test organisms were propagated with previously published [6] culture methods at Missouri State University and the University of Georgia. Juvenile fatmucket metamorphosed on largemouth bass (*Micropterus salmoides*) and black sandshell juveniles metamorphosed on walleye (*Sander vitreus*). Glochidia were <24 h old at the start of each acute toxicity test and juveniles ranged in age from 4 to 6 weeks at the time of testing.

Partial life-cycle test

Brooding female fatmucket collected from Perche Creek (Boone County, MO) in March 2009 were shipped overnight to the University of Georgia. Mussels were packaged in individual plastic bags with 1 L of water in a cooler with ice. Upon delivery, water temperature was 9 to 11°C and mussels were unpacked and scrubbed with a non-abrasive sponge to remove any visible periphyton or debris from the periostricum. Mussels were held in a 530-L Living Stream (Frigid Units Inc.) with natural pond water and approximately 5 cm of quartz/silica sand (Playsand, Quickcrete Products Corp). In the Living Stream, pond water was renewed once per week and holding temperatures were maintained at 5 to 10°C to inhibit parturition of glochidia

[19].

Brooding female mussels (in marsupia exposure) were exposed to PFOS (0, 1, 100 µg/L) in water for 36 d followed by extraction of the glochidia and then a 24-h aqueous PFOS exposure (0, 1, 100 µg/L) with the free glochidia (Fig. 1). We assessed the effects of PFOS on glochidia survival and duration of viability to a subset of the glochidia exposed at each stage. All remaining free glochidia at the end of the 24-h aqueous exposure were then infested on host fish (largemouth bass, *Micropterus salmoides*) to test metamorphosis success to the juvenile stage. We used this approach to reflect environmentally realistic conditions of exposure for glochidia in the different stages and to determine the stage at which glochidia were more sensitive to PFOS exposure.

Only adult fatmucket with initial glochidia viabilities > 70% were used in the partial-life-cycle study. We used a 19-guage hypodermic needle and dechlorinated city water to flush glochidia from two interlamelar spaces (i.e., watertubes) from the marsupial demibranchs [20] of each female. Ten subsamples of 30 to 50 glochidia were assessed for viability using the shell-closure response to NaCl [7]. Twelve adult mussels were identified as having adequate viability and four individuals were randomly assigned to each of three marsupial exposure treatment groups. Exposures (36 d) were conducted in 3.8-L glass jars with one adult mussel per jar. Each jar was aerated and all jars were maintained in an environmental chamber. Initial temperatures were similar to the holding temperature (mean = 7.5° C) for the first 7 d of the exposure, and then gradually increased 2° C per day to approximately 15° C and maintained for the remainder of the experiment. All glassware was triple washed with methanol, and preconditioned with PFOS treatment concentrations for 24 h prior to introduction of mussels. All in marsupia exposures were conducted in filtered, dechlorinated tap water held for > 24 h in the experimental

chamber to reach target temperature prior to water changes. Mean hardness (47.5 ± std. dev. 9.2 mg $CaCO_3$/L), and alkalinity (34.8 ± 4.1 mg $CaCO_3$/L) were measured by titration twice weekly (n = 8) prior to water changes. Water changes (100%) were conducted daily and water quality (pH, temperature, and dissolved oxygen) was measured prior to water change with a Hydrolab Quanta multiprobe (Hach Company) in one replicate of each treatment group. Replicates used for water quality measurements were changed daily to allow measurements from all four replicates every 4 d. For all treatments, water temperature ranged from 14.6 to 16.1°C, dissolved oxygen ranged from 6.1 to 7.3 mg/L, and pH ranged from 7.6 to 8.5 but did not differ across treatments.

After the 36-d exposure, adult mussels were removed from exposure vessels and approximately 120,000 glochidia were gently flushed (with a syringe) from each mussel. Subsamples of 200 to 250 glochidia from each mussel were used to determine viability immediately following the in marsupia exposure using the same procedure as above. Remaining glochidia were used for the 24-h aqueous PFOS exposure (see below). Length and wet weight of each female was recorded and two mussels from each treatment group were euthanized, removed from their shells and whole soft tissues were frozen at -80° C for later chemical analysis.

Glochidia were pooled from females within each in marsupia treatment to average individual effects among females. We then divided each group of free glochidia into approximately equal aliquots for the 24-h aqueous exposure to PFOS (Fig. 1). Glochidia from treatment groups of 1 μg/L and 100 μg/L (in marsupia exposures) were each divided into four aliquots for treatment at the corresponding PFOS concentration-and four aliquots to be treated with only dilution-water during the 24 h exposure. Glochidia from the control groups for the in marsupia exposure were divided into twelve aliquots, four of each treated with 0, 1, or 100 μg/L

of PFOS, respectively. Therefore, the exposure regimen resulted in four replicates for each of seven treatment groups (Fig. 1) for use in glochidia viability duration and metamorphosis experiments. Free glochidia PFOS exposures (24 h) were conducted in 150 ml glass beakers with 100 ml of dechlorinated tap water. All glassware was preconditioned with target PFOS concentrations for 24 h and a water change was performed prior to introduction of glochidia. Exposures were conducted in an environmental chamber at 20°C.

Following the 24-h free glochidia exposure, a subsample of glochidia from each replicate was inoculated on host fish, largemouth bass, to determine if PFOS exposure affected the ability of glochidia to metamorphose to the juvenile stage. Among all treatments, host fish weights ranged from 4.7 to 12.5 g and lengths ranged from 6.4 to 9.2 cm and fish size did not differ among treatments. Inoculations were conducted on individual fish in 1 L of filtered dechlorinated tap water in a 1.5 L glass aquarium for 15 min. Each aquarium was aerated to suspend glochidia and facilitate attachment to host fish. Immediately prior to inoculation, glochidia density was adjusted to approximately 1,000 viable glochidia/L, based on glochidia viability assessed on a subsample of 50 to 100 glochidia using the NaCl shell-closure method [7]. After a 15-min inoculation period, the fish were rinsed to remove unattached glochidia and were placed in individual 3-L tanks in a recirculating aquaculture system (Aquatic Habitats, Inc.). The outflow of each tank was fitted with a 150-μm mesh cup to collect sloughed glochidia and metamorphosed juveniles. Cups were monitored every 24 to 48 h for 19 d following inoculation. Metamorphosis success was calculated for each fish as the proportion of successfully metamorphosed juveniles to the total number of juveniles and glochidia sloughed off each fish.

We also tested duration of viability for glochidia from each treatment. Aliquots of approximately 2,000 glochidia from each treatment were placed in 100 ml of dechlorinated tap

water in 150-ml glass beakers. All aliquots were kept in an environmental chamber at 20° C and 50% of the water was replaced every 48 hr. Viability, as determined by valve closure response to NaCl, was determined every 48 hr for up to 8 d for five subsamples of 30 to 50 glochidia from each aliquot. Viability duration was assessed at 48 h time points rather than every 24 h because this was more logistically feasible given that metamorphosis success was also assessed every other day, thus allowing for alternating efforts on these two assays. Twenty-four hour time points of viability duration would add more precision to the data and are recommended for future studies if resources are available.

Acute toxicity tests

Standard acute toxicity tests of PFOS and PFOA with glochidia and juvenile fatmucket and black sandshell were carried out at North Carolina State University. For tests with glochidia, the organisms were shipped from Missouri State University in coolers via overnight courier. Upon arrival, viability of glochidia was assessed by exposing three sub-samples of 50 to 100 glochidia (each) to a saturated NaCl solution, which initiates shell closure in viable glochidia. We used glochidia for toxicity tests only if initial viability exceeded 90%, in accordance with standard guidelines [7]. The average temperature of culture shipping water at the time of receipt was 19.2 °C (range 17.1 to 21.4 °C). Tests were 48-h non-aerated static experiments. At 24 and 48 h, viability (i.e., shell closure) was assessed with the addition of a saturated NaCl solution to a subsample of 50 of the 150 glochidia from each of three replicates per treatment.

Juvenile mussels were also shipped in coolers via overnight courier, and average shipping water temperature was 22 °C (range 18.2-23.5 °C) at the time of receipt. Upon arrival mussels were acclimated to the test temperature and test water by adjusting their shipping temperature

with three 50% water replacements using dilution water held at 20°C. Mussels were given a 48 h acclimation period once the target test temperature of 20°C was reached. Acute toxicity tests consisted of 96 h non-aerated static tests with 90% water renewal at 48 h. Survival (based on foot movement inside or outside of the shell) was assessed visually (7 mussels per treatment replicate and 10 mussels per control replicate) in each of three replicates per treatment at 48 and 96 h with an Olympus SZ61 microscope [7, 21].

Each acute test consisted of three replicates of each of six test chemical concentrations (0.005, 0.05, 0.5, 5, 50, 500 mg/L PFOS or PFOA) and a dilution-water control. A dilution factor of 10 was used because no previously published tests of acute toxicity of PFAAs on freshwater mussels were available at the time of study design. A narrower range of concentrations is recommended for acute toxicity studies when a preliminary range has previously been established [7].

Sodium chloride (NaCl) was used as a reference toxicant for quality assurance purposes; reference toxicant tests have been used in previous studies with glochidia to serve as a measure of relative health and condition of test organisms [3, 21]. The reference toxicant tests were conducted by preparing treatment concentrations ranging from 0.25 to 8.0 g NaCl/L with 7 concentrations and a dilution factor of 0.5. Concentrations of NaCl were confirmed at the start of the test with a salinity meter (YSI 30, Yellow Springs Instruments) and were within 0.05 g/L of target in all treatments. Glochidia viability was assessed at 24 and 48 h, as described for PFOS and PFOA.

Reconstituted hard water (hardness 160-180 mg/L as CaCO3) was used as dilution water for all toxicity tests [24] and was prepared by adding reagent-grade salts ($CaSO_4$, $2H_2O$, $MgSO_4$, KCl, and $NaHCO_3$; Fisher Scientific) to deionized water [24]. Standard methods were used for

measurement of all water quality parameters as described elsewhere [21]. For all PFAA acute tests, alkalinity ranged from 97 to 110 mg $CaCO_3$/L with a mean of 104.4 mg $CaCO_3$/L, hardness ranged from 132 to 162 mg $CaCO_3$/L with a mean of 149.6 mg $CaCO_3$/L, conductivity ranged from 514 to 643 µs/cm with a mean of 556.5 µs/cm, pH ranged from 8.05 to 8.56 with a mean of 8.46, and dissolved oxygen ranged from 8.16 to 9.46 mg/L with a mean of 8.62 mg/L (n = 12 for alkalinity and hardness, n = 55 for all other parameters).

Test chemicals & quantification

Chemicals (PFOS, >98% purity; PFOA, 96% purity) were purchased from Fisher Scientific or Sigma Aldrich. Certified NaCl (ACS grade, Fisher Chemical) was used in reference toxicant tests. Analyses of PFAA concentrations in water and tissue samples were conducted at the United States Environmental Protection Agency's (U.S. EPA) National Exposure Research Laboratory (NERL) in Research Triangle Park, NC, USA.

During the 36-d in marsupia exposure, water samples for PFOS analysis were collected on day 10 and 11 of the exposure. We collected 15 ml from two replicates of each treatment, one immediately prior to renewal and another at 15 min following the water change. Samples were stored in 15-ml conical centrifuge tubes (BD Falcon; Becton, Dickinson & Company) at 4°C. Water samples were shipped overnight to NERL for solid-phase extraction (SPE) and high-performance-liquid-chromatography/mass-spectrometry (HPLC/MS) analysis following the protocol of Nakayama et al. [22] for water samples with target concentrations less than 0.1 µg/L PFOS. For target PFOS concentrations over 1.0 µg/L, water samples were directly injected due to sufficiently high analyte concentrations. Two standard curves were used to quantify PFOS water concentrations during the partial-life-cycle experiment: low range (0.5, 0.25, 0.5, 0.75, 1.0,

2.5, 5.0 µg/L) and high range (1, 5, 10, 25, 50, 100, 150 µg/L). Two replicate samples were measured at each standard concentration. Accuracy (recovery) of PFOS in the low range standard curve ranged from 89.5 to 123% (n=7) and for the high range standard curve accuracy was 85.3 to 123% (n=7).

Adult mussel tissue samples were collected immediately following the 36-d in marsupia exposure from two replicates in each treatment and stored at -80° C. Samples were shipped to NERL for PFOS analysis. Whole tissue homogenization, SPE, HPLC/MS, and quality assurance procedures followed were based on modifications of the methods previously described by Ye et al. [23] and Delinsky et al. [24]. Recovery of PFOS in standards used for tissue analysis ranged from 97.2 to 104.5% (n=7).

At the start of each acute toxicity test, water samples were composited from all replicates of each treatment concentration and were shipped overnight to NERL for PFOS or PFOA analysis as described for water samples in the partial lifecycle test. Samples were stored in 15-ml conical centrifuge tubes (BD Falcon; Becton, Dickinson & Company) or 1-L Nalgen HDPE bottles at 4° C until analysis. Standard curves for PFOS and PFOA ranged from 5 to 500,000 ng/ml (ten-fold dilution series). Recovery of PFOS standards ranged from 95.2 to 102% (mean 98.3%, n=6) and recovery of PFOA standards ranged from 91.2 to 108% (mean 98%, n=6). All tissue and water samples were analyzed in duplicate and relative standard deviation was <20% for all samples.

Statistical analyses

For the partial life-cycle test, we used a mixed-model logistic regression framework with

multi-model inference to assess the effects of exposure type (i.e., in marsupia or free glochidia) and PFOS concentration (0, 1, 100 µg/L) on viability duration and metamorphosis success. This approach is different from traditional ANOVA tests in that it allows for mediation of random-effects of nested variables. Additionally, data are not subject to the assumptions of a normal distribution as in ANOVA and allow for more predictive measurements of effect than nonparametric statistics [25]. We incorporated Akaike's information criteria (AIC) to assess the relative importance of competing regression models. Model selection through AIC is an established approach that is gaining popularity in analysis of ecological data, and incorporates the principle of parsimony to determine the most efficient explanations in likelihood models [26].

All statistical tests were conducted in the R statistical programming platform [27]. Count data from viability assessments and host-fish metamorphosis experiments were expressed as a binary response where 0 is a failure and 1 is a success (i.e., viability: 0 = nonviable, 1 = viable; metamorphosis: 0 = untransformed glochidia, 1 = metamorphosed juvenile). This approach resulted in a hierarchically structured dataset, where an individual glochidia or juvenile response is nested within a group (i.e., aliquot beaker for viability assessment and fish during a host-fish transformation). To account for variation among each group within a treatment, we used a mixed-model logistic regression with the lme4 package [28] to model an intercept that varies randomly among groups (i.e., a random effect) unrelated to treatment. The lme4 package allows modeling of more than one random effect [25], and in the viability duration analysis, we used a second random effect parameter to assess changes in slope variation among groups at each day post removal of glochidia from the parent mussel.

We fit generalized linear models (GLM) and mixed models of the response parameter and

used AIC [26] to compare relative support for the same models with random effects. We used the model (mixed effects or GLM) with the lowest AIC value for further model selection procedures. AIC values were considered directly comparable between GLM and mixed models because the functions used to calculate the maximum likelihood estimates for each model used equivalent algorithms and both model types use the Laplace approximation to determine the log-likelihood and AIC values (personal communication: Dr. Douglas Bates, University of Wisconsin, Madison). With the most accurate model (mixed-effects or GLM), we constructed several competing candidate models to assess the relative importance of each descriptive variable on the response variable and used AIC and relative importance weights (w_i) to determine the most parsimonious model (Table 1) [26]. We used the parameter estimates (i.e., logits, or log odds) of fixed-effects from the best fitting model to assess the effect of that parameter on the response. Wald statistics (z) and standard errors were also calculated, and the level of significance was considered $p < 0.05$. Wald statistics are a standard statistic on the statistical significance of a logit from the null model in a logistic regression and are similar in interpretation to an F-value in an ANOVA [25, 29]. We converted logits to probability estimates using the logit link function [29] and calculated 95% confidence intervals around the probability estimate using the Wald standard error of the logit multiplied by 1.96.

For acute toxicity tests, nominal concentrations of PFOS, PFOA, and NaCl were used to calculate the median effective concentrations and 95% confidence intervals (CIs) using the Trimmed Spearman-Karber method with ToxCalc statistical software (version 5.0.231, Tidepool Scientific Software). Estimates of EC50s were considered significantly different within a test when 95% CIs for treatment groups did not overlap.

RESULTS

Chemical quantification

Measured test concentrations of PFOS and PFOA were within 10% of target in water from acute tests; measured concentrations from the partial life-cycle test were not as close to target (Table 2). Calculations for PFOS concentration in the control and low dose were derived from the low range standard curve and the high range standard curve was used to calculate the PFOS concentration for the 100 μg/L target concentration. In the partial life-cycle test, the control treatment contained measurable PFOS concentrations in water; however, whole body tissue concentrations of PFOS in adult mussels increased in a dose-dependent manner in other treatment groups (Table 2). Target PFOS treatments of 1 and 100 μg/L in water are subsequently referred to by the measured concentrations (4.5 and 69.5 μg/L, respectively).

Partial life-cycle exposure to PFOS

There were no mortalities to adult *L. siliquoidea* during the 36-d partial life-cycle test. Immediately following in marsupia exposure (day 0 post-removal), glochidia viability ranged from 50 to 91% in all treatments and did not significantly differ among treatments (Fig. 2A); however, viability of PFOS-treated mussels declined precipitously beginning at 1-d post-removal from females. By day 7 post-removal, probability of glochidia viability from females exposed to both PFOS levels was approximately 10%, whereas glochidia from control females had >50% probability of viability at this time point (Fig. 2A). Compared to the relatively constant rate of decline in viability of the control group, there was rapid reduction in viability in the 4.5 and 69.5 μg/L treatment groups. There was no statistical difference between viability of the 4.5 and 69.5 μg/L PFOS treatment groups, though viability of glochidida from the two treatments decreased

substantially after test day 1 post-removal and were significantly lower than the viability in the control on test days 3,5, and 7 (Fig. 2A).

The mixed-effects model had greater explanatory weight (AIC = 22849) than the GLM (AIC = 25510) for the partial life-cycle assay; therefore, the candidate set of a priori models (Table 1) was carried out with terms to account for random-effects. Of the candidate models, the in marsupia exposure model held the greatest weight of evidence and explained 78% of the variability in glochidia viability (AIC = 22843, $w_i = 0.78$). The in marsupia X Glochidia Exposure model was the next best model with 17% support (AIC = 22847, $w_i = 0.17$) and all other models had less than 10% support. Therefore, the in marsupia exposure model was used to generate parameter estimates (Table 3). Logit estimates from the in marsupia exposure model were all statistically significant at $p < 0.0001$ with negative relationships (Table 3), suggesting that PFOS exposures of 4.5 and 69.5 μg/L, number of days post-exposure, and interactions between exposure treatment and days post-exposure have significant negative effects on the viability of glochidia. For example, interactions between PFOS treatment and time can be explained as a change in the rate of decrease across days with an associated change in treatment, similar to an interaction in a traditional ANOVA [29].

Metamorphosed juvenile fatmucket were first collected six days post-inoculation, peaked on day nine, and were not collected after day 17. In marsupia PFOS treatment levels of both 4.5 and 69.5 µg/L reduced the probability of metamorphosis; however, only the 100 μg/L treatment was significantly different at the $\alpha = 0.05$ level of significance (Fig. 2B). The AIC values from mixed-effect models (AIC = 21961) were lower than simple GLM (AIC = 22580) so we accounted for the random variation among fish using the mixed-effect model for the remainder of the candidate models. Of the candidate models, the in marsupia exposure model had 83%

support as the most explanatory model (AIC = 21955, $w_i = 0.83$) and was used for parameter estimation (Table 3). All other candidate models had 13% support or less (Table 3).

Acute toxicity tests

All acute tests with glochidia and juveniles met acceptability criteria of >90% survival in control treatments [9]. Both PFOS and PFOA caused acute toxicity to fatmucket and black sandshell glochidia, but in both species, PFOS was more toxic to glochidia than PFOA (Table 4). Glochidia of both species were more sensitive to PFOS than juveniles; for juvenile tests, we could not calculate an EC50 for PFOA within the range of tested concentrations (EC50 > 500 mg/L) because fewer than 50% of juveniles were affected in even the highest test concentrations. Results of NaCl reference toxicant tests (Table 4) were consistent with reference values in the literature for these and other freshwater mussel species [21, 30].

DISCUSSION

The primary objective of the present study was to develop a novel partial-life-cycle assay for the effects of chemical contaminants on freshwater mussels. The method we describe can be a valuable tool for assessing environmentally relevant exposures to several life-stages of freshwater mussels (e.g., adult, brooding, and free glochidia). Indeed, our study revealed effects of PFOS at concentrations lower than previously described for mussels and other taxa. Application of this approach should increase our knowledge of the contaminant effects on freshwater mussels because it (1) facilitates the identification of the most sensitive life-stage to individual toxicants, (2) incorporates environmentally realistic exposure concentrations, and (3) is adaptable to include further endpoints on adults (e.g., behavioral or physiological

measurements) and metamorphosed juveniles (e.g., time to death, physiological parameters, behavior, etc.). Adult exposures also allow for investigation of sublethal contaminant-induced effects including, but not limited to, changes in reproductive status, physiological parameters, molecular markers, and behavior, although these were not assessed in the present study.

Few studies have compared the effects resulting from contaminant exposures within the brooding female and to free glochidia. In an investigation of the most sensitive unionid life-stage to copper toxicity, Jacobson et al. [31] exposed brooding female *Villosa iris* to 0, 8, and 17 ug Cu/L for 30 d and compared glochidia viability and attachment rates on largemouth bass. Viability was assessed immediately following removal from copper exposure, and no significant difference was found between treatment and control groups. Because viability was only assessed once, and duration of viability was not measured, no conclusions can be drawn regarding the in marsupia toxicity of Cu compared to PFOS in the present study. In marsupia exposures and duration of viability will likely be a useful tool to compare freshwater mussel sensitivity among different classes of toxicants which may differ, among other things, in their ability to cross marsupial gill membranes. Jacobson et al. [31] also compared viability and attachment rates in released (i.e., free) *V. iris* glochidia exposed to 0, 24, 42, 59 ug Cu/L for 24 h. The concentrations tested had no effect on attachment success of in marsupia or free glochidia. However, in the Jacobson et al. study [31], copper concentrations tested were not the same at both exposures (in marsupia vs. free glochidia). Thus, the method could not provide an adequate comparison of sensitivity between life-stages. The exposure design in the present study improves on the previous method because it uses the same contaminant concentrations across exposure types and therefore allows direct identification of the most sensitive period of exposure. Use of metamorphosis as an endpoint may also be more sensitive than glochidia viability or

attachment as metamorphosis is a complex process that may be interrupted through a variety of biochemical pathways.

In the present study, freshwater mussels were sensitive to PFAAs in both acute and chronic toxicity tests. Acute toxicity data of PFOS and PFOA presented in the present study suggest that freshwater mussel glochidia are among the most sensitive organisms tested to date with PFAAs [32]. Qi et al. [32] reviewed the currently published acute toxicity values for PFOS to aquatic organisms and found that only three organisms (96-h LC50s, shrimp – *Mysidopsis bahia* = 3.6 mg PFOS/L, *Neocardina denticulate* = 10 mg PFOS/L, fish – *Pimephales promelas* = 9.1 mg PFOS/L) had mean LC50s below those of 24- and 48-h glochidia tests reported in the current study. Prior to the present study, only one other study had examined the effects of PFOS on freshwater mussels, but it was not published in peer-reviewed literature [33] (reviewed by Qi et al. [32] and Beach et al. [34]). In that study, *Unio complamatus* were exposed to PFOS for 96 h and the LC50 was reported at 59 mg/L, which is lower than the 96 h LC50s for juvenile mussels in the current study (e.g., *Ligumia recta*, Table 4). The life-stage of animals used in the *Unio* test was not reported, but length ranged from 45 to 55mm.

We found that 36-d in marsupia exposure to PFOS was significantly more harmful than exposure of free glochidia for both viability duration and metamorphosis. Duration of viability was significantly reduced compared to the control at PFOS concentrations 3,000 times lower (4.5 µg/L) than the most sensitive acute endpoint (glochidia 24-h EC50 = 13.5 mg/L). The in marsupia exposure model was the best explanatory model for both endpoints and free glochidia exposure (24 h) did not significantly affect either endpoint at the concentrations tested. In marsupia exposures were not only more diagnostic of adverse effects of PFOS on glochidia survivorship and metamorphosis, but are also environmentally realistic because mussels brood

glochidia for several weeks to nearly a year, depending on species [3]. Conduct of toxicity tests that incorporate in marsupia exposures is highly desirable for environmental relevance but exposure of brooding adult mussels is often impossible or impractical because many species are imperiled and large numbers of suitable test organisms are not available. Relationships between acute and chronic (in marsupia) glochidia and juvenile exposures should be further examined to determine if chronic toxicity could be predicted from acute test results.

Our dataset for probability of glochidia viability exposed in marsupia failed to suggest a linear concentration-response relationship (Fig. 2A). A threshold response may be present because responses were similar at 4.5 and 69.5 μg/L. We did not test for mechanism(s) of PFOS toxicity in the present study; however, reductions in glochidia viability duration and metamorphosis success may be due to behavioral avoidance of an irritant (i.e., shell closure) by the brooding female rather than mechanistic toxicity of the pollutant. Cope et al. [3] previously showed that in the presence of Cd, *L. siliquoidea* reduced filtering activity and oxygen consumption. Therefore, the decrease in survival in our study may have been caused by a physiological consequence (e.g., asphyxia of brooding glochidia) of shell closure behavior by the adult female. Nevertheless, the resulting reduction in viability/survival is still a response to the presence of PFOS.

Most existing data on the toxicity of PFAAs to freshwater organisms has focused on PFOS and PFOA [35-37]. In acute tests prior to the present study, the most sensitive taxa tested were daphnids with a PFOS 48-h LC50 of 17 to 35 mg/L and 199 to 476 mg/L for PFOA. Sub-chronic exposures (21-28 d) of fathead minnow (*Pimephales promelas*) to 0.3 mg/L PFOS resulted in increased plasma levels of sexual steroids testosterone, ketotestosterone, and estradiol [36, 37], suggesting PFAAs may act as endocrine disruptors. Further, Japanese medaka (*Oryzias*

latipes) exposed to 0.01 mg/L PFOS had reductions in gonadosomatic-index (GSI), progeny, and growth of offspring [35]. Although PFAA concentrations used in previous tests were several orders of magnitude greater than typical levels found in the environment, exposures in laboratory studies are typically short (3-28 d) and may not adequately represent exposure duration in the environment. In a partial life-cycle test of the effects of PFOS on the damselfly *Enallagma cyathigerum,* metamorphosis was significantly reduced at 10 μg/L [18], a concentration also associated with significantly increased oocyte death rates in the amphipod *Monoporeia affinis* [38]. Such reductions in reproduction and recruitment at PFAA concentrations at or near environmentally relevant levels could cascade to population level effects.

Species sensitivity distributions have been used to derive a predicted no effect concentration (PNEC) for PFOS [32, 34] and EPA tier II benchmarks for PFOS and PFOA have been established at 0.6 and 1.2 μg/L, respectively. In the present study, reductions in metamorphosis success of glochidia exposed in marsupia were seen at PFOS concentrations 50 to 100 times greater than the established benchmarks. Duration of glochidia viability was reduced at concentrations < 10 times greater than established benchmarks after a 36 d in marsupia exposure. However, the ecological relevance of glochidia viability duration is not currently understood because glochidia are typically only exposed to host fish - outside of the marsupium - for a critical window of hours to a few days [3, 5]. We also attempted a 28 d aqueous exposure of juvenile (90 days post metamorphosis) *L. siliquoidea* to 0, 0.1, 1.0, 10 & 100 μg/L of PFOS (Hazelton et al. unpublished data) but those results were inconclusive due to an unexplainable low survival rate in control animals (~60 %). To better understand the effects of PFAAs on early life-stages of freshwater mussels, future research objectives should include a 28-d juvenile chronic toxicity test, and recalculation of acute EC50's at dilution factors of less

than 10 [7].

We observed some inaccuracy in our target concentrations of PFOS in the 36-d in marsupia exposure. First, these samples were taken at day 10 to 11 of the 36-d exposure, and although a 90% water change was conducted daily, some residual PFOS may have been on the glassware or the mussel and thus accumulated within the sample jar throughout the experiment. Second, measureable PFOS concentrations in our control group may have been due to cross-contamination from water quality instruments transferred between treatment groups. We did not analyze tissue from brooding females upon collection from the field; mussels may have had residual PFOS concentrations at the start of our study. Nevertheless, we measured a dose-dependent increase in whole tissue concentrations of PFOS across treatments (Table 2). Bioconcentration factors (BCF = [mean tissue PFOS ng/g]/[mean water PFOS ng/ml]) were consistent across treatments (Control = 4.30, 4.5 µg/L = 3.32, 69.5 µg/L = 3.56), but were 2 to 90 times lower than those reported in marine mussels from polluted ecosystems (BCF = 138-297 [14]), and resident freshwater mussels from PFOS polluted reaches of the Conasauga River, GA (BCF = 11-300, R. Bringolf unpublished data). The low BCF values in the present study suggest that adult *L. siliquoidea* exposed to PFOS for 36 d did not reach equilibrium, even though test concentrations were 4 to 70 times greater than normally found in natural systems [11].

Duration of the free glochidia phase in nature is difficult to estimate experimentally and therefore the appropriate duration of the free glochidia exposure is a major concern for the relevance of acute glochidia tests. Currently the U.S. EPA does not accept acute glochidia toxicity test data for establishing water quality criteria for chemical contaminants [39] because an appropriate duration has not been established. In marsupia exposures used in the present study are ecologically relevant because the brooding length of many Unionid species is known,

or easier to quantify than duration of the free glochidia stage in situ. Brooding duration and host infection strategies of Unionids are quite diverse [3, 5], and differences in sensitivity to in marsupia exposures will likely differ among species and contaminant type. Further testing of the partial life-cycle test with different toxicants and life history strategies is necessary to determine the utility of this assay for chemical risk assessment. In addition, incorporation of the partial life-cycle assay into existing toxicity testing procedures will be useful in contaminant risk assessment for freshwater mussel populations. The assay could be followed by chronic juvenile toxicity tests [8, 9] to further understand the effects of contaminants throughout the early life-stages of freshwater mussels.

The partial life-cycle test used in the present study is adaptable for monitoring a number of endpoints in various mussel life-stages and may be improved by further standardization of techniques (e.g., use of moderately hard water in adult exposures, additional water sample collection for chemical validation [7]). The assay described does have some disadvantages compared to the currently accepted standard toxicity tests for freshwater mussels [7]. First, this assay is logistically more demanding and may require more financial and temporal resources than standardized methods. However, the assay allows for collection of more data on a wider range of endpoints that may be adapted to a particular mussel species, contaminant, or research objective. Another disadvantage is this assay requires the use of brooding adult mussels. Collection and use of reproductively mature individuals from wild populations for toxicity tests on numerous contaminants may not be the best conservation strategy given the widespread decline in freshwater mussel populations [1]. Nevertheless, careful application of this assay in combination with contaminant screening and established acute and chronic toxicity test methods [7], along with continued advancement and availability of animals from freshwater mussel

propagation [7, 40], should allow for the responsible use of adult mussels in toxicity testing.

CONCLUSION

The partial life-cycle assay was useful for identifying the most sensitive life-stage of freshwater mussels. This approach is adaptable to new endpoints, and, when combined with current acute [7] and chronic [7]toxicity assays, will be useful in further understanding of environmental toxicology and risk assessment to freshwater mussels. Data generated in the partial life-cycle analysis is probabilistic (e.g. probability of survival, metamorphosis) and may be useful in informing in situ responses of freshwater mussels to contaminants. The effects of chronic exposures to low concentrations of PFAAs are not fully understood [34] and further research on the effects in aquatic systems is warranted, particularly for estimation of population level changes caused by exposure to PFOS.

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FIGURE CAPTIONS

Fig. 1. Exposure regimen of PFOS in a partial life-cycle test with freshwater mussel glochidia. In marsupia exposures were conducted as static renewal (100% daily) with four replicates of each treatment, one brooding *L. siliquoidea* per replicate. Glochidia from all mussels in each in marsupia PFOS treatment group were pooled and 4 aliquots of the glochidia were used for 24 h glochidia PFOS exposures.

Fig. 2. Fatmucket (*Lampsilis siliquoidea)* glochidia viability over time (A), and probability of metamorphosis (B) following 36-d PFOS exposure in marsupium. Error bars represent 95% confidence intervals. Levels of significance: $*p < 0.05$, $***p < 0.0005$ when compared to control.