Reproductive & Developmental Toxicity of "Forever Chemicals" to Matagorda Bay's Prey Fishes

Final Report Submitted by:

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Executive Summary

Populations of several ecologically and economically important fish species in Matagorda Bay have declined sharply over recent decades, concurrent with increasing industrialization, urban development, and wastewater discharge within the watershed. Per- and polyfluoroalkyl substances (PFAS), a class of persistent synthetic chemicals known as 'forever chemicals,' are ubiquitous environmental contaminants detected in surface waters, sediments, and biota worldwide[1-3]. PFAS enter estuarine systems through a variety of pathways, including wastewater treatment plant effluent, industrial discharge, and stormwater runoff[2]. Their persistence and bioaccumulative potential make them a particular concern for estuarine fish populations[4], yet data on the occurrence and toxicity of PFAS in Gulf Coast estuaries are largely absent.

The present study addressed this data gap through three integrated phases of work conducted over three years (2023–2026). Phase 1 characterized the environmental occurrence and distribution of PFAS across four Matagorda Bay sites using paired sediment and water samples analyzed by EPA Methods 1633 and 537.1. Perfluorooctane sulfonate (PFOS) emerged as the most consistently detected PFAS across all sites and matrices, with the highest sediment concentrations observed near the Palacios Wastewater Treatment Plant. Perfluorononanoic acid (PFNA) was the second most abundant compound in sediment, while perfluorohexane sulfonic acid (PFHxS) and perfluorodecanoic acid (PFDA) were additionally detected in water samples. These environmentally detected compounds, particularly PFOS and PFNA, guided all subsequent experimental work.

Phase 2 focused on development and optimization of PFAS tissue extraction methods for fish muscle, liver, and gonad tissue, using EPA Method 1633 as a baseline. Because this method was originally designed for biosolids rather than biological tissues, significant protocol modifications were required. While consistent recoveries were achieved across tissue and solvent samples, recoveries remained below EPA-approvable thresholds (~30%), and further method refinement is ongoing.

Phase 3 comprised four laboratory toxicity experiments using commercially sourced sheepshead minnow (*Cyprinodon variegatus*; SHM), an EPA model commonly used for toxicity testing[5]. First, the developmental effects of five individual PFAS detected in Phase 1 (PFOS, perfluorooctanoic acid (PFOA), PFNA, PFDA, perfluorooctanesulfonamide (PFOSA)) were evaluated in embryo-larval SHM across environmentally relevant concentration ranges, revealing compound-specific and dose-dependent effects on larval length, body condition, eye area, and yolk sac utilization. Second, a direct embryo-larval co-exposure to 10 µg/L PFOS + 10 µg/L PFNA produced a significant increase in relative yolk sac area in exposed larvae, indicating impaired yolk mobilization. Third, a chronic 21-day adult co-exposure to 10 µg/L PFOS + 10 µg/L PFNA assessed tissue-level physiological endpoints; PFAS-exposed males exhibited a significant reduction in gut mass while females showed no significant differences. Fourth, a second adult co-exposure was conducted in which exposed adults were bred within their treatment groups, with resulting embryos and larvae maintained in parental treatment water; morphometric analysis of F1 larvae revealed no significant differences relative to controls. Analytical water chemistry across all co-exposure experiments confirmed a consistent pattern of PFAS mixture interaction, with measured concentrations substantially below nominal spike levels, consistent with competitive partitioning in seawater.

Collectively, this study provides the first systematic characterization of PFAS contamination in Matagorda Bay and the first assessment of PFAS mixture toxicity in a Gulf Coast estuarine prey fish. Findings suggest that environmentally relevant PFAS concentrations can disrupt energetic physiology and digestive function in SHM, with implications for population-level fitness in wild fish. These data lay essential groundwork for future regulatory risk assessments and conservation efforts in Matagorda Bay.

Background

Per- and polyfluoroalkyl substances (PFAS) are a broad class of synthetic organic chemicals characterized by highly stable carbon-fluorine bonds that render them resistant to environmental degradation. As they do not break down under typical environmental conditions, PFAS persist and accumulate in aquatic systems, sediments, and the tissues of living organisms[1, 6]. These properties have earned them the designation of 'forever chemicals.' PFAS are widely used in industrial applications, non-stick coatings, fire-fighting foams, and consumer products, and they enter estuarine environments primarily through wastewater treatment plant effluent, industrial discharge, stormwater runoff, and atmospheric deposition[6-8].

Matagorda Bay is an economically and ecologically important Texas estuary that supports commercially and recreationally significant fish populations, including red drum (*Sciaenops ocellatus),* spotted seatrout (*Cynoscion nebulosus*), and southern flounder (*Paralichthys lethostigma*). These and other estuarine-dependent species use Matagorda Bay as a critical nursery habitat during early life stages. However, population trends for several of these species have declined sharply over the past five decades. Over the same period, the Matagorda Bay watershed has experienced substantial industrialization, including operations by Formosa Plastics Corporation, Dow Chemical, and multiple wastewater treatment facilities — all known or suspected sources of PFAS contamination.

Despite growing awareness of PFAS as environmental contaminants of concern, very little data existed prior to this study on PFAS occurrence or toxicity in Texas coastal estuaries. Nationally, PFAS research has focused heavily on freshwater systems and mammalian toxicology, leaving substantial data gaps for marine and estuarine fish — particularly Gulf Coast species. The present study was designed to address these gaps systematically by (1) characterizing PFAS contamination in Matagorda Bay, (2) developing methods for quantifying PFAS in fish tissues, and (3) evaluating the reproductive and developmental toxicity of environmentally relevant PFAS in a native estuarine prey fish.

Phase 1: Environmental Characterization of PFAS in Matagorda Bay

Methods

Study sites and Sample Collection

Paired sediment and water samples were collected from four locations within the Matagorda Bay system, each selected to represent potential PFAS point sources or reference conditions (Figure 1):

- Palacios Wastewater Treatment Plant (WWTP) effluent site
- Point Comfort (downstream of the Point Comfort WWTP, Formosa Plastics, and ALCOA wastewater outfalls)
- Chocolate Bay (downstream of the Port Lavaca WWTP)
- Reference site (upstream location near Matagorda Island)

At each location, samples were collected from three replicate transects (Sites A, B, C) spaced along a 20-meter gradient (example shown in Figure 2). At each transect, triplicate water samples were collected in PFAS-free bottles, followed by collection of five 5-cm sediment cores. Environmental parameters recorded at each site included latitude/longitude, salinity, dissolved oxygen, pH, and temperature. All samples were placed on ice immediately after collection and stored at -20°C until analysis.

Figure 1. Expected point sources for introduction of PFAS into the Matagorda Bay system and the proposed sampling sites for characterization of PFAS in the Bay.

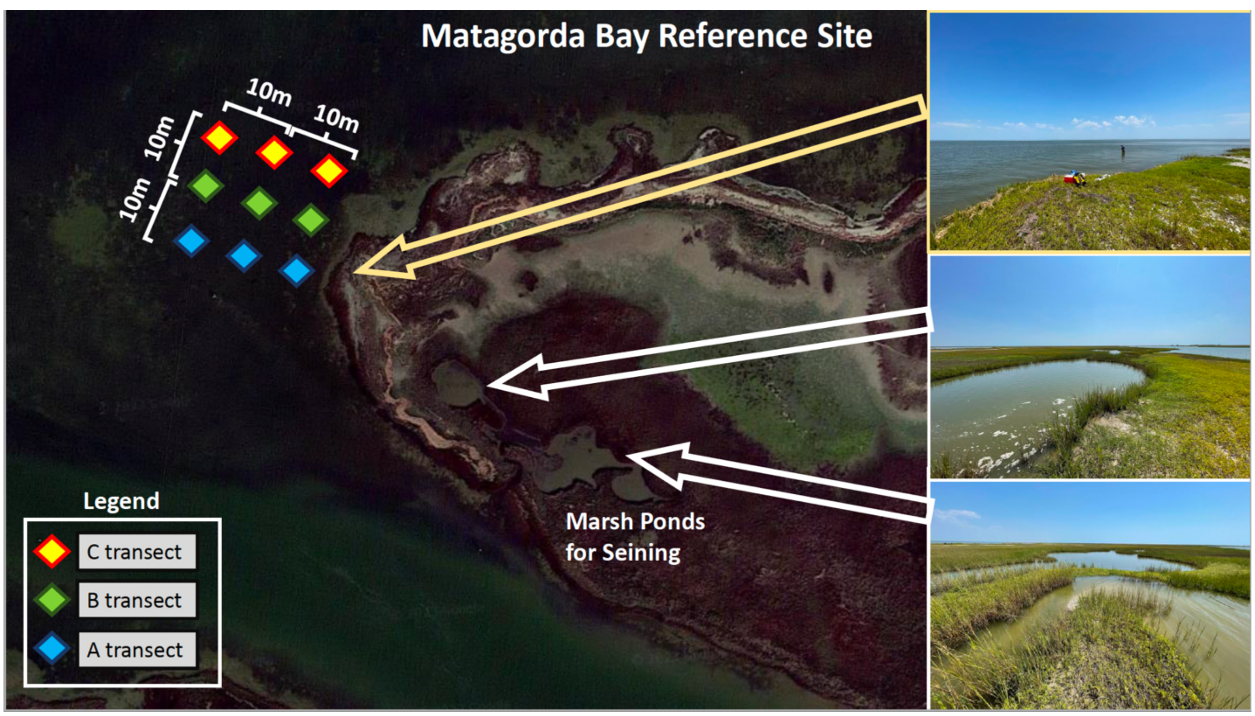

Figure 2. Site sampled as the 'Reference Site' for PFAS characterization of Matagorda Bay.

Analytical Methods

Sediment Analysis

All sediment samples (n = 9 per site; 36 total) were analyzed for 40 PFAS compounds by two EPA-certified commercial laboratories – SGS AXYS Analytical Services, Ltd. and Eurofins Environmental Testing Northern California, LLC – using EPA Draft Method 1633. This method provides quantification of the most prevalent perfluorocarboxylic acids (PFCAs), perfluorosulfonic acids (PFSAs), fluorotelomer substances, and sulfonamide compounds.

Water Analysis

Water samples (n = 1 per site; 4 total) were analyzed for 40 PFAS compounds by Pace Analytical, an EPA-certified commercials laboratory using EPA Method 1633. This method provides quantification of the most prevalent PFCAs, (PFSAs), fluorotelomer substances, and sulfonamide compounds.

Results

Sediment PFAS Profiles

PFAS contamination was detected at three of four sampling locations. The Palacios WWTP effluent site showed the most diverse PFAS profile and the highest overall concentrations, with PFOS (765–908 ppt), PFNA (117–236 ppt), PFDA (127–168 ppt), PFOA (66–97 ppt), PFUnA (~75–101 ppt), and several sulfonamide compounds detected across transects. PFOS and PFOA were the only PFAS detected at the Point Comfort site (57 and 62 ppt, respectively, in one transect). The Reference site showed detectable PFOS (35–200 ppt) and occasional PFNA, N-MeFOSA, and 6:2 FTS, suggesting background contamination. No PFAS above detection limits were found in Chocolate Bay sediments, despite PFOS being present in paired water samples from that location. Concentrations are summarized in Table 1.

Table 1. PFAS detected in sediment samples taken from all four sampling locations in Matagorda Bay. n.d. = not detected. Only compounds detected in at least one sample are shown.

Site	PFOA (pg/g)	PFOS (pg/g)	PFNA (pg/g)	PFDA (pg/g)	PFUnA (pg/g)	PFDoA (pg/g)	PFOSA (pg/g)	N-MeFOSA (pg/g)	6:2 FTS (pg/g)
Palacios A	71	857	137	127	101	n.d.	49	47	n.d.
Palacios B	66	908	236	168	75	44	n.d.	n.d.	n.d.
Palacios C	97	765	117	148	77	n.d.	0.06	43	n.d.
Reference A	n.d.	188	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	151
Reference B	n.d.	35	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4960
Reference C	n.d.	200	39	n.d.	n.d.	n.d.	n.d.	39	n.d.
Point Comfort A	57	62	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Point Comfort B	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Point Comfort C	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Chocolate Bay A	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Chocolate Bay B	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Chocolate Bay C	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Water PFAS Profiles

Water samples revealed a less complex PFAS profile relative to sediment, with only three compounds detected across sites: PFOS, PFOA, and PFBA. PFBA was the dominant compound and was detected three of our sampling sites (range: 7.7-19.9 ng/g), including Chocolate Bay where no PFOS was found in paired sediment samples. PFOA was detected only in one Palacios transect (2 ppt), and PFOS was detected at Palacios and Chocolate Bay (2.0-5.2 ng/g). Results are summarized in Table 2.

Table 2. PFAS detected in water samples from four Matagorda Bay sites. n.d. = not detected. Only compounds detected in at least one sample are shown.

Site	PFBA (ng/L)	PFOS (ng/L)	PFOA (ng/L)
Point Comfort	7.7	n.d.	n.d.
Palacios	11.8	5.2	2.0
Chocolate Bay	n.d.	2.0	n.d.
Reference Site	19.9	n.d.	n.d.

Phase 1: Conclusions and Implications for Subsequent Phases

Phase 1 established that PFAS contamination is present throughout the Matagorda Bay system. PFOS was the most consistently detected compound in sediment and was detected in water at two of the four sites, confirming its status as a priority contaminant. PFNA was the second most abundant compound in sediment. Together, PFOS and PFNA were selected as the compounds of focus for all subsequent Phase 3 mixture exposure experiments. The environmentally relevant concentrations detected – particularly PFOS water concentrations in the low µg/L range – were used to anchor exposure concentrations for laboratory toxicity studies.

Phase 2: Optimization of EPA Method 537/1633 for Estuarine Fish Tissue

The goal of Phase 2 was to develop a validated, reproducible extraction method for quantifying PFAS body burdens in sheepshead minnow (SHM) tissues, including liver, muscle, and gonad. This method is required to assess the extent of PFAS bioaccumulation in SHM collected from Matagorda Bay and from laboratory exposure studies.

Methods

Optimization of EPA Method

EPA Method 1633, originally designed for biosolids and sediment matrices, was used as the baseline extraction protocol. Because this method was not developed for biological tissues — which have substantially higher lipid content and more complex matrices than biosolids — direct application produced poor and inconsistent PFAS recoveries. Method optimization was conducted iteratively using seatrout (*name*) muscle as a surrogate matrix, prior to application to SHM tissues. Protocol modifications explored included:

- Optimization of homogenization parameters (duration, speed, and solvent volume ratios) for fish muscle
- Evaluation of modified extraction solvent combinations (methanol, acetonitrile, formate-buffered solvents) to improve recovery consistency
- Testing of cleanup steps (dispersive SPE with Quenchers salts) to reduce matrix interference from lipids and pigments
- Assessment of PFAS stability across freeze-thaw cycles and storage conditions

PFAS analysis was performed on both the IM Q-TOF LC-MS and the Shimadzu 8060 LC-MS/MS Triple Quadrupole at UT MSI. Quantification used naïve PFAS internal standards spiked prior to extraction for surrogate recovery monitoring.

Results

PFAS Extraction from Marine Fish Tissue

After multiple rounds of optimization, our most recent extraction trials achieved consistent recoveries across both solvent and tissue replicates, representing a marked improvement over initial attempts. The modified protocol successfully accounts for the high lipid content of fish tissues relative to biosolids matrices. However, overall PFAS recoveries currently remain in the range of approximately 30%, below the minimum threshold required for EPA Method 1633 certification. Ongoing efforts are focused on fine-tuning the extraction protocol to reduce analyte loss during the liquid-liquid partitioning and cleanup steps.

Once an acceptable recovery level is achieved, the finalized method will be applied to: (1) liver, muscle, gonad, gut, and brain tissues from SHM adults exposed during Phase 3 chronic exposure studies, and (2) archived tissue composites from both the first and second adult co-exposure rounds. These analyses will provide the first quantitative PFAS bioaccumulation data for SHM from Matagorda Bay.

Phase 3: Reproductive and Developmental Toxicity Studies

Phase 3 comprised four experimental components, all conducted with commercially sourced sheepshead minnow (SHM; *Cyprinodon variegatus*): (1) embryo-larval exposures to five individual PFAS detected in Phase 1; (2) a direct embryo-larval co-exposure to PFOS + PFNA; (3) a chronic 21-day adult co-exposure to PFOS + PFNA with tissue-level physiological endpoints; and (4) a second adult co-exposure to PFOS + PFNA in which exposed adults were bred to assess reproductive outcomes and F1 larval development. All experimental protocols were approved by the University of Texas at Austin Institutional Animal Care and Use Committee (IACUC; AUP-2021-00225; AUP-2024-00248).

Animals and General Experimental Conditions

Commercially sourced adult SHM broodstock were maintained at the Fisheries and Mariculture Laboratory (FAML) at UT MSI in Port Aransas, TX. For embryo-larval exposures, embryos were collected within 24 hours of spawning, assessed for viability, and staged for developmental consistency prior to use. All embryo-larval experiments were conducted at 25°C with daily 50% water changes using pre-spiked exposure water, in an environmental chamber. Adult exposure tanks contained filtered saltwater at 25 ppt salinity, 20°C, pH ~8.0, with 100% air saturation; each tank housed one male and three reproductively active females. Fish were fed daily to satiation, with excess food and waste removed. Water quality parameters (temperature, salinity, pH, ammonia, nitrite, nitrate) were recorded daily.

Individual PFAS Embryo-Larval Exposures

Rationale and Methods

To establish compound-specific dose-response relationships for environmentally detected PFAS, five individual PFAS identified in Phase 1 – PFOS, PFOA, PFNA, PFDA, and PFOSA – were evaluated in separate embryo-larval SHM exposures. Exposure concentrations (0, 2, 6, 16, and 44 µg/L) were anchored to the average PFOS concentration detected in Matagorda Bay water samples (~15 µg/L) and spanned a biologically relevant range encompassing and exceeding field concentrations.

For each compound, embryos (n = 10/dish, 5 replicates per dose) were introduced at approximately 24 hours post-fertilization (hpf) and exposed through hatch (~7 dpf). At test termination, a subset of larvae (n = 5/dish) were humanely euthanized in buffered MS-222, imaged in methylcellulose on glass slides using a Nikon SMZ800N microscope, and assessed for a suite of morphological parameters (Figure 3) using ImageJ software:

- Total length
- Standard length
- Relative body area (body area / standard length)
- Relative eye area (eye area / body area)
- Relative yolk sac area (yolk area / body area)

Statistical analyses were performed in R Studio using linear mixed models with compound and dose as fixed effects. All tests used $\alpha = 0.05$.

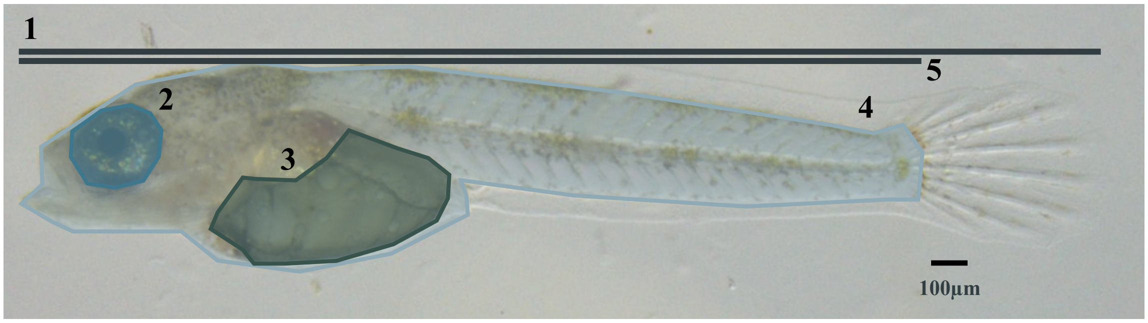

Figure 3. Morphological measurements of yolk-sac larval sheepshead minnow (*Cyprinodon variegatus*). Morphological parameters measured for sheepshead minnow include: 1. Total length; 2. Eye area; 3. Yolk sac area; 4. Body area; 5. Standard length.

PFAS Specific Effects on SHM Larval Morphological Development

All five PFAS produced compound-specific and dose-dependent effects on SHM larval development, with no two compounds producing identical response profiles. Full statistical analyses are included at the end of this report (Table S1).

Key findings are summarized below:

- **PFNA (Figure 4):** Larvae exposed to 16 and 44 µg/L were significantly longer than controls. Relative yolk area decreased significantly with increasing PFNA concentration, indicating altered yolk utilization. Relative eye area was elevated at 2, 16, and 44 µg/L.

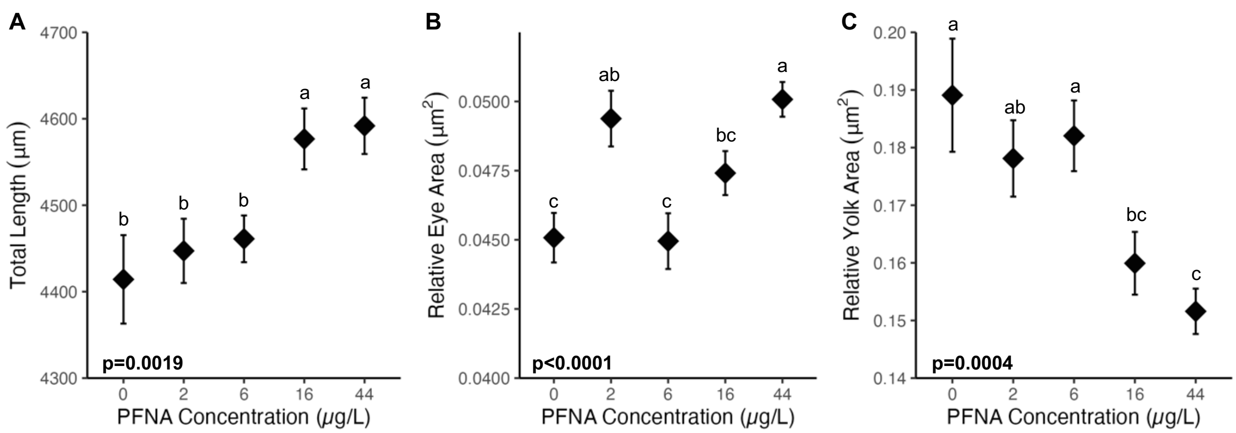

Figure 4. Impacts of PFNA on larval sheepshead minnow (*Cyprinodon variegatus*). Sheepshead minnow total length (A), relative eye area (B), and relative yolk area (C) following exposure to PFNA as embryos. Results are presented as averages ± SE. per treatment. Statistically significant differences from control are denoted using distinct letters (α = 0.05).

- **PFOS (Figure 5):** Larvae at 44 µg/L were significantly larger than lower-dose groups. No significant effects on relative yolk area were detected, suggesting that PFOS alone at these concentrations does not substantially disrupt energetic allocation in early larvae.

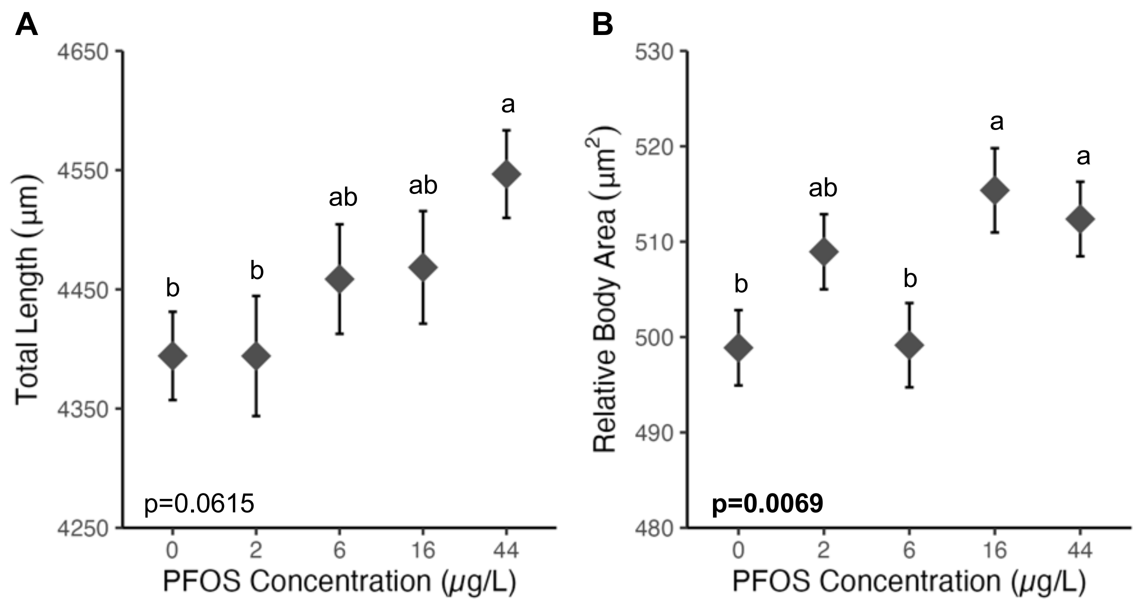

Figure 5. Impacts of PFOS on larval sheepshead minnow (*Cyprinodon variegatus*). Sheepshead minnow total length (A) and relative body area (B) following exposure to PFOS as embryos. Results are presented as averages ± SE. per treatment. Statistically significant differences from control are denoted using distinct letters (α = 0.05).

- **PFOA (Figure 6A):** Exposure to 16 and 44 µg/L resulted in significantly shorter larvae with decreasing relative body area at higher concentrations, suggesting growth inhibition or developmental delay.
- **PFOSA (Figure 6B):** Standard length increased dose-dependently. Relative yolk area decreased significantly with increasing PFOSA exposure, consistent with PFNA and supporting a shared energetic mechanism for these sulfonamide-class compounds.
- **PFDA (Figure 6C):** Larvae at 16 and 44 µg/L had significantly larger relative body areas and relative eye areas compared to controls. No significant effects on relative yolk area were observed.

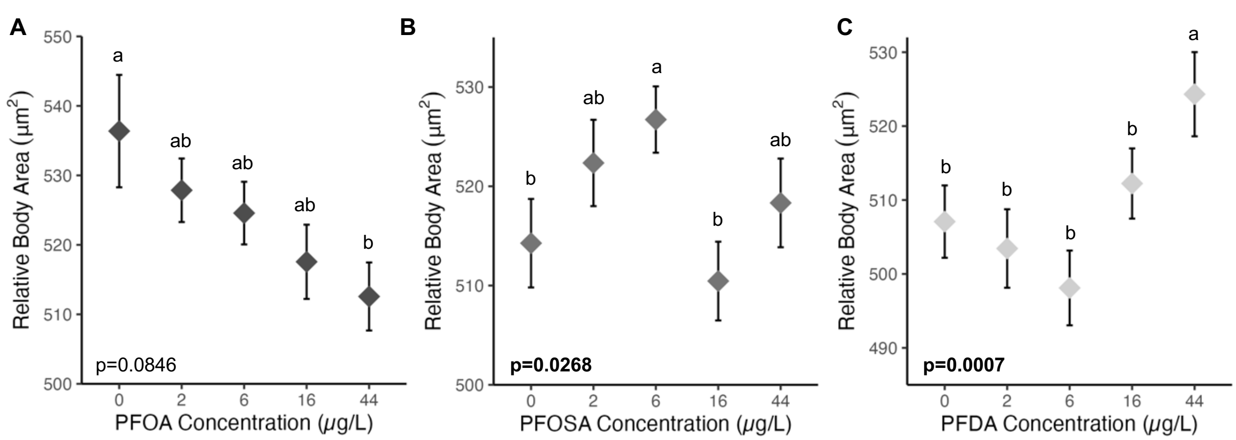

Figure 6. Impacts of PFOA, PFOSA, and PFDA on larval Sheepshead minnow (*Cyprinodon variegatus*). Impacts on sheepshead minnow total length following exposure to PFOA (A), PFOSA (B), and PFDA (C). Results

are presented as averages ± SE. per treatment. Statistically significant differences from control are denoted using distinct letters ($\alpha = 0.05$).

PFAS Specific Effects on SHM Larval Survival

Across all five compounds, survival was not significantly affected at any dose tested, indicating that the observed morphological responses occur below acutely lethal thresholds. The distinct compound-specific response profiles highlight the importance of evaluating individual PFAS separately before drawing generalized conclusions about class-level toxicity. Survival was not affected by PFNA dose ($F_{4,24}$=1.3427; p=0.2889), PFOS dose ($F_{4,24}$=1.6852; p=0.1928), PFOSA dose ($F_{4,24}$=0.4557; p=0.7671), or PFDA dose ($F_{4,24}$=0.4763; p=0.7527). PFOA did significantly impact survival across doses ($F_{4,24}$=3.2737; p=0.0322), though only 6 ppb significantly differed from control. Although a statistically significant reduction in survival was observed at 6 µg/L PFOA compared to control, survival remained high (>80%) and no monotonic or concentration-dependent trend was observed across treatments. Given the absence of a dose-response relationship, this isolated statistical difference is likely not indicative of a biologically relevant effect of PFOA.

Direct Embryo PFOS + PFNA Co-Exposure

Rationale and Methods

To complement the individual PFAS embryo-larval dataset (Section 3.1) with a mixture-level assessment, a direct embryo-larval co-exposure was conducted using commercially sourced SHM embryos exposed to 10 µg/L PFOS + 10 µg/L PFNA from fertilization through ~10 dpf. This experiment used embryos obtained directly from commercial broodstock and was conducted independently of the adult co-exposure studies described in Sections 3.3 and 3.4. Embryos were collected within two hours of spawning, rinsed, and held for 24 hours to reach consistent developmental stages. Embryos were then distributed into exposure dishes:

- Control seawater (5 replicate dishes, 10 embryos per 250 mL)
- 10 µg/L PFOS + 10 µg/L PFNA co-exposure (5 replicate dishes, 10 embryos per 250 mL)

Survival, hatch success (~7 dpf), and developmental progression were assessed daily. Water removed during daily 50% renewals was archived as composite samples. At ~10 dpf, larvae were humanely euthanized in buffered MS-222 and imaged for morphometric analysis using the same morphometric parameter suite described in Section 3.1 (Figure 3).

Analytical Water Chemistry

Composite water samples from PFAS-exposed and control dishes were analyzed using modified EPA Method 537.1. Day 0 measured PFNA concentrations were approximately 1.83 µg/L (nominal: 10 µg/L), while PFOS was 0.046 µg/L. By Day 8, PFNA had increased to approximately 3.84 µg/L and PFOS to 0.20 µg/L across composite samples (Table 3). The substantial discrepancy between nominal and measured concentrations – particularly for PFOS – reflects competitive partitioning between the two compounds in the seawater matrix, consistent with dynamics subsequently observed during the adult co-exposure (Section 3.3).

Table 3. PFAS levels in composite water samples from direct SHM embryo-larval PFOS + PFNA co-exposure. Analyzed using modified EPA Method 537.1.

Sample	PFNA Conc. (µg/L)	PFOS Conc. (µg/L)
PFOS+PFNA Day 0	1.8268	0.0464
Control Day 0	0.0000	0.0000
PFOS+PFNA Day 8	3.8397	0.1952
Control Day 8	0.0000	0.0000

Effects of PFOS + PFNA on Adult Growth and Tissue Indices

Morphometric analysis at ~10 dpf revealed no significant effects of the PFOS + PFNA co-exposure on standard length, relative body area, or relative eye area. However, a clear and statistically significant increase in relative yolk sac area was observed in PFAS-exposed larvae relative to controls, indicating impaired yolk mobilization and disrupted energetic allocation during the larval period (Figure 7).

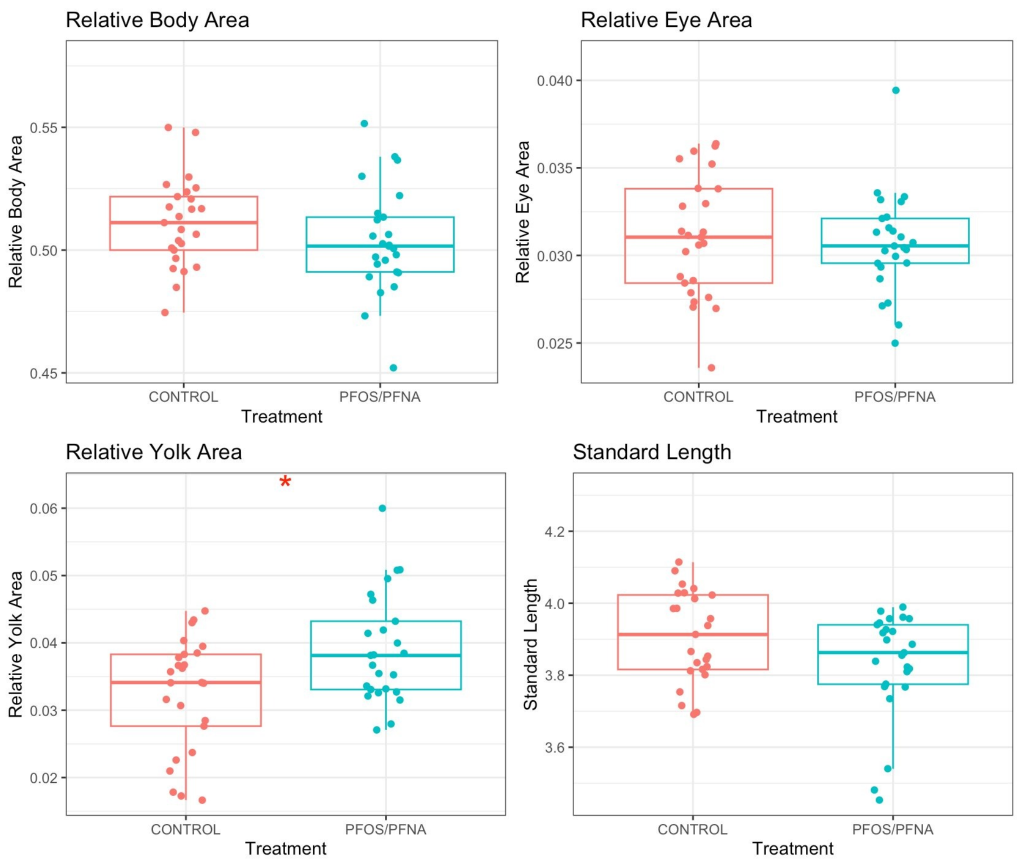

Figure 7. Morphometric parameters measured in SHM larvae (~10 dpf) from the direct PFOS + PFNA embryo-larval co-exposure (10 µg/L each). Relative yolk area was significantly elevated in PFOS/PFNA-exposed larvae compared to controls (asterisk; $p < 0.05$), indicating reduced yolk utilization. No significant differences were observed in relative body area, relative eye area, or standard length. Points represent individual larvae; boxes show interquartile range with median.

Chronic Adult PFOS + PFNA Co-Exposure

Rationale and Methods

The first adult co-exposure experiment was designed to evaluate the effects of chronic PFOS + PFNA mixture exposure on adult SHM body condition and tissue-specific physiology, with endpoints focused on whole-body mass, organ indices, and tissue weights. PFOS and PFNA were selected as the mixture compounds based on their consistent detection and highest concentrations across Phase 1 Matagorda Bay samples.

Five control tanks and five co-exposure tanks were established. Following a two-week acclimation period, PFAS tanks were dosed to 10 µg/L PFOS + 10 µg/L PFNA. Water changes were performed as needed for water quality maintenance using pre-spiked water to sustain exposure concentrations; water samples were collected prior to each change. The exposure ran for 21 days. At termination, all fish were weighed, measured, and humanely euthanized in buffered MS-222. Tissues isolated included liver, muscle, gonad, gut, and brain, each individually weighed. Female liver and muscle tissues were composited by tank to provide sufficient material for future PFAS extraction analyses. Data were analyzed using linear mixed-effects models with TankID as a random effect and post-hoc comparisons via estimated marginal means ($\alpha = 0.05$).

Effects of PFOS + PFNA on Adult Growth and Tissue Indices

No significant differences were observed in whole-body mass, hepatosomatic index (HSI), gonadosomatic index (GSI), liver mass, gonad mass, muscle mass, or brain mass in either females or males (Figures 8-9).

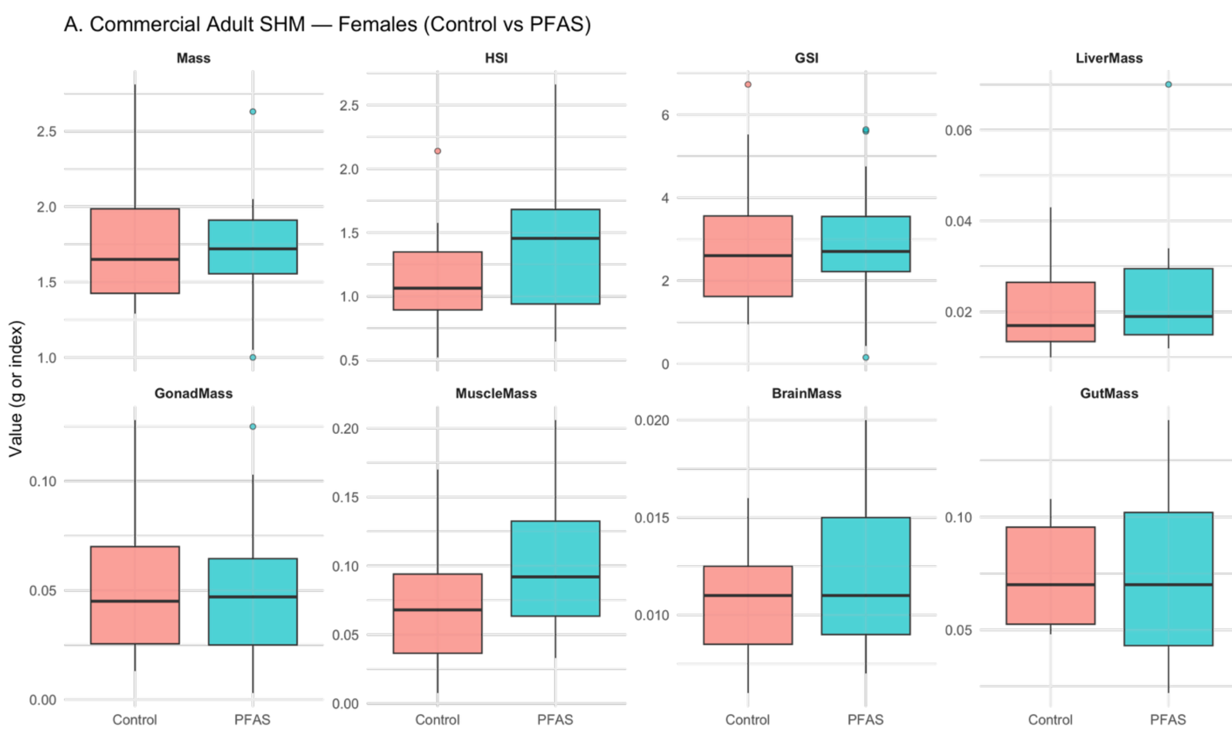

Figure 8. Adult SHM morphology following exposure to PFNA + PFOS mixtures. Results for whole-body mass, hepatosomatic index (HSI), gonadosomatic index (GSI), liver mass, gonad mass, muscle mass, and brain mass in adult female fish.

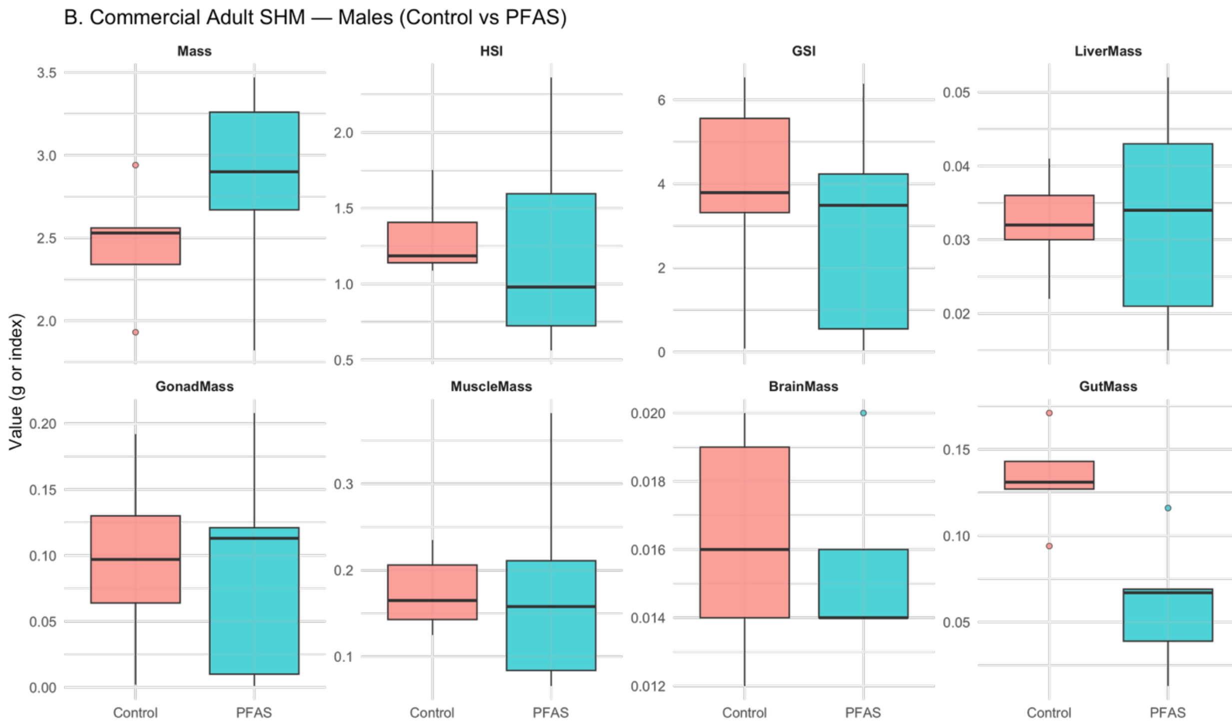

Figure 9. Adult SHM morphology following exposure to PFNA + PFOS mixtures. Results for whole-body mass, hepatosomatic index (HSI), gonadosomatic index (GSI), liver mass, gonad mass, muscle mass, and brain mass in adult male fish.

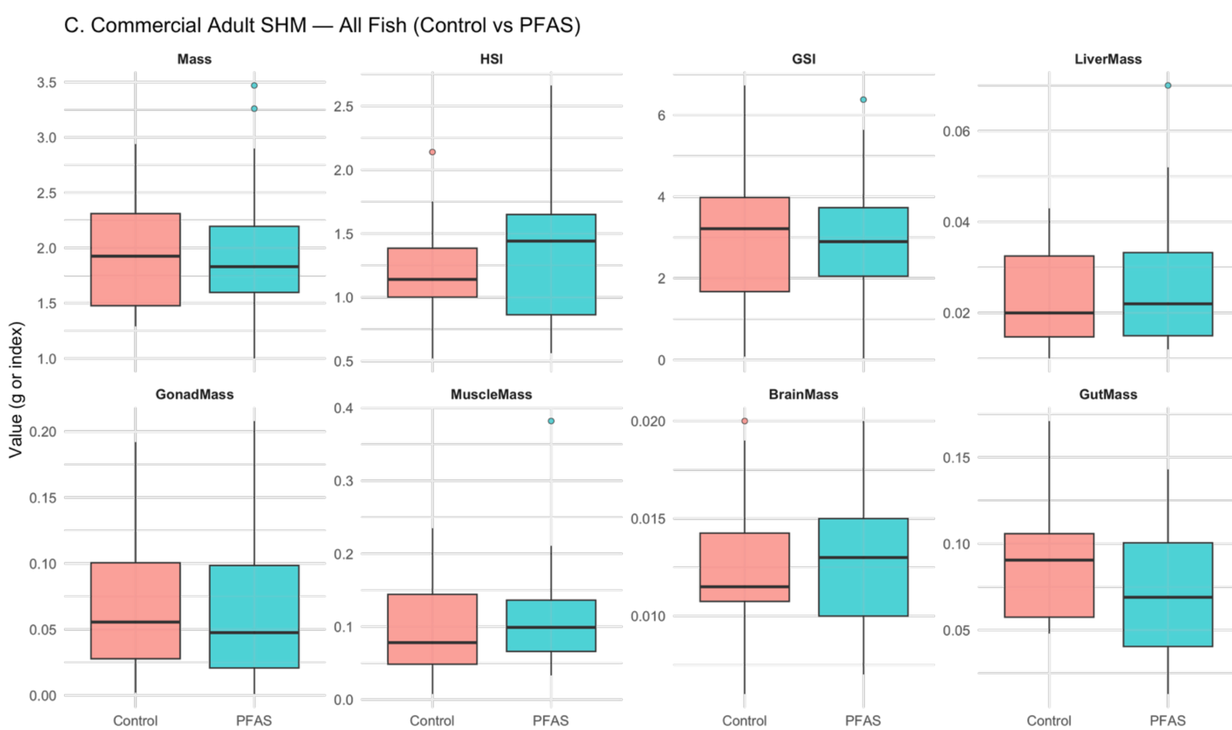

Figure 10. Adult SHM morphology following exposure to PFNA + PFOS mixtures. Results for whole-body mass, hepatosomatic index (HSI), gonadosomatic index (GSI), liver mass, gonad mass, muscle mass, and brain mass in adult male and female fish.

However, PFAS-exposed males exhibited a statistically significant reduction in gut mass compared to controls ($p = 0.0010$; Figure 10). This sex-specific finding suggests that the PFOS + PFNA mixture may disrupt digestive physiology, nutrient assimilation, or feeding behavior in males, potentially through metabolic or endocrine mechanisms. Female tissues showed no significant differences across any measured parameter.

Water chemistry measurements across the 21-day exposure revealed dynamic and inconsistent concentrations of both PFOS and PFNA within treatment tanks, with PFNA declining substantially from nominal levels within the first week. This pattern is consistent with active PFNA uptake by fish and/or partitioning to tank surfaces and organic matter, as well as interactive effects between the two compounds in the co-exposure matrix. All dissected tissues were archived at -20°C pending PFAS body burden quantification following completion of Phase 2 method development.

Parental and F1 Generation Embryo-Larval PFOS + PFNA Co-Exposure

Rationale and Methods

The second adult co-exposure experiment was specifically designed to assess whether parental PFAS exposure affects the development of F1 offspring when embryos and larvae are also continuously maintained in the parental treatment water. This design allows evaluation of both parental conditioning effects and direct F1 embryo-larval exposure acting in concert. As in the first adult round (Section 3.3), five control tanks and five PFAS-exposed tanks were established with one male and three females per tank. Following a two-week acclimation period, PFAS tanks were dosed to 10 µg/L PFOS + 10 µg/L PFNA and the exposure ran for 21 days.

At the conclusion of the 21-day exposure, all adults were evaluated for body weight and standard length; no significant differences were observed between PFAS and control groups within either sex. Adults were then maintained in their tanks and allowed to spawn naturally over one week. Spawns were collected daily and kept separate by spawn date and parental tank. Two replicate control tanks and three PFAS-exposed tanks successfully produced viable spawns; the remaining tanks experienced adult mortalities due to aggressive SHM spawning behaviors — a known challenge when housing SHM at the study density (3 females + 1 male per 5-gallon tank). Embryos from PFAS-exposed parents were maintained in PFAS-spiked tank water drawn from their respective parental tank, and control embryos were maintained in control tank water, ensuring continuous PFAS exposure of embryos and larvae from fertilization through termination. Embryos were reared through hatch (~7 dpf) and terminated at 1-day post-hatch, at which point larvae were humanely euthanized and imaged for morphometric analysis (Figure 2). Statistical analyses included a linear mixed-effects model, ANOVA, and Wilcoxon test ($\alpha = 0.05$; n = 15 control larvae from 2 tanks; n = 52 PFAS larvae from 3 tanks).

Analytical Water Chemistry

Analytical chemistry was completed on archived tank water samples from this second adult and F1 larval exposure round using a modified EPA Method 537.1 approach. As shown in Table 4, the pattern of PFAS mixture interaction was again consistent with prior exposure rounds. When both compounds were co-spiked in seawater, measured PFOS concentrations ranged from approximately 5.5–6.0 µg/L and PFNA from approximately 1.8–2.0 µg/L, substantially below nominal levels of 10 µg/L each. By contrast, individual compound spikes in seawater recovered 6.9–7.8 µg/L for PFOS and 3.1–3.4 µg/L for PFNA, indicating that the presence of the second compound suppresses recovery of both — consistent with competitive partitioning or direct intermolecular interactions in the seawater matrix. Blank samples returned zero detections for both analytes, confirming no background contamination.

Table 4. Measured PFOS and PFNA concentrations (µg/L) in tank water samples from the second adult SHM co-exposure and F1 larval rearing. Analyzed using modified EPA Method 537.1. NA = not analyzed for this compound; — = no data.

Sample ID	Compound Added	Spike Conc. (µg/L)	PFOS Conc. (µg/L)	PFNA Conc. (µg/L)
PFOS_STD_1_SW	PFOS added to SW	10 PFOS	7.755	NA
PFOS_STD_2_SW	PFOS added to SW	10 PFOS	6.876	NA
PFNA_STD_1_SW	PFNA added to SW	10 PFNA	NA	3.415
PFNA_STD_2_SW	PFNA added to SW	10 PFNA	NA	3.071
PFNA/PFOS_STD_1_SW	PFNA + PFOS added to SW	10 PFNA + PFOS	6.021	1.990
PFNA/PFOS_STD_2_SW	PFNA + PFOS added to SW	10 PFNA + PFOS	5.521	1.774
SPE_Blank	Blank	0	0	0
PFOS_STD_3	PFOS Stock in LCMS Water	10 PFOS	2.473	—
PFNA_STD_3	PFNA Stock in LCMS Water	10 PFNA	NA	1.214
PFNA/PFOS_STD_3	PFOS + PFNA stocks in LCMS Water	10 PFNA + PFOS	2.278	1.189

F1 Morphological Findings

Morphometric analysis of F1 SHM larvae revealed no statistically significant differences between PFAS and control groups across all measured parameters, including total length, standard length, body area, body area relative to standard length, eye area relative to total length, and eye area relative to body area (Figure 11).

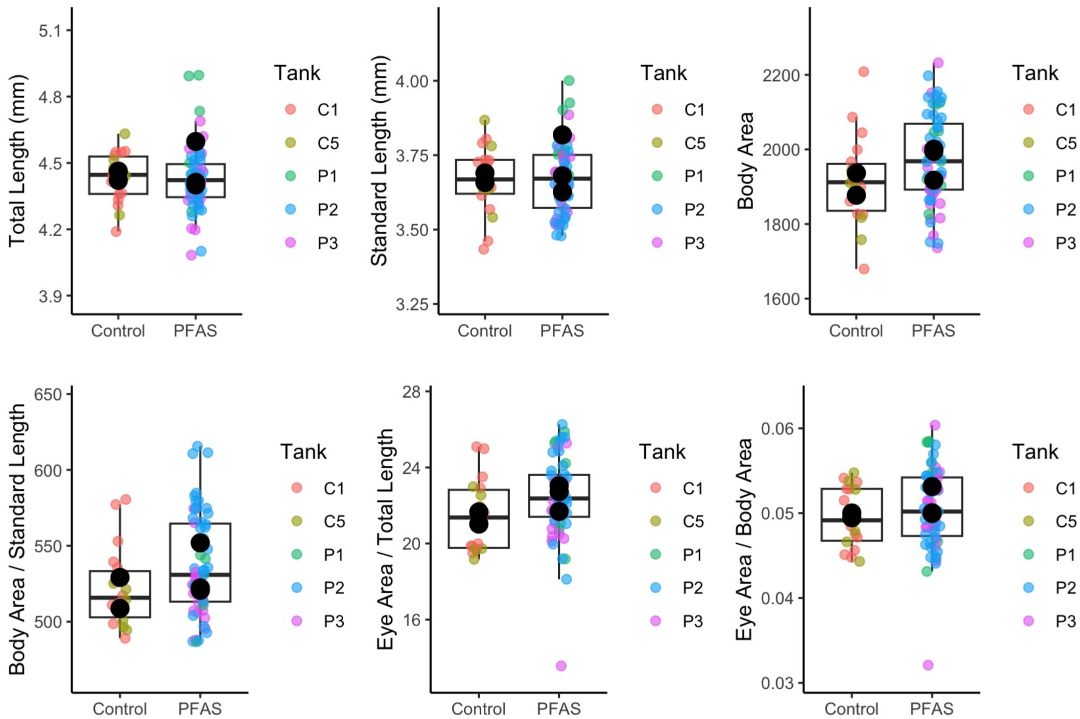

Figure 11. Morphometric parameters measured in F1 SHM larvae born to PFAS-exposed (10 µg/L PFOS + 10 µg/L PFNA) or control parents and reared in their respective parental treatment water. No statistically significant differences were observed between treatment groups for any parameter. Points represent individual larvae colored by replicate tank (C1, C5 = control; P1, P2, P3 = PFAS). Large black dots indicate tank means. n = 15 control, n = 52 PFAS larvae.

The absence of gross morphological effects in F1 larvae is a notable complement to the elevated yolk sac retention observed in the direct embryo-larval co-exposure (Section 3.2), where embryos were exposed from fertilization through ~10 dpf. One important distinction between these experiments is the developmental window: F1 larvae in this study were assessed at 1 day post-hatch, whereas the direct co-exposure assessed larvae at ~10 dpf. It is possible that yolk sac differences would have emerged at a later timepoint in the F1 cohort had rearing continued. Additionally, the relatively small number of control tanks that successfully spawned (n = 2) limits statistical power and warrants cautious interpretation. Future work with greater replication and extended larval rearing would help clarify whether parental PFAS exposure affects offspring development at later stages or through subtler physiological pathways not captured by gross morphometrics.

Publications and Presentations

Peer-Reviewed Publications

Ackerly KL, Walsh G, Roark KJ, DiBona E, Ostentowski R, Rivera T, Lin L, Nielsen KM. *In review.* PFAS contamination in Texas Gulf Coast estuarine sediments and impacts of environmentally informed concentrations on early life stage sheepshead minnow (*Cyprinodon variegatus*). Journal: *Environmental Research*.

DiBona E, Ackerly KL, Roark KJ, Nielsen KM. *In prep*. Effects of chronic PFNA and PFOS Mixtures on the Growth and Reproduction of Adult Sheepshead Minnow (*Cyprinodon variegatus*). Target Journal: *ES&T Toxicology.*

Conference Presentations

DiBona E, Ackerly KL, Roark KJ, Walsh G, Neill C, Nielsen KM. 2025. Oral Presentation. *South-Central SETAC Regional Chapter Annual Meeting*. San Marcos, TX.
DiBona E, Ackerly KL, Roark KJ, Walsh G, Neill C, Nielsen KM. 2025. Oral Presentation. *SETAC Annual Meeting*. Portland, OR.
Ackerly KL, Walsh G, Roark KJ, Rivera TM, Nielsen KM. 2024. Oral Presentation. *SETAC Annual Meeting*. Fort Worth, TX.
Walsh G, Ackerly KL, Roark KJ, Rivera TM, Nielsen KM. 2024. Poster Presentation. *SETAC Annual Meeting*. Fort Worth, TX.
Ackerly KL, Roark KJ, Esbaugh A, Rodriguez Gil JL, Palace V, Ussery E, Nielsen KM. 2024. Invited Oral Presentation. The Society of Experimental Biology (SEB) Conference. Prague, Czech Republic.

Synthesis and Conclusions

This three-year study provides the first integrated assessment of PFAS contamination and toxicity in Matagorda Bay and its resident prey fish community. Taken together, the findings across all three phases yield several important conclusions:

- **PFAS are present throughout Matagorda Bay.** PFOS was detected at every sampling site and in every matrix examined, with concentrations in water reaching levels relevant to toxicological effects in sensitive species. The detection of multiple PFAS compounds – including PFNA, PFBA, PFDA and various sulfonamides – reflects diverse and ongoing sources of contamination.
- **PFOS demonstrates unexpected mobility in the Bay system.** The detection of water-column PFOS concentrations at sites where sediment PFOS was absent (most notably Chocolate Bay) suggests that PFOS is not fully partitioning to sediments as would be predicted from published partition coefficients at salinities of ~30 ppt. This may reflect elevated dissolved organic carbon or other matrix factors and has significant implications for the biological availability of PFOS to water-column species such as SHM.
- **Individual PFAS produce distinct, compound-specific developmental effects in SHM larvae.** At environmentally relevant concentrations, PFNA and PFOSA produced the most consistent energetic disturbances (decreased relative yolk area), while PFDA increased body and eye size, PFOA inhibited growth, and PFOS produced modest changes in body size without yolk disruption. These divergent profiles underscore the importance of evaluating PFAS individually and as mixtures.
- **The PFOS + PFNA mixture disrupts energetic physiology across life stages.** In directly exposed embryo-larval SHM, the PFOS + PFNA mixture caused significant yolk sac retention. In adult males from a separate chronic exposure, the same mixture produced a significant reduction in gut mass. These convergent findings across two

independent experiments and two life stages support a consistent hypothesis that PFOS + PFNA co-exposure disrupts metabolic and digestive processes in SHM.

- **PFOS and PFNA interact in the seawater matrix.** Analytical chemistry consistently demonstrated that co-exposure conditions yield substantially lower measured concentrations of both compounds compared to single-compound spikes, indicative of competitive partitioning or physicochemical interactions. This finding has methodological implications for future PFAS mixture toxicity studies in marine systems.
- **No gross morphological effects were detected in F1 larvae from PFAS-exposed parents.** In the second adult exposure, in which exposed adults were bred and F1 offspring maintained in parental treatment water, no significant morphometric differences were observed in F1 larvae relative to controls. This is an informative contrast to the yolk sac retention seen in directly exposed larvae, though differences in assessment timing (1 dpf vs. ~10 dpf) and control replication limit direct comparison. Subtler effects at the molecular, physiological, or behavioral level – or effects detectable at later developmental stages – warrant future investigation.

Collectively, these data establish a critical scientific foundation for evaluating the ecological risk of PFAS contamination to Matagorda Bay fish populations. The presence of bioavailable PFAS at multiple sites, combined with demonstrated toxicological effects at environmentally relevant concentrations, suggests that PFAS represent a meaningful but poorly characterized stressor for prey fish in this system. Future work should prioritize PFAS tissue bioaccumulation quantification, assessment of wild-caught versus commercially reared SHM sensitivity, expanded transgenerational studies, and evaluation of PFAS effects on reproductive endpoints including fecundity, fertilization success, and offspring quality.

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Supplemental Materials:

Supplemental Table 1. Sheepshead minnow statistical analyses and morphological measures

PFOS				
Concentration (µg/L)	**Total Length (µm)**	**Relative Body Area (μm^2)**	**Relative Eye Area (μm^2)**	**Relative Yolk Area (μm^2)**
Statistical Outputs	$F_{4,4} = 2.314$	$F_{4,4} = 3.721$	$p > 0.05$	$p > 0.05$
	$p = 0.061$	**$p = 0.007$**		
0	4394.199 ± 37.027	498.873 ± 3.948	0.047 ± 0.001	0.207 ± 0.006
2	4394.099 ± 50.369	508.942 ± 3.936	0.047 ± 0.001	0.201 ± 0.009
6	4458.619 ± 45.941	499.155 ± 4.422	0.047 ± 0.001	0.196 ± 0.007
16	4468.458 ± 47.225	515.383 ± 4.417	0.046 ± 0.001	0.203 ± 0.007
44	4546.669 ± 36.723	512.378 ± 3.909	0.048 ± 0.001	0.186 ± 0.005
PFOA				
Concentration (µg/L)	**Total Length (µm)**	**Relative Body Area (μm^2)**	**Relative Eye Area (μm^2)**	**Relative Yolk Area (μm^2)**
Statistical Outputs	$p > 0.05$	$F_{4,4} = 2.104$	$p > 0.05$	$p > 0.05$
		$p = 0.085$		
0	4505.386 ± 54.516	536.373 ± 8.088	0.045 ± 0.001	0.182 ± 0.010
2	4497.987 ± 50.181	527.858 ± 4.572	0.061 ± 0.015	0.178 ± 0.006
6	4512.523 ± 53.153	524.571 ± 4.512	0.044 ± 0.002	0.188 ± 0.008
16	4429.223 ± 57.951	517.564 ± 5.344	0.047 ± 0.001	0.238 ± 0.047
44	4367.461 ± 64.773	512.566 ± 4.896	0.044 ± 0.002	0.197 ± 0.011
PFDA				
Concentration (µg/L)	**Total Length (µm)**	**Relative Body Area (μm^2)**	**Relative Eye Area (μm^2)**	**Relative Yolk Area (μm^2)**
Statistical Outputs	$p > 0.05$	$F_{4,4} = 5.224$	$p > 0.05$	$p > 0.05$
		$p = 0.0007$		
0	4422.127 ± 54.424	507.081 ± 4.894	0.047 ± 0.007	0.198 ± 0.007
2	4457.213 ± 50.301	503.442 ± 5.309	0.048 ± 0.010	0.197 ± 0.010
6	4382.043 ± 58.839	498.106 ± 5.058	0.047 ± 0.001	0.219 ± 0.011
16	4499.140 ± 35.700	512.235 ± 4.747	0.049 ± 0.001	0.193 ± 0.006
44	4498.578 ± 54.001	524.315 ± 5.694	0.049 ± 0.001	0.185 ± 0.008
PFNA				
Concentration (µg/L)	**Total Length (µm)**	**Relative Body Area (μm^2)**	**Relative Eye Area (μm^2)**	**Relative Yolk Area (μm^2)**
Statistical Outputs	$F_{4,4} = 4.552$	$p > 0.05$	$F_{4,4} = 7.480$	$F_{4,4} = 5.490$
	$p = 0.0019$		**$p < 0.0001$**	**$p = 0.0004$**
0	4414.213 ± 51.183	502.459 ± 4.091	0.045 ± 0.001	0.189 ± 0.010
2	4449.212 ± 35.728	507.373 ± 2.514	0.050 ± 0.001	0.179 ± 0.006
6	4461.113 ± 27.030	501.490 ± 4.081	0.045 ± 0.001	0.182 ± 0.006

16	4576.679 ± 35.218	502.913 ± 4.452	0.047 ± 0.001	0.160 ± 0.005
44	4591.814 ± 32.564	506.805 ± 3.767	0.050 ± 0.001	0.152 ± 0.004
PFOSA				
Concentration (μg/L)	**Total Length (μm)**	**Relative Body Area (μm^2)**	**Relative Eye Area (μm^2)**	**Relative Yolk Area (μm^2)**
Statistical Outputs	$p > 0.05$	$F_{4,4} = 2.854$ **$p = 0.027$**	$p > 0.05$	$p > 0.05$
0	4551.821 ± 66.115	514.268 ± 4.460	0.048 ± 0.001	0.187 ± 0.009
2	4619.145 ± 45.779	522.353 ± 4.348	0.049 ± 0.001	0.179 ± 0.008
6	4663.761 ± 29.637	526.724 ± 3.341	0.048 ± 0.001	0.172 ± 0.004
16	4663.111 ± 35.348	510.451 ± 3.965	0.050 ± 0.001	0.169 ± 0.006
44	4682.660 ± 44.067	518.326 ± 4.469	0.045 ± 0.003	0.171 ± 0.008