Assessing the threat of tire leachate and urban runoff on Matagorda Bay fish populations

Final Report Submitted by:

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Submitted to:

Matagorda Bay Mitigation Trust

Contract Number 018

Executive Summary

Urban runoff has long been linked to large-scale freshwater fish kills, particularly in the Pacific Northwest United States, where 40-90% of migrating salmon die annually from Urban Runoff Mortality Syndrome (URMS). These large-scale fish kills typically occur after rainfall events and are attributed to chemicals leaching from tire wear particles (TWPs). Normal wear and tear on tires leads to deposition of TWPs on roadways, where they become weathered by environmental processes before being washed into nearby bodies of water by stormwater runoff. A previously unknown transformation product present in TWPs called 6PPD-quinone was recently linked to URMS in salmonids; however, almost nothing is known about its environmental occurrence and/or potential to cause URMS in other species, including estuarine-dependent sportfish.

Populations of several ecologically and economically important sport fish species in the Matagorda and San Antonio Bays have declined sharply in the last five decades, concurrent with a marked increase in population growth and manufacturing operations in the area. Coastal urbanization is naturally accompanied by an increase in vehicular traffic and tire wear, contributing to the presence of TWP contamination within local estuaries. This could have important implications for fish populations in these ecosystems, as TWPs contain a range of chemicals (including 6PPD-quinone) that are known or suspected to be toxic to aquatic biota and may leach into seawater.

While studies examining the toxic effects of chemicals leaching from TWPs exist, they primarily utilize small-bodied freshwater fish as models. Therefore, the potential risks of TWP contamination to fish populations within urbanized estuaries in the Texas Coastal Bend region remain unknown. To address this data gap, the present study sought to establish a holistic analytical protocol of quantifying 6PPD-quinone in natural waters, (2) evaluate the environmental fate and occurrence of TWP pollution, including 6PPD-quinone, in Matagorda/San Antonio Bays, and (3) investigate the relative toxicities of TWP leachate and 6PPD-quinone to native fish species.

An analytical protocol aiming at enhancing the extraction efficiency and analysis of 6PPD-quinone in natural seawater was established, including the selection of solid phase extraction (SPE) cartridges, whether to apply filtration, the effects of salinity, and other parameters. Incubation experiments demonstrated that 6PPD-quinone is relatively stable in seawater for at least 14 days under dark conditions. These results suggest there is minimal microbial degradation of this compound, which is of importance when evaluating fate and toxicity of contaminants in natural waters. Results of the field characterization efforts indicated that concentrations of 6PPD-quinone in local south and central Texas estuaries and rivers ranged from non-detect (ND) to > 4 ng/L, with the highest concentrations found near a stormwater drainage site in Corpus Christi.

Results of toxicity testing indicated that current environmental concentrations of 6PPD-quinone are unlikely to exert toxicity on any of the native species tested, including sheepshead minnow *(Cyprinodon variegatus)*, red drum *(Sciaenops ocellatus)*, or Southern flounder *(Paralichthys lethostigma)*. By contrast, TWP leachate was found to be toxic to early life stage (ELS) red drum and southern flounder, with flounder demonstrating a greater relative sensitivity. Developmental leachate exposure led to adverse effects on the survival and development of both species through a combination of phototoxic and non-phototoxic mechanisms. However, it remains unclear which constituents of the TWP leachate mixture are responsible for the observed effects and/or whether current environmental concentrations are sufficient to adversely impact ELS fish populations.

Background

Populations of several ecologically and economically important sport fish species in the Matagorda and San Antonio Bays declined sharply in the last five decades (Figure 1A).[1] Population trends in the study area also coincide with a marked increase in manufacturing operations over the same period of time (Figure 1B), which includes several industries known to contribute to environmental contamination at the local and global levels (e.g., chemical manufacturing, petrochemical facilities, smelting and refining).[2-4] Increasing urbanization within the Matagorda and San Antonio Bay watersheds has also had adverse impacts on both the quantity and quality (e.g., higher contaminant concentrations) of freshwater inflows.[5] This has important implications for fish populations in these ecosystems, as exposure to urban and municipal contamination can reduce survival, recruitment, growth, and reproductive output of freshwater and marine fish.[6-10]

Pacific coho salmon *(Oncorhynchus kisutch)* migrating through urbanized watersheds of the Northwestern United States provide a dramatic example of how contaminant exposure can lead to population level impacts. For decades, these fish populations have been subject to a phenomenon known as "urban run-off mortality syndrome" (URMS), whereby adult Coho returning to coastal freshwaters to spawn are killed *en masse* in the hours following storms.[11] Though stormwaters contain a mixture of toxic chemicals, no causative agent could be identified at sufficiently high concentrations to explain the recurrent fish kills.

Recently, a quinone photo-degradation product of the common tire antiozonant N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine was finally identified as the driver of URMS in salmon.[11, 12] This chemical, called 6PPD-quinone, has a 24-hour median lethal concentration of 0.79-µg/L for juvenile salmon (i.e., 50% of fish are expected to die within 24 hours of being exposed to less than one part per billion of this chemical).[11] In addition to its acute lethality, its parent compound (6PPD) accounts for 0.4 - 2% of tire formulations by mass globally, indicating that 6PPD-quinone represents a potentially serious threat to aquatic life everywhere.[13] Moreover, it is expected to be especially prevalent in urbanized watershed that receive intense solar radiation, as it is generated in the presence of oxygen and ultraviolet radiation (UV).[11, 13]

The Matagorda and San Antonio Bay watersheds are both highly urbanized and subject to intense UV.[5, 14, 15] Thus, it is reasonable to assume that chemicals from TWPs, as well as their photo-transformation products (e.g., 6PPD-quinone), are abundant in these systems. It is also important to note that TWPs contain a range of chemicals with confirmed or suspected photo-dynamicity (i.e., the potential to absorb certain wavelengths of light) that are often lipophilic and will preferentially partition from the water column into biota. The toxicity of such constituents to transparent early life stage (ELS) aquatic organisms increases dramatically in the presence of solar radiation – often by an order of magnitude or more – typically causing mortality. This is due to the interaction between photodynamic constituents present in the tissues of transparent biota with solar radiation that penetrates through these organisms due to their lack of pigmentation. This phenomenon, known as photo-induced toxicity, leads to the generation of reactive oxygen species that damage tissues in a self-propagating reaction known as lipid peroxidation.[13, 16, 17]

Estuaries in the Texas Coastal Bend region regularly receive intense solar radiation and also contain highly concentrated numbers of ELS estuarine-dependent sportfish, which passively drift to seagrass nurseries during early development. [10, 14, 15, 18-22] Thus, the presence of photodynamic constituents in TWPs may pose a risk to fish populations via a photo-toxic mechanism, which is distinct from URMS.

Phase 1: Establish Analytical Protocols & Characterize the occurrence, nature, and extent of TWP contamination in Matagorda & San Antonio Bays

Methods

Analysis of TWP Leachate and 6PPD-quinone

For the untargeted analysis of leachate from TWPs, 0.2 μm filtered leachate samples were acidified to a pH of 2 with trace metal grade HCl to facilitate the extraction process, following standard SPE of dissolved organic matter.[23] Specifically, the PPL-SPE cartridges used for the extraction were first conditioned with LC/MS grade methanol (4 cartridge volumes), followed by another 2 cartridge volumes of pH 2 LC/MS water (acidified with trace metal grade HCl). After conditioning, filtered leachate samples were passed through the SPE cartridges at a speed less than 10 mL/min to maximize the extraction efficiency. Samples were eluted from the cartridge with four cartridge volumes of LC/MS grade methanol. The solvent was then gently blown by N_2 gas to a final volume of 2 mL. One mL of the sample was then transferred to HPLC vials for subsequent high-resolution LC/MS analysis, following our previous work under ESI+ ionization mode (e.g., Lu et al.[24]).

For 6PPD-quinone related analysis, standards were created using commercially obtained standards of 6PPD-quinone and deuterium labeled 6PPD-quinone (D5-6PPD-quinone). A standardization curve was constructed for both chemicals by dissolving them in acetonitrile (ACN) to create initial stock solutions. Working solutions were created from these stock solutions by dissolving them in LC/MS methanol to create the following concentrations: 0.01 μg/L, 0.05 μg/L, 1.0 μg/L, 5 μg/L, 10 μg/L, 20 μg/L, 50 μg/L, and 100 μg/L. All eight standards were wrapped in aluminum foil to protect them from light and stored at 4 °C until instrumental analysis. Blanks of pure methanol were also used in LC-MS analysis as a 0.0 μg/L reference point.

Cartridge resin and filtration tests

Cartridge resin tests were performed to determine which resin provided the highest percent yield of 6PPD-quinone. Duplicates of Bond Elut C8, Bond Elut C18, Bond Elut PPL (Agilent, Santa Clara, CA), Oasis HLB, Oasis PRiME MCX, and Oasis PRiME HLB (Waters, Milford, MA) were analyzed. For the cartridge resin tests, surface seawater was collected from the Port Aransas ship channel (Figure 1) using a 4 L amber glass bottle and filtered through a 5.0 μm Whatman™ Polycap™ HD 75 filter. This filtered water was then separated into 200 mL aliquots in 250 mL pre-combusted amber glass bottles. The aliquots were spiked with D5-6PPD-quinone to a final concentration of 20 μg/L. Each cartridge was conditioned with LC/MS methanol (ca. 10 mL/cartridge) and LC/MS H_2O (ca. 10 mL/cartridge) before being loaded with the water samples at a rate of ca. 5 – 10 mL/min. To ensure a higher recovery, each sample bottle was further rinsed with LC/MS H_2O and swirled well to cover the sides of the bottle at least three times (ca. 60 mL). The rinsing water was also passed through the column. To remove any residual salts, each column was further rinsed with 2 to 3 cartridge volumes of LC/MS H_2O before drying for another 10 – 20 min. Samples were finally eluted from the cartridge with 16 mL of LC/MS methanol, and the eluents were concentrated under a gentle nitrogen flow and volumized to 5 mL for analysis. Extracts were stored at -20 °C until instrumental analysis. The three best performing cartridges from the cartridge test were then selected to determine the effect of filtration on 6PPD-quinone

recovery. Seawater was collected and further filtered through a 0.2 µm Whatman™ Polycap™ AS filter, and this filtered water was processed as described above.

Salinity experiments

An experiment was run to evaluate the impact of salinity on 6PPD-quinone recovery after LC-MS analysis. Artificial seawater was created to the following salinities: 0, 10, 20, 30, and 40. Samples were spiked with D5-6PPD-quinone to an initial concentration of 20 µg/L and extracted via Oasis PRiME HLB cartridges. All samples were analyzed in duplicates using the methods described in the cartridge resin section.

Stock solution pretreatment experiments

A suite of experiments was conducted to evaluate the effectiveness of upstream treatments on 6PPD-quinone recovery. Specifically, the effects of solvent (ACN vs. dimethyl sulfoxide [DMSO]), freeze-thaw, sonication, and bottle type (plastic vs. glass) on 6PPD-quinone recovery were investigated. To investigate these effects, six stock solutions (concentrations of ca. 100 mg/L) were made. Stock solutions were further diluted into working solutions with concentrations of 5 µg/L, 50 µg/L, and 500 µg/L in methanol, and were run in the LC-MS. The six working solutions were as follows:

1) 6PPD-quinone dissolved in ACN in glass bottle, sonicated for 30 min.
2) 6PPD-quinone dissolved in fresh DMSO in glass bottle, sonicated for 30 min.
3) 6PPD-quinone dissolved in DMSO in glass bottle, sonicated for 30 min, frozen for 24 h at -20 ℃ and thawed;
4) 6PPD-quinone dissolved in DMSO in glass bottle, no sonication (wait for 30 min.), sample taken from the surface;
5) 6PPD-quinone dissolved in DMSO in glass bottle, no sonication (wait for 30 min.), sample taken from the bottom; and
6) 6PPD-quinone dissolved in fresh DMSO in plastic bottle, sonicated for 30 min.

Working solutions 1 & 2 were used to observe the choice of solvent (i.e. the effect of ACN vs. DMSO); working solutions 1, 2, & 3 were used to observe the effects of freeze-thaw; working solutions 2, 4 & 5 were used to observe the effects of sonication; and working solutions 1, 2, & 6 were used to observe the effects of using a plastic bottle vs. glass bottle. Glass treatments used pre-combusted 250 mL amber glass bottles, whereas plastic treatments used 250 mL plastic bottles. Samples were extracted via Oasis PRiME HLB cartridges and analyzed using the methods described in the cartridge resin section.

Incubation Experiments

An incubation experiment was performed to evaluate the stability of 6PPD-quinone in natural seawater. Surface seawater from the Port Aransas ship channel (Figure 1) was collected using a 4 L amber glass bottle and filtered through a 5.0 µm filter to remove larger particulates and ensure the retention of the microbial community, before being divided into 100 mL aliquots in pre-combusted 125 mL amber glass bottles. Bottles were spiked to a final concentration of 1 µg/L 6PPD-quinone. Samples were stored at room temperature in the dark and frozen at -20 ℃ at each respective time point prior to extraction and instrumental analysis. Control samples were not spiked with 6PPD-quinone. After the final time point, all samples were removed from the freezer

and thawed. Samples were then spiked with deuterated 6PPD-quinone (D5-6PPD-quinone) to a final concentration of 20 µg/L and extracted using Oasis PRiME HLB cartridges. The following sample pretreatment continued as described in the cartridge test section. All time points were analyzed in triplicates.

Environmental Characterization of 6PPD-quinone

Surface water samples were collected from south and central Texas rivers and bays in order to determine the environmental occurrence of 6PPD-quinone under runoff and base streamflow conditions. Briefly, 4 L water samples were collected from each station and put on ice prior to returning to the lab within the same day. Samples were then spiked with D5-6PPD-quinone to a final concentration of 20 µg/L. Extraction of 6PPD-quinone occurred in a manner similar to that described previously, although the environmental samples were neither filtered prior to extraction nor subdivided into smaller aliquots. Samples were collected in September 2023 and June 2024. A map of all sampling locations with geographic coordinates is shown in Figure 1B.

Mass Spectrometry Analysis

Quantification of the 6PPD-quinone in samples was carried out using an HPLC (Shimadzu LC-2040C) coupled to a triple-quadrupole mass spectrometer (Shimadzu LCMS-8045). The chromatograph used a Phenomenex reverse phase C18 column (Luna 3 µm C18 100 Å, 150 × 3 mm) and C18 guard column at a column temperature of 45 °C. Mobile phase A was LC/MS grade H_2O with 0.1% formic acid (v/v). Mobile phase B was a 1:1 mixture of (v/v) LC/MS grade methanol and LC/MS grade ACN with 0.1% formic acid (v/v). The injection volume of the sample was 10 µL, and the flow rate of the pump is 0.2 mL/min. The binary gradient was set as follows: 50% B 0−0.5 min, 50%−100% B 0.5−10.5 min, 100% B 10.5−12 min, 100%−50% B 12−15 min; 50% B 15−20 min. Detection used electrospray ionization (ESI+) and multi-reaction monitoring (MRM) modes. Nebulizing gas (nitrogen gas) flow was 3.0 L/min. Heating gas (nitrogen gas) flow was 10 L/min and drying gas (zero grade air) flow was 7.5 L/min. Interface temperature was set to be 300 °C, and the desolvation line (DL) temperature was also 300 °C. With a dwell time of 400 ms, mass to charge ratios (*m/z*) of 299.2→215.1 (14 eV) and 299.2→187.1 (26 eV) were selected as the qualitative and quantitative ion transitions of 6PPD-quinone, respectively. Similarly, *m/z* of 304.2→220.1 (14 eV) and 304.2→192.1 (26 eV) were used for the qualitative and quantitative analyses of D5-6PPD-quinone, respectively. Concentrations of 6PPD-quinone in samples were then estimated from a calibration curve that was derived based on an estimation of the sample concentrations, with D5-6PPD-quinone as the isotopic internal standard for response normalization.

Results

Untargeted Analysis of TWP Leachate

Over 1,100 compounds were detected and assigned with a CHONS formula from the leachate, dominated by aromatic-like compounds. However, none of the EPA-priority polycyclic aromatic hydrocarbons (PAHs) were detected in the analysis (i.e., concentrations were below method detection limits). It is not uncommon for tires to produce PAHs, but only under specific conditions such as when tires skid or when they are subjected to high friction. The tire particles used to make TWP leachate for the present study were generated from new tire via cryo-mill; therefore, these results are not surprising. Given the nature of untargeted analysis, identification of any detected compound from high-resolution MS would require additional information, such as tandem MS and/or comparison with known standards. Therefore, the untargeted analysis result itself would

not be sufficient to conclude the presence of specific chemicals until targeted analysis. Nevertheless, the results demonstrated the high diversity of the chemical compounds of the leachate, offering a useful database for toxicity evaluation.

Cartridge Resin and Filter Tests

Oasis PRiME HLB provided the highest percent yield of D5-6PPD-quinone at 80.0 ± 6.0 %, followed by Oasis Prime MCX (78.1 ± 3.2 %), Bond Elut C18 (70.3 ± 1.0 %), Oasis HLB (62.8 ± 0.7 %), Bond Elut C8 (56.1 ± 11.6 %), and Bond Elut PPL (26.9 ± 0.7 %) (Figure 3A). There were no significant differences in recovery rates amongst cartridge types (one–way analysis of variance [ANOVA]; $p > 0.05$), with the notable exception of Bond Elut PPL, which significantly different from other types of cartridges (one–way ANOVA; $p < 0.05$). Solid phase extraction enables the separation of components in a sample mixture based off the differential affinities between the sorbent material and analytes in the mobile phase. Retention of analytes to the stationary phase is achieved with strong but reversible interactions between the analyte and the surface of the sorbent.[25] Typical interactions are hydrophobic (van der Waals forces), polar (hydrogen bonding and dipole–dipole forces) or ion-exchange interactions.[25, 26]

The optimization of solid phase extraction is a crucial step to ensure extraction with a high efficiency and yield, and it is accordingly helpful to consider various physio-chemical considerations of the analyte and cartridge sorbents. Bond Elut PPL's base material is composed of a functionalized polystyrene-divinylbenzene stationary phase, and its extreme hydrophilicity ensures the extraction of highly polar analytes.[25] Given 6PPD-quinone's amphiphilic nature, it is expected that Bond Elut PPL was not able to extract the compound as effectively. On the other hand, HLB cartridges are specifically designed to interact with both polar and nonpolar functional groups, which can explain the superior performance of HLB PRiME observed in this study. These results agree with past research observing the impact of SPE cartridge type on 6PPD-quinone recovery rate. For example, Zhang et al.[27] found that Oasis PRiME HLB resulted in superior recoveries of 6PPD-quinone present in various marine organisms in comparison to Bond Elut C18. Additionally, using Oasis Prime HLB as a binding agent, Ren et al.[28] were able to construct diffusive gradients in thin films (DTG) capable of accurately measuring in-situ concentrations of 6PPD-quinone in aquatic environments. Accordingly, we recommend using Oasis PRiME HLB for 6PPD-quinone analysis in natural water samples.

After the cartridge test, the three-best performing cartridges (Oasis PRiME HLB, Oasis PRiME MCX, and Bond Elut C18) were selected to determine if filtration of water samples prior to extraction had any effect on the final recovery rate. There was no significant difference in recovery rate amongst cartridge types (one–way ANOVA; $p > 0.05$), and recovery rates were similar to those in the cartridge tests, suggesting that filtration does not have a significant impact on the final recovery rate and observed concentration of 6PPD-quinone (Figure 3B). This indicates that only a minor fraction of 6PPD-quinone is adsorbed to particles in the water column. Therefore, filtration is recommended before the solid phase extraction.

Influence of Salinity

The salinity of the initial solution had no effect on the final D5-6PPD-quinone recovery rate (one–way ANOVA; $p > 0.05$) (Figure 4). As the salinity of the initial solution increased, recovery rates slightly decreased (99.7 ± 32.8 % at 0 PSU, 74.2% ± 9.5 % at 40 PSU), although all recovery rates were within the standard deviation of each solution salinity. This finding is in line with previous work, which have observed acceptable recovery rates of 6PPD-quinone (e.g. > 75%) in aqueous

samples across a range of different salinities (Johanssen et al., 2021; Zeng et al., 2023).[29, 30]

Influence of Upstream Analytical Techniques

It was determined that sonication was necessary to completely dissolve 6PPD-quinone in DMSO, as undissolved 6PPD-quinone particles were present in stock solutions without sonication. Stock solution taken from the bottom of the vial (i.e., near the undissolved 6PPD-quinone particles) had a higher measured concentration compared with solution taken from the surface at 500 ppb (up to 27% higher). Additionally, the concentrations from the unsonicated solutions were greater than those in the sonicated fresh DMSO stock solution (Figure 5). Considering that these stock solutions were unsonicated, it is likely that 6PPD-quinone was not homogeneously distributed throughout the solution, explaining the observed higher concentration in comparison to the sonicated solution.

The measured peak intensity of 6PPD-quinone dissolved in DMSO was up to 50% higher than that in ACN at 500 ppb. This finding is in line with previous research comparing these two solvents. ACN was initially selected as a solvent due to its use in other 6PPD-quinone studies.[27, 31, 32] However, ACN does not lend itself to aquatic toxicological analyses of 6PPD-quinone exposure due to its observed toxicity in a variety of model species.[33] Accordingly, DMSO was selected as an appropriate solvent for use in a toxicological context due to its history as a nontoxic solvent at low concentrations.[34]

Results of our analyses suggested a clear solvent effect of DMSO on 6PPD-quinone. DMSO can cause charge-state coalescence of precursor ions and was reported to enhance electrospray response of protein and peptide by over 20%.[35, 36] Therefore, we recommend that if DMSO is used as the carrier solvent in toxicological test, the same solvent should be used in the subsequent chemical analysis.

Plastic bottles also affected the measured concentration of 6PPD-quinone. The measured concentration of 6PPD-quinone in DMSO in plastic bottles was 94.3% of that in glass bottles at 500 ppb. This is likely due to the significant sorptive capacity of plastics, due to their porous nature and thus high surface area to volume considerations which lend to various hydrophobic interactions in comparison to glass.[37] Accordingly, when given the option between plastic and glass, we recommend using glass as an appropriate container. Lane et al. [32]reported similar findings in a comparison between high density polyethylene (HDPE), polystyrene (PS), polypropylene (PP), and glass containers, and observed significant sorption in all plastic containers with improved outcomes (less sorption) as container size increased and the surface-to-volume ratio decreased.

Biodegradation Experiments

The concentration of 6PPD-quinone remained relatively stable over the 14-day incubation period, ranging from 0.94 ± 0.21µg/L at Day 0 to 0.74 ± 0.09 µg/L at Day 14 (Figure 6). There was no significant difference in 6PPD-quinone concentration over the time period analyzed (two-sample t-test; $p > 0.05$). These results are somewhat consistent with other experiments that observed the biodegradation of 6PPD-quinone in aqueous solutions. Di et al.[38] reported the half-life of 6PPD-quinone to be 13 to 16 days in river water, and Yan et al.[39] found that 6PPD-quinone remained stable in river water until the end of their experiment at 5 days. However, Hiki et al.[40] reported the half-life of 6PPD-quinone in dechlorinated drinking water at room temperature to be 33 hours, although the 6PPD-quinone in this study was exposed to a 16 h/8 h light/dark cycle for 5 days, in which the light is likely contributed to the shorter half-life through indirect and/or direct photolysis. These studies collectively demonstrate that the half-life of 6PPD-quinone in water varies widely,

influenced by factors such as temperature, sunlight exposure, water composition, and the presence of reactive species.

Field Results

6PPD-quinone was detected in almost all of the river surface water samples obtained (Figure 7). Concentrations ranged from 0.00 to 0.00127 µg/L, and concentrations at Cole Park in Corpus Christi Bay during high flow after storms were 0.00437 µg/L. These concentrations are in the range of those observed in Brisbane River, Australia (0.38–88 ng/L[41]), Highland Creek and Don River, Canada (< LOD – 720 ng/L[30]), and in the Zhujiang and Dongjiang Rivers in Guangdong, China (0.26–11.3 ng/L and 0.29–8.12 ng/L, respectively[27]). All observed concentrations were lower than those reported in Seattle-region roadway runoff (0.8–19 µg/L) and river water concentrations in this study were lower than those in Seattle-region watersheds after various storm events (<0.3–3.2 µg/L[11]). Despite samples being collected immediately after a storm event in June 2024, concentrations at all sites were below the LC50 for adult coho salmon (0.095 µg/L[42]). The low values obtained, particularly in relation to those observed in the Seattle region, are likely due to a combination of factors. Firstly, our sample sites are located in rural areas that receive lower volumes of traffic. Temperature and sunlight exposure also likely play a role. Although this study observed the presence of aqueous 6PPD-quinone concentrations, it should be noted that 6PPD-quinone favorably partitions into sediments or organic matter. 6PPD-quinone is amphiphilic and has a log octanol-water partition coefficient of 3.4 and is therefore likely to be found in greater concentrations in sediments.[43] Accordingly, further work should be done to assess the relative distribution and presence of 6PPD-quinone in water vs sediment samples.

Phase 2: Toxicity Testing with Native Fish Species

The initial goal of Phase 2 was to use two native estuarine fish models, including ELS sheepshead minnow (*Cyprinodon variegatus*) and red drum (*Sciaenops ocellatus*), to investigate the comparative toxicities of whole TWP mixtures and 6PPD-quinone. We hypothesized that TWP leachate would be more toxic to ELS estuarine fish than 6PPD-quinone alone, due to the potentiating effects of mixtures, combined with the presence of multiple photodynamic constituents in leachate.

Methods

Animal Care and Use

All experimental protocols were approved by The University of Texas at Austin Institutional Animal Care and Use Committee (IACUC; AUP-2021–00225). Red drum and Southern flounder embryos were produced via induced spawning of wild-caught broodstock maintained at The University of Texas at Austin Marine Science Institute (UTMSI) in Port Aransas, TX. Prior to use, all embryos were assessed for fertilization and quality using a NIKON SMZ800N (Nikon Metrology, Inc., Brighton, MI) microscope. Fertilized embryos were subsequently disinfected in a 1-part per thousand (‰) formalin wash for 1 hour with constant aeration, followed by a clean seawater rinse. Thereafter, embryos used for embryo-larval testing were immediately loaded into test chambers.

Embryos designated for use in post-settlement exposures were reared in static tanks containing filtered and conditioned natural seawater, biofilters and a source of continuous aeration. Salinity was maintained at 30 ± 2‰ on a 14 light: 10 dark light cycle at 27.5 ± 0.5 °C. Each day, fish were

fed Otohime marine fish larval and weaning feed (B1 – C2) (Marubeni Nisshin Feed Co., Ltd, Tokyo, Japan) to satiation, followed by removal of excess food and waste.

6PPD-quinone Exposures

Preparation of 6PPD-quinone Test Solutions

As described in Ackerly et al. (2024), a 1,000-mg/L master stock was first prepared by dissolving commercially produced 6PPD-quinone (HPC Standards, Atlanta, GA, USA; Item #: 687855) in DMSO. Thereafter, conditioned natural seawater was spiked with the master stock solution to achieve target nominal concentrations (the % DMSO was held constant across all treatments).

Preliminary Rangefinder Study with 6PPD-quinone (only) and Adult Sheepshead Minnow

Due to the lack of published toxicity values for 6PPD-quinone, we first conducted a rangefinder study to determine the 48-hour LC50 for adult sheepshead minnow (i.e., the concentration that is expected to lead to mortality in 50% of exposed organisms within a pre-selected test duration). Adults were determined to be the most appropriate life stage for preliminary testing, as the only other LC50 values published in the literature at the time were for adult life stages of other species. Moreover, comparative toxicity studies have demonstrated important sensitivity differences between wild caught and lab cultured fish populations within the same species.[44] Thus, we chose to utilize wild caught sheepshead minnow to ensure environmental relevance.

Briefly, adult sheepshead minnow were exposed to either 0, 5, 10, 15, or 20 µg/L 6PPD-quinone (n = 10 per treatment) for 48-hours. This concentration range incorporates the maximum reported measured values present in multilane roadway runoff (19 µg/L), receiving waters in urbanized watersheds of the Pacific Northwest (3.5 µg/L) that were sufficient to cause URMS in Coho salmon,[45] as well as the values measured by Co-PI Liu in local freshwater inflows.

Individual fish were housed in 1 L beakers filled with 500 mL of filtered seawater spiked with 6PPD-quinone in DMSO, or a solvent control containing only seawater and concentrations of DMSO equal to those in other treatments. Beakers were constantly aerated and water quality was checked every 24 hours. At test hour 24, 50% of the test solution in each chamber was replaced to ensure water quality was maintained within ideal ranges. Survival was assessed at both 24- and 48-hours (i.e., test termination), with all fish humanely euthanized in buffered MS-222 at test termination.

Despite using a high treatment concentration that was approximately equivalent to the highest measured values reported in the literature for multi-lane highway runoff, no mortality was observed at any concentration. Sheepshead minnow are a notoriously tolerant EPA model species that demonstrate a remarkable tolerance to multiple environmental stressors, including some toxicants. Therefore, we opted to replace sheepshead minnow with Southern flounder (*Paralichthys lethostigma*) for all subsequent testing.

Acute Toxicity Testing of 6PPD-quinone (only) with ELS Red Drum

LC50 values can vary considerably across species and life stages; therefore, initial testing with ELS red drum was conducted using concentrations ranging from 0 to 500 µg/L. As our exposures exceeded the maximum concentrations measured in direct roadway runoff by a full order of magnitude, we are confident that our study design surpassed the range of potentially relevant exposure scenarios for wild fish populations.

Red drum transition to exogenous feeding around 96-hpf; thus, the US EPA standard 96-hour

toxicity test protocol is not feasible for this species without conducting feedings (which can influence test results). Therefore, a 72-hour test duration was used for early development toxicity tests with this species. This test duration is routinely used for toxicity testing with this species and is considered equivalent to the 96-hour test protocol used for slower developing model fish species.[46, 47]

Toxicity tests with embryo-larval red drum were carried out in 250-mL borosilicate exposure vessels containing 20 embryos or larvae per dish and 200-mL of either a 0, 10, 50, 100, 300, or 500 µg/L seawater solution spiked with 6PPD-quinone (n = 3 replicates per treatment). Successful hatch was evaluated at test hour 24, with larval survival evaluated at test hours 48 and 72. At test termination, larvae were humanely euthanized in buffered MS-222, placed in methylcellulose on a glass microscope slide, and then imaged using a Nikon SMZ800N microscope (Nikon Metrology, Inc., Brighton, MI). ImageJ software (National Institutes of Health, version 1.8.0_172) was subsequently used to analyze images for a suite of morphological parameters, including standard length, body area, relative pericardial area, and relative brain and eye size.

Early results of mechanistic studies and anecdotal evidence from studies examining lethality in sensitive species suggest that the toxicity of 6PPD-quinone may be due to effects on oxygen transport.[48] Because red drum utilize cutaneous respiration prior to settlement (rather than relying on gas exchange at the gills),[49] studies conducted exclusively with pre-settlement larvae may miss important effects on oxygen transport processes that occur in later life stages. Therefore, in an effort to account for potential sensitivity differences across ontogeny, we also conducted additional rounds of testing with more developed stages of red drum, including 14-day post hatch (DPH) fish and 50 - 80 DPH fish.

Methods used for these exposures were consistent with those used for embryo-larval testing, with three modifications. These included an increase in the number of replicates (n = 6 replicate dishes per treatment), a reduced maximum exposure concentration (100 µg/L), and a test duration of 48 hours. Survival was evaluated every 24 hours and fish were humanely sacrificed in buffered MS-222 at test termination.

Acute Toxicity Testing of 6PPD-quinone (only) with ELS Southern Flounder

Methods utilized for embryo-larval Southern flounder tests were consistent with those described previously for red drum, including the use of survival and larval morphology as endpoints.

At 30 – 40 DPH, Southern flounder larvae begin to metamorphose into the familiar “flatfish” juvenile/adult phase over the course of 2 to 3 weeks. This process is characterized by a series of dramatic structural rearrangements that are energetically costly. Thus, to consider potential sensitivity differences that may exist between pre- and post-metamorphosis fish exposed to 6PPD-quinone, we also conducted toxicity testing with post-metamorphosis Southern flounder. Testing was conducted over the course of 96-hours with concentrations of 6PPD-quinone ranging from 0 to 100 µg/L, with survival assessed every 24 hours.

At test termination, post-metamorphosis fish were humanely sacrificed and gill baskets were dissected for histopathology. Gills were fixed in buffered 10% formalin and sent to the Fish Health and Pathology Lab at Texas A&M, where stained slides were prepared. Finished slides were subsequently sent to Dr. Morag Clinton (University of Alaska Fairbanks), who specializes in fish gill histopathology. All slides were blinded prior to evaluation to avoid the potential introduction of unconscious bias.

Statistical Analyses for 6PPD-quinone (only) Tests

Hatch success was calculated as the proportion of living hatched larvae relative to the number of embryos at test initiation (n = 20). Similarly, 48- and 72-hour survival data was calculated as the proportion of living larvae relative to the number of embryos at test initiation. Morphological measures based on larval surface area (i.e., body, brain, pericardial, and eye areas) were normalized to length prior to statistical analyses.

All statistical analyses for tests initiated with embryos were conducted with JMP Pro V 15.0, while exposures using post-settlement and/or post-metamorphosis stages were conducted in R (2022.02.03) using the packages *afex*, *emmeans*, and *fsa.* Data were tested for normality and homogeneity of variance using Shapiro-Wilk's and Levene's tests, respectively. Normally distributed data were analyzed with parametric tests, while non-parametric tests were used for non-normally distributed data.

Due to the high percentage of hatch success and survival for ELS red drum and Southern flounder exposed to 6PPD-quinone (Tables 1 & 2; Figure 8), survival data was not statistically analyzed. As 6PPD-quinone tests initiated as embryos included a small number of replicates (n = 3 dishes per treatment), a linear mixed effects model (with concentration as a fixed effect and replicate nested within concentration as a random effect) was used to evaluate morphological data for this life stage. For post-hatch larval exposures with greater replication (n = 6 per treatment), representative means were calculated for each parameter (total and notochord lengths, relative areas, heart rate measures), which were subsequently analyzed using an ANOVA or Kruskal-Wallis test, followed by a Dunn's post-hoc. An $\alpha = 0.05$ was used for all statistical tests.

Tire Wear Particle Leachate Exposures

Generation of TWP Leachate for Toxicity Testing

TWP leachate stock was generated by adding 100-g of cryo-milled TWPs to 1-L of saltwater, which was agitated for 10-days at 22°C. The resulting mixture (i.e., our 100% TWP leachate stock) was further diluted in seawater to nominal concentrations of 0 (control seawater), 5, 9, 14, and 18% leachate for acute toxicity tests, and 0, 0.5, 1, 2.5 and 5% leachate for sub-lethal testing.

Acute Toxicity Testing of Leachate with ELS Red Drum & Southern Flounder

The goals of acute toxicity testing with TWP leachate were to (1) develop of a 48-hour LC50 for ELS red drum and Southern flounder, (2) determine the relative sensitivities of two species of estuarine-dependent sportfish exposed to TWP leachate under ambient laboratory lighting conditions, (3) to determine whether photodynamic compounds present in leachate may potentiate toxicity to ELS sportfish via a phototoxic mechanism, and (4) determine whether ELS sportfish demonstrated a different relative susceptibility to TWP leachate exposure in the presence of solar radiation.

Fertilized embryos (n = 20 per dish) were exposed to 200 mL of test solution containing 0, 5, 9, 14, or 18% (nominally) TWP leachate in 250 mL borosilicate crystallizing dishes (n = 10 replicate dishes; Figure 9). Replicates for each concentration were then evenly divided into two cohorts based on the presence/absence of UV. Testing with the UV negative (UV-) cohort was conducted under ambient laboratory lighting conditions (i.e., visible light spectrum only), while testing with the UV positive (UV+) cohort was conducted under ambient laboratory lighting

augmented by 6 hours of artificial UV-A (AgroMax 4ft T5 UV-A Plus Bulbs). Toxicity testing for both cohorts (UV+ and UV-) was conducted simultaneously using a fully factorial study design that included 5 replicate dishes for every lighting and leachate combination (Figure 9). Exposures were carried out in an environmental chamber, with all other environmental parameters held constant (Figures 9 & 10).

Regardless of cohort, a 14 light: 10 dark light cycle was used both days of the test (Figure 10), which was intended to simulate conditions present in the Gulf of Mexico during red drum spawning season. For the entirety of the test, UV intensity was monitored at the 380 nm wavelength (UV_{380}) using an Ocean Optics© SR4 High Sensitivity Spectrometer. The average UV intensity was 0.017 $mW/cm^2/s$, which is approximately half that of incident surface UV measured in the Gulf of Mexico during late summer.[14, 15, 20, 50] A reduced target UV intensity was selected for this study to account for the fact that ELS red drum are primarily present in seagrass nurseries and are thus unlikely to continuously experience intensities as high as those measured at the water's surface. Hatch was evaluated at test hour 24 and larval survival was assessed at test hours 48 and 72.

Sublethal Toxicity Testing of Leachate with ELS Red Drum & Southern Flounder

The goals of sublethal toxicity testing with red drum and Southern flounder included (1) identifying TWP leachate concentrations that reduce fitness via changes in larval fish morphology, (2) to determine whether sub-lethal effects occur at lower relative exposure concentrations when UV is also present, and (3) to evaluate the relative sensitivity of red drum and Southern flounder larvae to sublethal concentrations of TWP leachate.

Sublethal testing was conducted using the same fully factorial study design described previously for acute testing; however, nominal exposure concentrations were reduced to 0, 0.5, 1, 2.5 and 5% TWP leachate (n = 5 replicates per treatment in each cohort; Figure 9). At the conclusion of sublethal tests, endogenously feeding larvae were imaged (n = 10 fish imaged per replicate dish) for analysis of additional endpoints including standard length, relative body size (surface area normalized to standard length), and relative brain, eye, pericardial, and yolk sac size (normalized to body area). These endpoints were evaluated using Image J (National Institutes of Health).

Statistical Analyses for TWP Leachate Tests

As described previously, hatch success and larval survival data were calculated by dividing the number of living hatched larvae by the initial number of embryos (n = 20). Morphological data based on larval surface area (i.e., body, brain, pericardial, and eye areas) were once again normalized to length prior to statistical analysis. All TWP leachate data was analyzed using JMP Pro V 18.0, at an $\alpha = 0.05$. The distribution of data and homogeneity of the variance were again evaluated using Shapiro-Wilk's and Levene's tests, respectively, prior to further analysis.

If data were found to be normally distributed, treatment effects were assessed using an ANOVA followed by a Tukey's post hoc. Non-normally distributed survival data were evaluated using a Kruskal-Wallis with a Dunn post hoc. LC50 values for acute toxicity tests were calculated using a logistic regression of survival by nominal treatment concentration, followed by an inverse prediction of 50% mortality.

Confirmatory Chemistry for 6PPD-quinone Exposures

At the end of each experiment, all fish were removed from each replicate and a composite water

sample was also taken from each concentration and immediately placed at -20°C for quantification of 6PPD-quinone concentrations at test end. Aliquots (100 mL) of test solutions from each treatment were collected in duplicate for confirmatory chemical analyses. Each water sample was spiked with D5-6PPD-quinone as a surrogate standard, to a final concentration of 25 µg/L. The solid phase extraction was performed using Oasis PRiME HLB extraction cartridges (200 mg, 6 mL). Cartridges were conditioned with LC/MS grade methanol (10 mL) and LC/MS grade H_2O (10 mL) before loading unfiltered water samples at a rate of 5 – 10 mL/min. To ensure a higher recovery, each sample bottle was further rinsed with LC/MS grade H_2O and swirled well to wash sides of the bottle at least three times, before loading onto cartridges. To remove residual salts, each cartridge was further rinsed with 2 to 3 cartridge-volumes of LC/MS grade H_2O before being dried for 10 – 20 min. Cartridges were eluted using LC/MS grade methanol and concentrated under a gentle nitrogen flow. Samples were reconstituted with methanol to a designated volume and stored at -20 °C until further analysis.

6PPD-quinone in samples was analyzed using high performance liquid chromatograph coupled to a triple-quadrupole mass spectrometer (HPLC/MS, Shimadzu LCMS-8045). A reverse phase C18 column (Phenomenex, Luna 3 µm C18 100, 150 × 3 mm) was used with a C18 guard column (at 45 °C). Mobile phase A was LC/MS grade H_2O with 0.1% formic acid (v/v). Mobile phase B was 1:1 mixture of (v/v) LC/MS grade methanol and LC/MS grade ACN with 0.1% formic acid (v/v). The injection volume of the sample was 10-µL, and the flow rate of the pump was 0.2-mL/min. The binary gradient was set as follows: 50% B: 0−0.5 min, 50%−100% B: 0.5−10.5 min, 100% B: 10.5−12 min, 100%−50% B: 12−15 min; 50% B: 15−20 min. Detection was performed using electrospray ionization (ESI+) and multi-reaction monitoring (MRM) modes. Nebulizing gas (nitrogen gas) flow was 3-L/min. Both heating gas (nitrogen gas) and drying gas (zero grade air) flow rates were 10-L/min. The interface and desolvation line (DL) temperatures were both set to 300 °C. A dwell time of 400-ms was used and mass to charge ratios (m/z) of 299.2→215.1 (14 eV) and 299.2→187.1 (26 eV) were selected as the qualitative and quantitative ion transitions of 6PPD-quinone, respectively. The m/zs of 304.2→220.1 (14 eV) and 304.2→192.1 (26 eV) were used for the qualitative and quantitative analyses of D5-6PPD-quinone. 6PPD-quinone concentrations were estimated from a calibration curve.

RESULTS

6PPD-quinone

Measured 6PPD-quinone concentrations

Select nominal versus measured concentrations of 6PPD-quinone are shown in Table 5. As nominal concentrations were generally within 10% of the measured concentrations for the embryonic and larval exposures, these data are presented and discussed using the nominal concentrations. However, the measured concentrations for the post-settlement exposures were within 50% of the nominal concentrations. Additional analytical analyses showed that 6PPD-quinone dissolved in DMSO can result in a heterogeneous distribution pattern in solution, providing a plausible explanation for the variance between targeted and measured concentrations (data not shown).

Rangefinder with Adult Sheepshead minnow

As previously stated, no treatment effects on survival were observed in wild caught adult sheepshead minnow exposed to 6PPD-quinone over the course of 48 hours.

Acute Toxicity Testing of 6PPD-quinone (only) with ELS Red Drum

Exposure to 6PPD-quinone did not result in significant acute mortality of red drum exposed as embryos, larvae, or post-settlement juveniles at any concentration tested (Table 1; Figure 11A). There were no significant treatment effects of 6PPD-quinone exposure on the total length, notochord length, relative brain area, relative pericardial area, relative eye area, or heart rate of larval red drum, although there was a significant decrease in the relative body size (Kruskal-Wallis; χ^2=11.24; DF = 5; p = 0.05) in the 10 µg/L and 50 µg/L treatments (Table 3; Figure 11A). Despite reaching the threshold for statistical significance, it is unlikely that these results are of biological significance, as the decrease was ≤ 2.6 % of overall body size and was not observed in the 100 µg/L treatment. Average values (± 1 standard error) and statistics on all red drum morphological parameters are available in Table 3.

Acute Toxicity Testing of 6PPD-quinone (only) with ELS Southern Flounder

Exposure to 6PPD-quinone did not result in significant acute mortality of embryonic, larval, or post-metamorphosis flounder at any of the concentration tested (Table 2; Figure 11B). There were also no significant treatment effects on any of the morphological parameters evaluated (Figure 11B). Average values (± 1 standard error) and statistics on all Southern flounder morphological parameters are available in Table 4.

Results of the histopathological analysis revealed small lamellar and filament tip bleeds in a subset of fish from all treatments; however, there was no evidence of erythrocyte degradation or fibrinogen deposition within petechial bleeds. Thus, these findings were not found to be of clinical significance and are likely an artifact of handling and/or sample collection rather than exposure to 6PPD-quinone. Gills from the control group demonstrated densely cellular filament tips, with a regenerative appearance. Gill structure was generally delicate (no interlamellar cell mass noted), and a small number of eosinophils were present at the base of lamellae within gill filaments. There was also no membrane damage or lysis observed in the control group, the epithelial pavement cells were well attached to the underlying pillar cells of lamellae, and there was a low prevalence of epithelial lifting artifacts. Collectively, these results indicate good fixation of gill tissues.

Pathological features were noted within the 50 µg/L treatment of varying potential clinical significance. Mild cellular hypertrophy was noted within gill sections (primarily with enlarged ionocyte/chloride cell types) and ionocyte/chloride cell hypertrophy was noted in a subset of fish. Hypertrophy of epithelial pavement cells was also noted for some individuals in the 50 µg/L treatment (Image 1). Particularly large goblet cells accompanied by increases in cellularity were also noted in a limited number of fish from this treatment relative to controls. Changes in the cellularity of chloride cells was also noted in one fish, as was an isolated foci of cell death (localized to a single lamellae) with pyknosis of epithelial pavement cell nuclei and basophilic fragmentation of cells (Image 2). Gill filaments of fish from the 50 µg/L treatment also demonstrated a higher prevalence of eosinophilic granular material relative to group 0 fish.

Similarly, pathological features of varying potential clinical significance were noted within the 100 µg/L treatment. Mild cellular hypertrophy - primarily with enlarged epithelial pavement cell types – was observed in several fish from the 100 µg/L treatment (Image 3). Goblet cells also demonstrated increased cellularity relative to the control group, while only one fish presented with increased cellularity of ionocyte/chloride cells.

Localized epithelial pavement cell disruption was noted in multiple fish that were similar in appearance to artifactual changes and epithelial cell lifting/loss of membrane structure was also observed in the gills of fish from the 100 µg/L group (Image 4). There was an increased presence of eosinophilic granular material noted in a subset of fish from this treatment, with a high presence of eosinophilic granular material within gill filaments and lamellar structures in two individuals (Images 5 & 6).

Interestingly, multiple infectious pathologies were noted within this treatment, including single celled parasitic organisms and basophilic stained, rod-shaped bacteria that were consistent in appearance between samples. These infectious agents provide a likely explanation for the epithelial pavement cell damage discussed previously (Images 7 & 8), as well as the subtle increase in inflammatory cells (lymphocytic cell types) noted in one fish from this treatment.

Collectively, these results suggest a possible immunotoxic effect of 6PPD-quinone in post-metamorphosis Southern flounder, although it is important to note that these effects were observed only in fish exposed to concentrations well above those with environmental relevance. Thus, it is unlikely that wild Southern flounder populations will experience widespread immunotoxicity due to 6PPD-quinone exposure during this life stage. However, subtle effects on the immune system can increase the adverse effects of exposure to other environmental contaminants, particularly those that also act through an immunotoxic mechanism of action.

Tire Wear Particle Leachate

Confirmatory Chemistry for TWP Leachate

In addition to TWP leachate analyses done by Co-PI Liu, TWP leachate used in experiments samples were also sent to ALS Environmental, an EPA certified analytical laboratory, for analysis of PAHs via Ultra LVI Semivolatiles by EPA Method 8270D. PAHs are toxic constituents of fossil fuels, a subset of which are the most abundant and well-studied photodynamic compounds ubiquitously detected in the aquatic environment (Table 6).[20, 50] These compounds are often found to be drivers of photo-induced toxicity in complex mixtures; thus, we suspected these compounds may be responsible for the phototoxic effects observed in TWP leachate toxicity testing.

Briefly, 100 mL TWP leachate samples (exposed to either ambient light or UV light) were collected in amber bottles and placed at 4°C. Samples were sent on ice overnight to ALS the day after collection, where they were analyzed for PAHs within 7 days of receipt. The analysis conducted by ALS included 18 PAH analytes: 1-Methylnaphthalene, 2-Methylnaphthalene, Acenaphthene, Acenaphthylene, Anthracene, Benz[a]anthracene, Benzo[a]pyrene, Benzo[b]fluoranthene, Benzo[g,h,i]perylene, Benzo[k]fluoranthene, Chrysene, Dibenz[a,h]anthracene, Fluoranthene, Fluorene, Indeno[1,2,3-cd]pyrene, Naphthalene, Phenanthrene, and Pyrene. This analyte list incorporates all compounds from Table 6, with the exception of benzo[a]fluoranthene and benzo[e]pyrene, both of which have a fairly low photodynamic activity relative to anthracene (i.e., RPA) and are therefore not expected to be among the most important drivers of phototoxic effects.[17, 51, 52]

Interestingly, none of the PAH analytes were present above method detection limits (which were sufficiently low to detect the range of phototoxic concentrations identified in the literature[9, 18-20, 50, 53]) in the ambient- or UV-exposed TWP leachate samples used for toxicity testing. This

indicates that any phototoxic responses observed in ELS red drum and Southern flounder co-exposed to UV and unweathered TWP leachate must be primarily driven by other unidentified photodynamic PAHs or non-PAH constituents.

Acute Toxicity Testing of Leachate to ELS Red Drum & Southern Flounder

Survival of red drum was significantly (ANOVA: $p < 0.001$) impacted by TWP leachate exposure after 48-hours; however, this effect was only observed in the presence of UV (Figure 12A). A nominal LC50 of 12.41% leachate was calculated for ELS red drum co-exposed to UV; however, we were unable to calculate an LC50 value for the UV- group, due to high survival of embryo-larval red drum in all treatments.

Survival of Southern flounder was significantly (ANOVA: $p < 0.001$) impacted in by TWP leachate exposure after 48-hours, with and without UV co-exposure. A nominal LC50 of 17.6% leachate was calculated for the UV- group, using the methods previously described. In the UV+ group, the nominal LC50 value for leachate was 11.6%, indicating that TWP leachate is likely more toxic under environmentally relevant conditions relative to the standard indoor lighting conditions used for laboratory toxicity testing. It is also important to note that these exposures were conducted using unweathered TWP leachate, which is likely to be less toxic than weathered leachate. This is due to the generation of oxygenated photoproducts during the weathering process that are typically more toxic than their parent compounds.

Sublethal Toxicity Testing of Leachate with ELS Red Drum & Southern Flounder

During subsequent sublethal testing with ELS red drum, survival was again significantly (ANOVA: $p < 0.001$) impacted by exposure to TWP leachate at test hour 48. However, lethal effects were observed at lower nominal concentrations (5% leachate) than those observed during acute toxicity testing (which also included a 5% treatment) and a potentiating effect of UV co-exposure was not observed (Figure 12B). These results may be at least partly explained by the use of nominal concentrations and/or inter-study variations in the relative concentrations of yet-to-be-identified phototoxic components present in the complex TWP leachate mixture. Regardless, these results indicate that ELS red drum are sensitive to TWP contamination, but that it is not exacerbated by the presence of UV below a certain exposure threshold. More work is needed to determine which specific components of the leachate mixture may be driving the phototoxic effects at higher relative concentrations.

Surviving red drum larvae also demonstrated significant effects of TWP leachate exposure on morphological parameters, including length and relative yolk area (Figure 13). Length was significantly reduced in ELS red drum exposed to 5% leachate, but only in the UV-exposed group (Mixed Model: $p = 0.045$; Figure 13A). ELS red drum exposed to 2.5% and 5% leachate in the presence of UV also had significantly larger relative yolk areas compared to all other treatment groups (Mixed Model: $p = 0.039$; Figure 13B), indicating that exposure to leachate at these doses in the presence of UV may be significantly impacting the fish's ability to utilize yolk energy reserves. This has potential implications for energetically costly developmental processes, as well as the transition to exogenous feeding, which is often associated with significant latent mortality.[44, 46]

Survival of ELS flounder included in chronic toxicity testing was again significantly (ANOVA: $p = 0.007$) impacted by exposure to TWP leachate at test hour 48; however, it was also impacted by UV exposure, even in control dishes (Figure 14). This indicates that ELS flounder are

sensitive to damage from UV radiation (alone), as well as to TWP contamination. Surviving fish also demonstrated significant effects of TWP leachate exposure on morphological parameters, including length and pericardial edema (Image 9). Length was significantly reduced in all TWP leachate exposure concentrations > 1% (Mixed Model; $p < 0.001$; Figure 15A), with even more significant reductions observed in the UV co-exposed group (Mixed Model; $p < 0.001$). This indicates that TWP contamination adversely affects the fitness of ELS flounder through both non-phototoxic and phototoxic mechanisms.

Similarly, the presence of pericardial edema was significantly higher (Mixed Model; $p < 0.001$; Figure 15B) in ELS flounder exposed to TWP leachate at exposure concentrations > 1%. While this effect occurred in both UV+ and UV- treatments, the effect was not significantly greater in the UV+ cohort. Thus, effects on pericardial edema appear to be driven by non-phototoxic mechanisms.

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Table 1. Red drum survival (6PPD-quinone)

	Embryonic Exposures			Larval Exposures		Post-settlement Exposures
Dose (PPB)	Hatch Success (%)	48h Survival (%)	72h Survival (%)	48h Survival (%)	72h Survival (%)	48h Survival (%)
0	97.92±1.14	92.92±2.42	92.22±3.02	100±0.00	100±0.00	100
10	94.17±1.83	92.50±2.72	92.50±1.44	96.67±2.47	95.83±2.39	-
50	94.17±1.82	91.25±2.05	91.25±2.22	100±0.00	100±0.00	100
100	94.17±1.93	93.75±1.52	93.33±2.36	100±0.00	100±0.00	100
300	97.08±1.79	94.17±2.20	94.17±1.67	100±0.00	97.50±1.12	-
500	94.17±2.45	92.50±2.58	91.11±2.74	95.83±1.54	95.83±1.54	-

Table 2. Southern flounder survival (6PPD-quinone)

	Embryonic Exposures			Larval Exposures		Post-settlement Exposures
Dose (PPB)	Hatch Success (%)	48h Survival (%)	72h Survival (%)	48h Survival (%)	72h Survival (%)	96h Survival (%)
0	93.33±1.67	91.67±4.41	91.67±4.41	96.00±1.88	95.00±3.16	100.00
10	98.33±1.67	100±0.00	95.00±5.00	98.00±2.00	96.00±2.45	-
50	96.67±3.33	96.67±3.33	91.67±4.41	93.33±3.16	90.83±4.18	75.00
100	100±0.00	93.33±4.41	88.33±4.41	92.50±4.06	85.00±10.41	87.50
300	96.67±1.67	91.67±3.33	90.00±2.89	96.25±1.25	86.25±5.16	-
500	88.33±1.67	80.00±5.77	78.33±6.01	94.17±2.00	91.67±2.45	-

Table 3. Red drum morphology (6PPD-quinone)

Dose (PPB)	Standard Length (µm)	Notochord Length (µm)	Relative Body Area (µm²)	Relative Brain Area (µm²)	Relative Pericardial Area (µm²)	Relative Eye Area (µm²)	Heart Rate Average (BPM)
Statistical Output	*$F_{5,30}$=1.84; p=0.14*	*$F_{5,30}$=1.32; p=0.28*	*χ^2=11.24; df=5; p=0.05*	*$F_{5,30}$=0.49; p=0.78*	*$F_{5,30}$=0.60; p=0.70*	*$F_{5,30}$=0.78; p=0.57*	*χ^2=7.19; df=5; p=0.21*
0	2574.58±23.75	2096.81±23.23	285.59±0.83	20.08±0.31	10.43±0.16	18.64±0.35	144.93±3.65
10	2563.48±18.49	2096.79±13.52	279.39±1.71	19.16±0.81	10.59±0.20	18.39±0.38	158.36±9.84
50	2591.98±16.30	2123.49±12.91	278.25±1.61	20.05±0.57	10.28±0.30	18.23±0.30	144.27±6.73
100	2590.52±19.97	2126.45±18.81	283.67±2.46	19.64±0.86	10.40±0.21	17.99±0.45	164.07±10.56
300	2527.20±29.29	2077.44±29.21	281.16±1.45	19.37±0.35	10.75±0.16	17.85±0.17	159.73±9.01
500	2516.71±30.33	2061.24±29.01	282.88±1.75	19.14±0.51	10.40±0.37	17.94±0.37	164.27±9.49

Table 4. Southern flounder morphology (6PPD-quinone)

Dose (PPB)	Standard Length (µm)	Notochord Length (µm)	Relative Body Area (µm)	Relative Brain Area (µm)	Relative Pericardial Area (µm)	Relative Eye Area (µm)	Relative Yolk Area (µm)
Statistical Output	*$F_{5,30}$=0.34; p=0.89*	*χ^2=3.40; df=5; p=0.64*	*χ^2=4.07;df=5;p=0.54*	*$F_{5,30}$=1.06; p=0.40*	*$F_{5,30}$=0.65; p=0.67*	*$F_{5,30}$=1.22; p=0.33*	*$F_{5,30}$=0.32; p=0.90*
0	3173.83±50.34	2816.15±52.65	264.79±2.20	14.21±0.18	7.58±0.35	9.52±0.12	21.86±1.45
10	3152.82±26.05	2825.46±23.43	255.81±5.44	14.27±0.54	6.89±0.36	9.65±0.26	21.42±0.60
50	3140.99±33.33	2801.05±39.25	260.61±1.78	14.26±0.35	7.44±0.27	9.63±0.19	21.71±1.71
100	3129.42±42.99	2774.48±37.94	258.63±2.84	14.79±0.50	7.49±0.34	10.11±0.27	20.63±0.65
300	3175.95±24.77	2838.58±14.83	263.16±2.21	15.17±0.26	7.47±0.33	9.82±0.14	22.30±0.73
500	3177.03±22.69	2798.99±12.37	257.16±5.02	15.04±0.53	7.24±0.19	9.95±0.19	22.27±0.99

Table 5. Nominal and measured concentrations of 6PPD-quinone for embryonic, larval, and post-settlement fish exposures

Exposure	Nominal Dose (PPB)	Measured Dose (PPB)	Sampling Timepoint
Embryonic	0	0.00	Final
	500	442.21	Final
Larval	0	6.10	Final
	100	98.62	Final
	500	449.85	Final
Post-settlement	0	0.00	Initial
	50	26.28	Initial
	100	53.50	Initial
	0	0.00	Final
	50	3.66	Final
	100	6.53	Final

Table 6. List of known photodynamic PAHs and their photodynamic activity relative to anthracene (RPA). Higher RPAs correlate with a greater phototoxic response in transparent aquatic biota. Compounds highlighted in orange were included in the PAH analysis conducted by ALS.

COMPOUND	RPA
Pyrene	1.41
Benzo[a]pyrene	1.08
Dibenz[ah]anthracene	1.05
Anthracene	1
C1-pyrene/fluoranthene	0.929
C2-pyrene/fluoranthene	0.929
C3-pyrene/fluoranthene	0.929
C4-pyrene/fluoranthene	0.929
Benz[a]anthracene	0.871
Chrysenes (C1, C2, C3, C4)	0.832
Fluoranthene	0.832
Benzo[a]fluoranthene	0.376
Benzo[b]fluoranthene	0.376
Benzo[k]fluoranthene	0.376
Benzo[e]pyrene	0.326
C1-anthracene/phenanthrene	0.182
C2-anthracene/phenanthrene	0.182
C3-anthracene/phenanthrene	0.182
C4-anthracene/phenanthrene	0.182

Table 7. Average values and statistics for morphological analyses conducted as part of sub-lethal TWP exposures with ELS red drum.

Nominal Dose (PPB)	Light Treatment	Standard Length (µm)	Relative Body Area (µm²)	Relative Brain Area (µm²)	Relative Eye Area (µm²)	Relative Yolk Area (µm²)	Relative Pericardial Area (µm²)
Statistical Outputs	Treatment	**p=0.0448**	p=0.7220	p=0.6126	p=0.7036	**p=0.0387**	p=0.4878
	Dose	p=0.1566	**p=0.0444**	**p=0.0006**	**p<0.0001**	**p=0.348**	**p=0.0217**
	Treatment:Dose	p=0.0910	p=0.9568	p=0.1235	p=0.6165	p=0.0571	p=0.9528
0	Ambient	2630.84±17.86	266.49±2.91	18.56±0.47	12.50±0.32	22.92±0.43	9.57±0.60
0.5	Ambient	2677.46±17.76	269.33±2.22	17.30±0.56	12.02±0.47	23.00±46	9.55±0.50
1	Ambient	2644.78±14.50	265.76±1.56	18.37±0.35	12.48±0.22	22.35±0.42	8.75±0.46
2.5	Ambient	2616.73±27.11	269.83±3.14	17.88±0.61	12.77±0.31	23.27±0.44	9.85±0.57
5	Ambient	2631.41±21.32	269.05±2.21	19.31±0.60	12.19±0.33	22.47±0.41	9.47±0.61
0	UV	2676.43±18.60	260.51±2.13	16.75±0.41	10.76±0.26	23.08±0.46	7.99±0.39
0.5	UV	2629.05±18.52	263.28±1.91	17.35±0.51	11.22±0.23	22.45±0.44	8.53±0.40
1	UV	2640.49±18.60	263.63±2.13	17.03±0.41	11.23±0.36	22.57±0.68	7.89±0.48
2.5	UV	2610.25±25.51	266.60±2.43	17.57±0.51	11.27±0.33	24.62±0.51	9.25±0.51
5	UV	2518.30±22.31	262.70±3.19	17.03±0.66	11.24±0.33	24.98±0.72	8.21±0.77

Table 8. Average values and statistics for morphological analyses conducted as part of sub-lethal TWP exposures with ELS Southern flounder.

Nominal Dose (PPB)	Light Treatment	Standard Length (µm)	Relative Body Area (µm²)	Relative Brain Area (µm²)	Relative Eye Area (µm²)	Relative Yolk Area (µm²)	Relative Pericardial Area (µm²)
Statistical Outputs	Treatment	**p=0.0011**	**p=0.0495**	p=0.1928	**p=0.0327**	**p<0.0001**	p=0.3665
	Dose	**p<0.0001**	**p<0.0001**	**p=0.0452**	p=0.4402	**p=0.0040**	**p<0.0001**
	Treatment:Dose	p=0.1322	p=0.4958	p-0.9292	**p=0.0303**	p=0.5710	p=0.3283
0	Ambient	3128.26±26.38	270.60±2.38	10.978±0.30	11.17±0.49	66.17±0.63	11.71±2.54
0.5	Ambient	3169.45±16.73	269.22±2.80	9.86±0.57	10.60±0.26	66.54±1.42	6.68±0.94
1	Ambient	3113.98±40.70	270.95±2.42	10.05±0.51	11.02±0.34	66.42±1.27	7.31±0.87
2.5	Ambient	3054.58±31.14	271.96±4.21	9.96±0.47	11.92±0.43	62.23±2.81	23.29±4.27
5	Ambient	2988.75±19.44	286.44±2.54	8.49±0.51	10.42±0.38	57.30±2.95	48.33±2.64
0	UV	3110.70±15.58	272.24±4.02	9.952±0.52	11.79±0.45	73.10±1.86	5.00±0.54
0.5	UV	3108.43±20.69	270.28±4.03	10.03±0.60	11.57±0.49	71.21±1.77	8.38±2.02
1	UV	3081.42±24.47	272.10±4.47	9.57±0.69	11.97±0.43	73.85±1.77	7.35±2.11
2.5	UV	2942.02±28.46	279.68±2.64	9.12±0.46	10.89±0.34	69.15±2.11	25.32±2.71
5	UV	2844.95±24.11	299.87±3.78	7.90±0.53	11.47±0.37	68.40±1.92	44.44±2.83

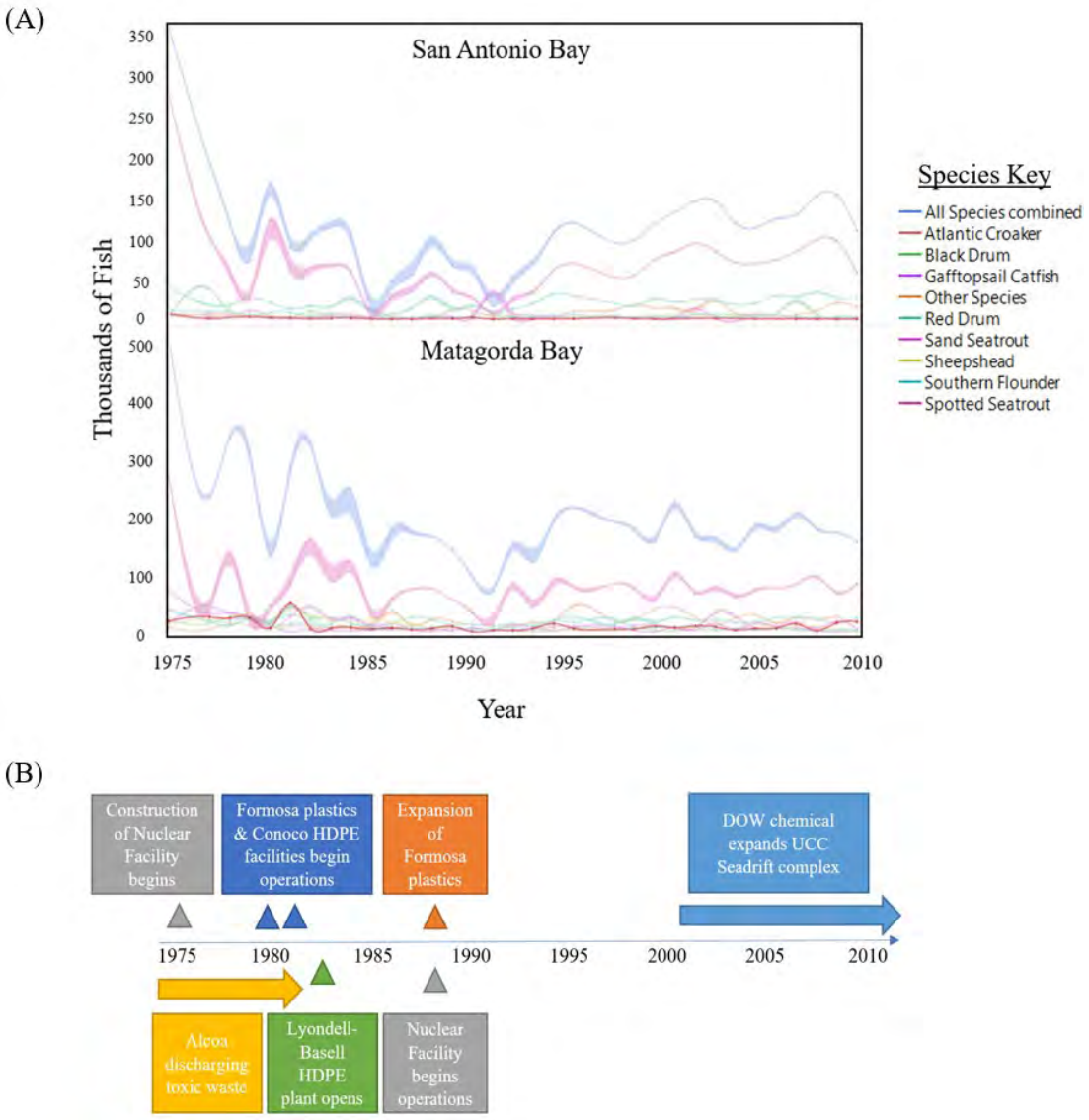
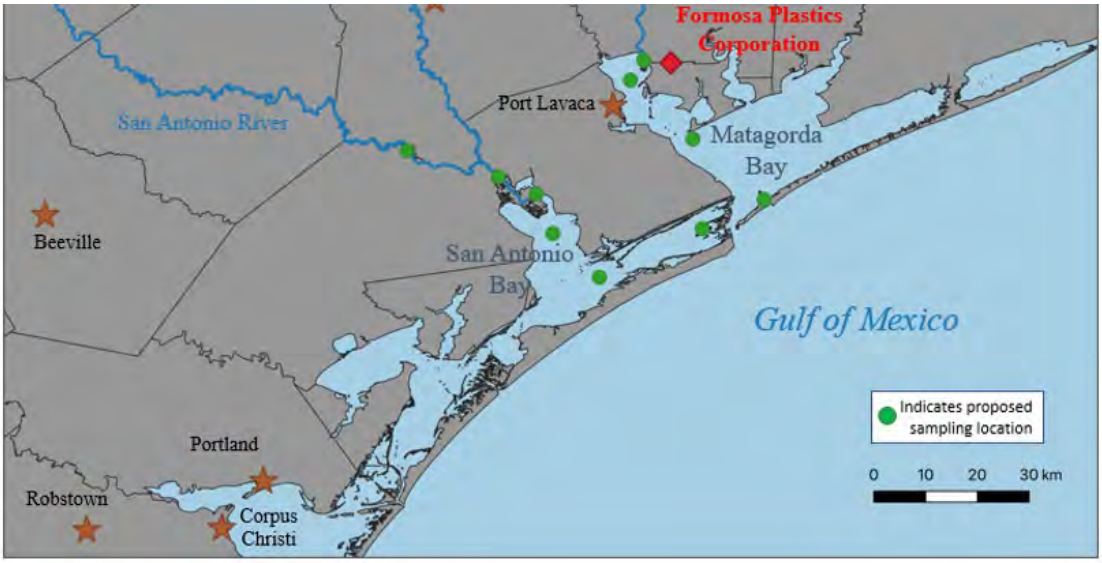
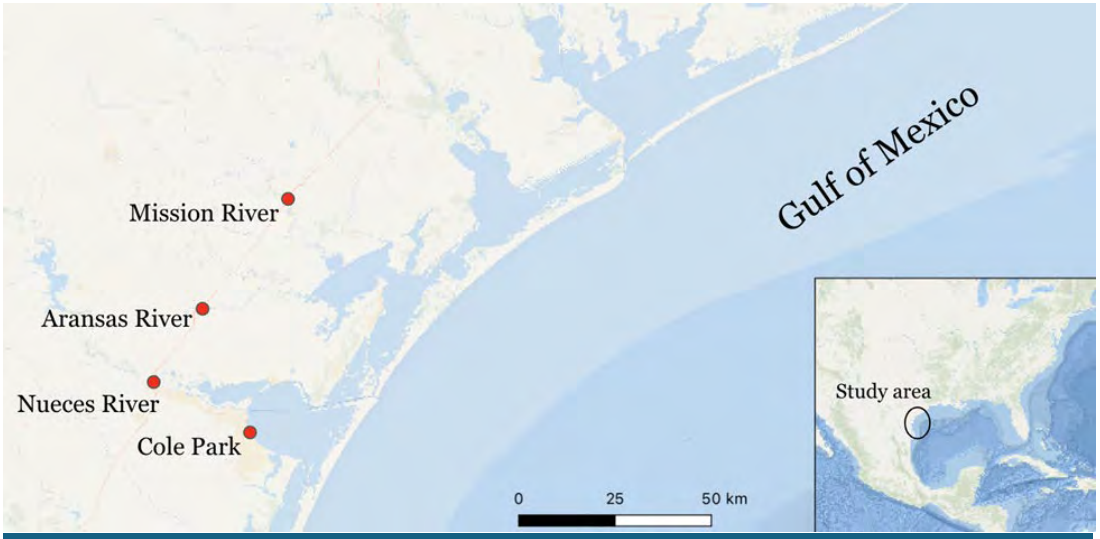


Figure 1. Visual comparison of **(A)** population trends for select fish species and **(B)** select events contributing to industrialization of the Matagorda and San Antonio Bay systems (1974 – 2008). Fish population levels represent annual finfish landings (in thousands of fish) reported by Texas Parks & Wildlife for private sport boat anglers in the study areas.

(A)



(B)



Site Name	Latitude	Longitude
Mission River	28.291327	-97.278431
Aransas River	28.127029	-97.427795
Nueces River	27.883556	-97.625629
Cole Park	27.771393	-97.2888461

Figure 2. (A) All sampling sites, and **(B)** sampling sites (including latitude and longitude) for which samples have already been analyzed and results are reported herein. Analysis of remaining environmental samples is still underway.

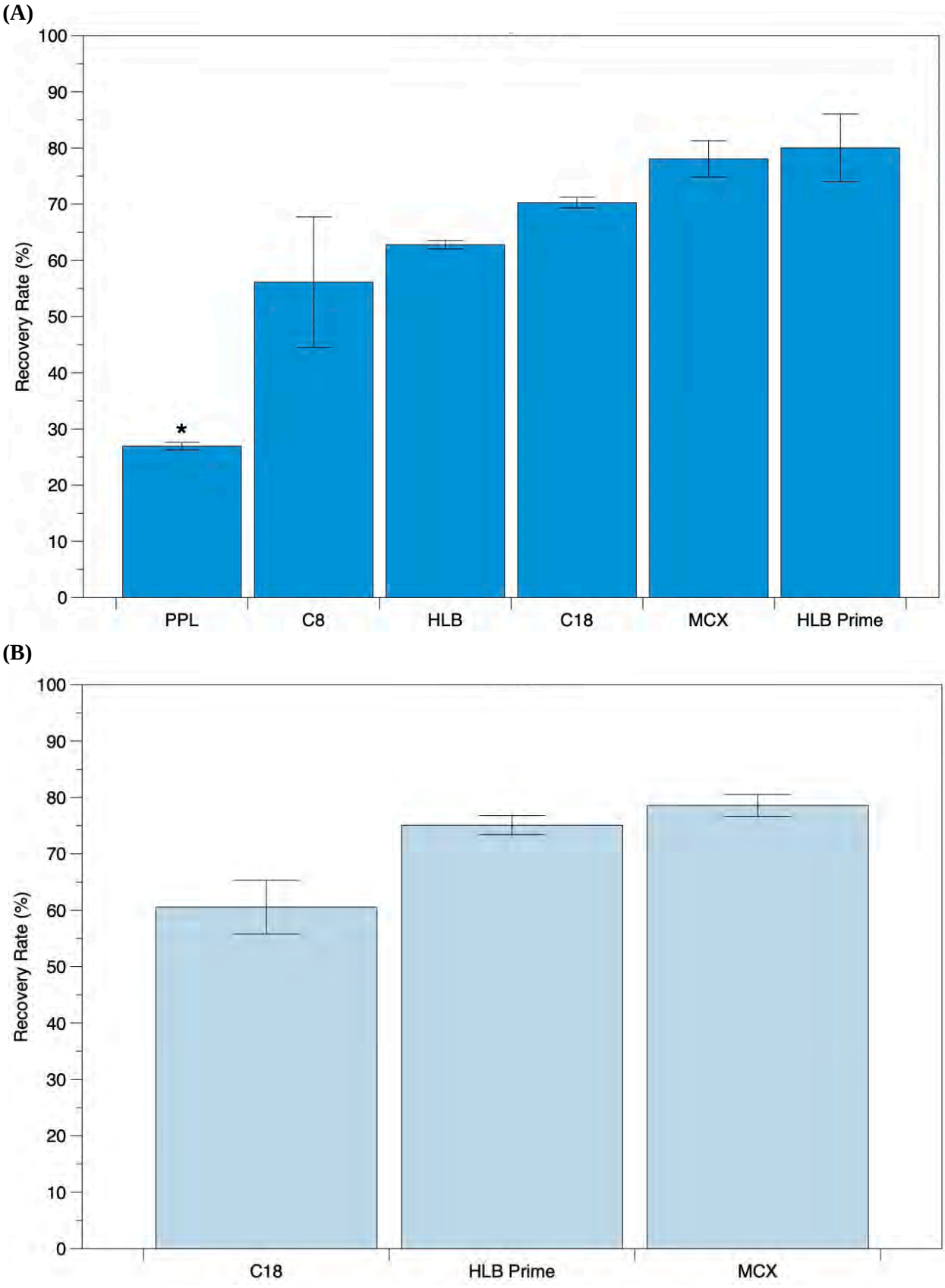


Figure 3. D5-6PPD-quinone recovery rate **(A)** by cartridge type and **(B)** in filtered samples (also by cartridge type).

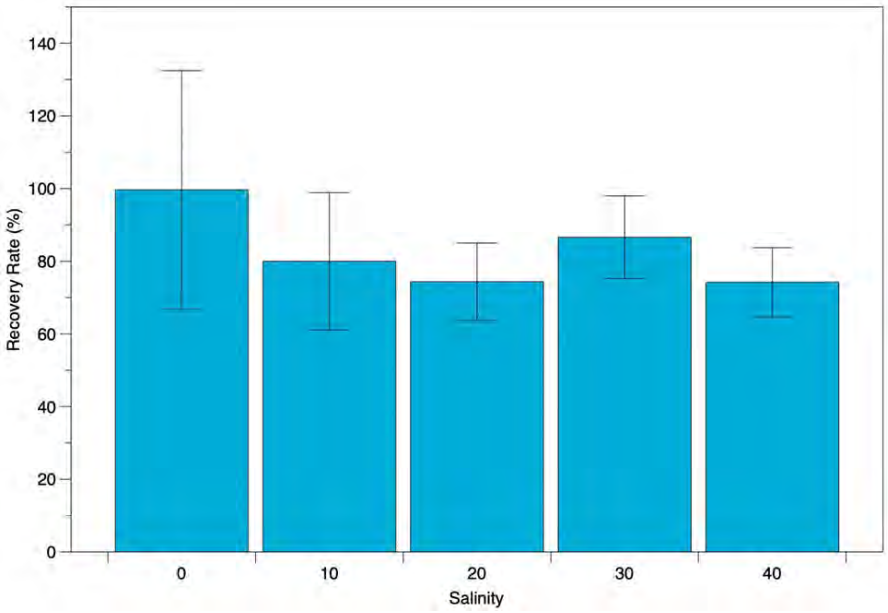


Figure 4. D5-6PPD-quinone recovery rate based on initial salinity.

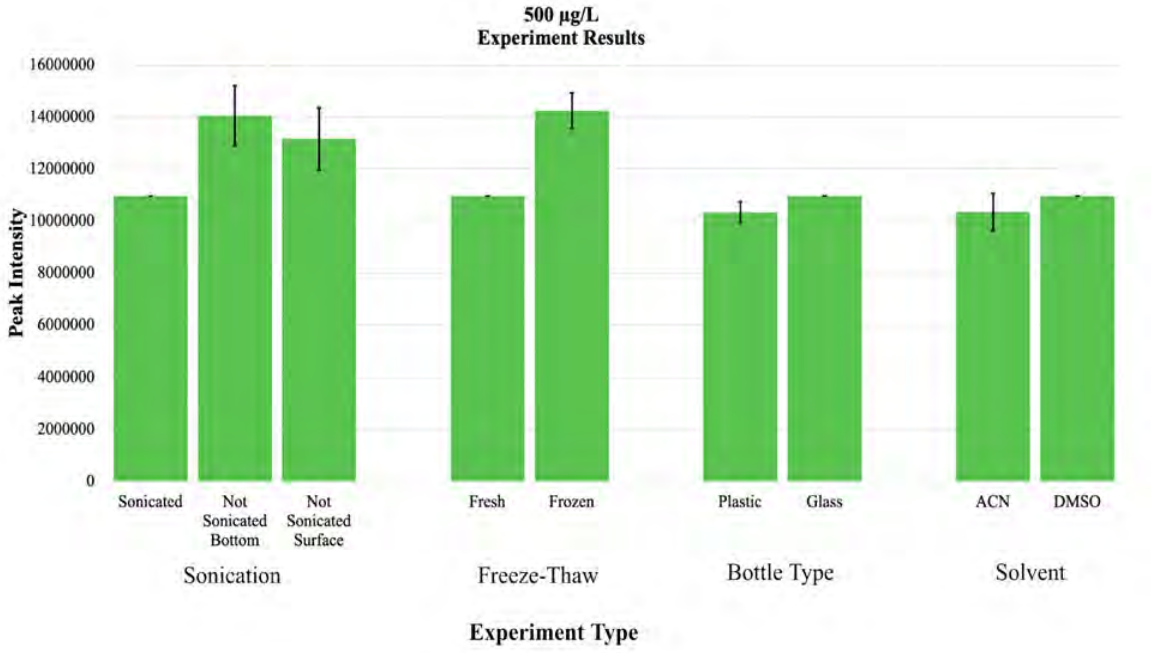


Figure 5. Impact of upstream techniques on 6PPD-quinone peak intensity.

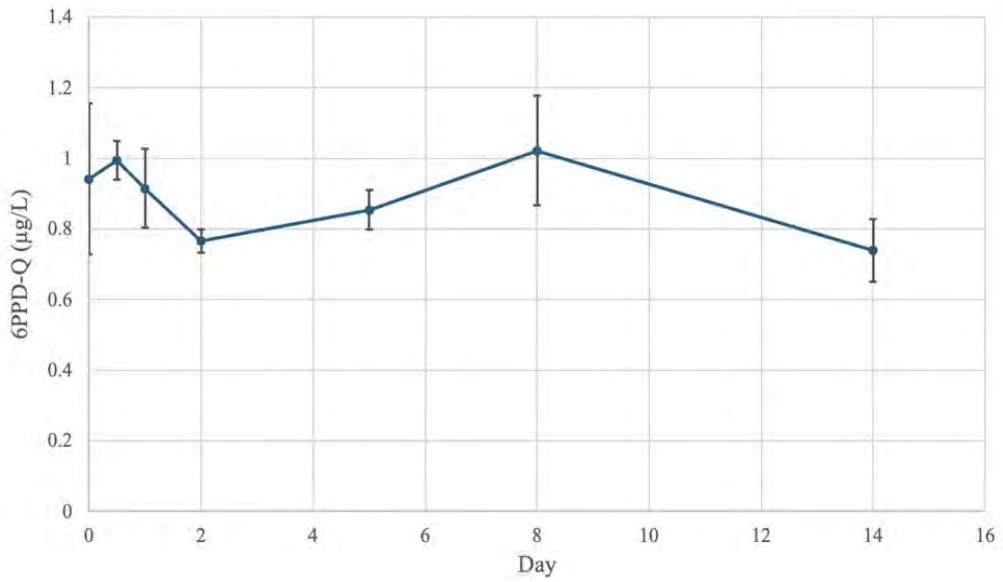


Figure 6. Results of a biodegradation experiment conducted in 5.0 µm filtered seawater indicates that 6PPD-quinone (µg/L) is relatively stable.

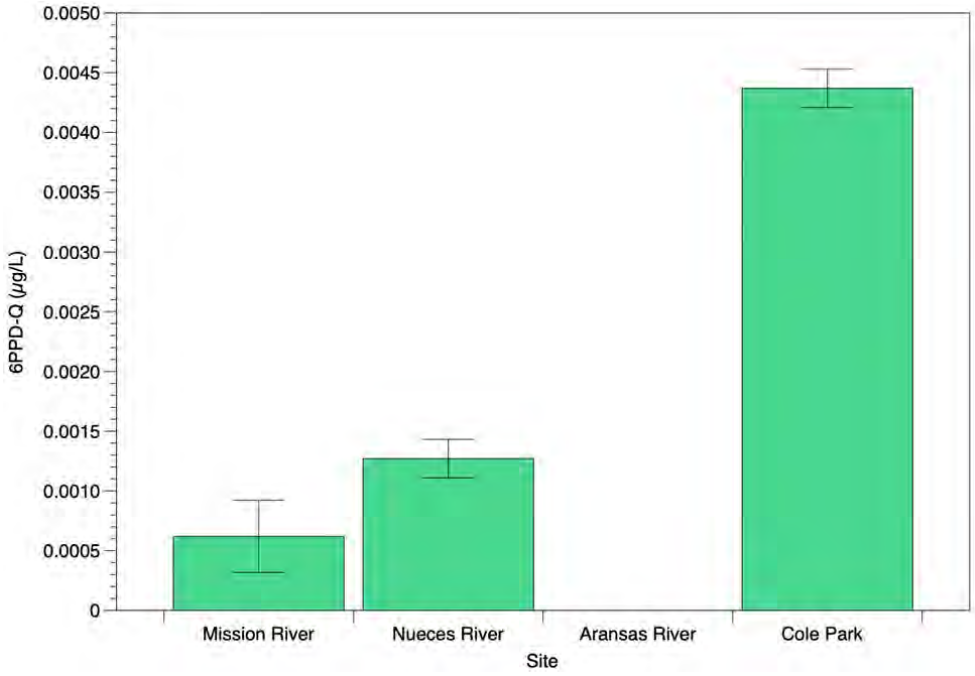


Figure 7. 6PPD-quinone concentrations (µg/L) in surface water samples for select sampling sites.

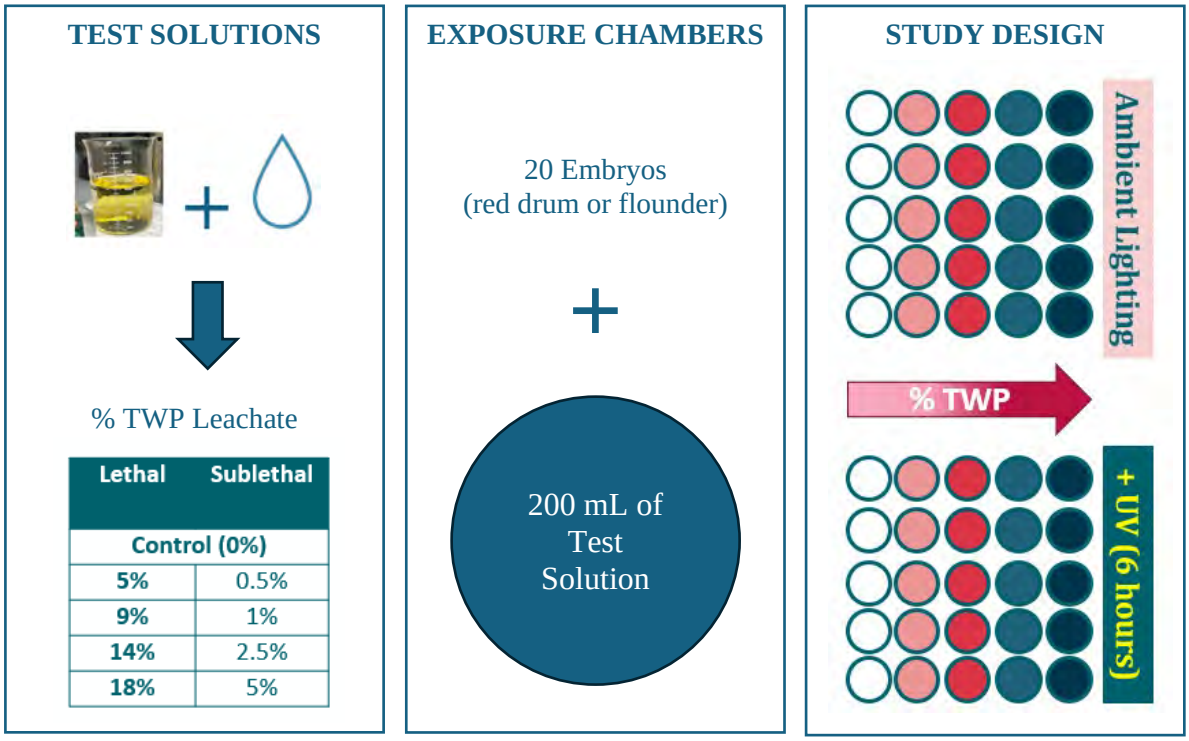


Figure 9. Summary of the fully factorial study design used for acute and sublethal TWP leachate exposures with ELS red drum and Southern flounder.

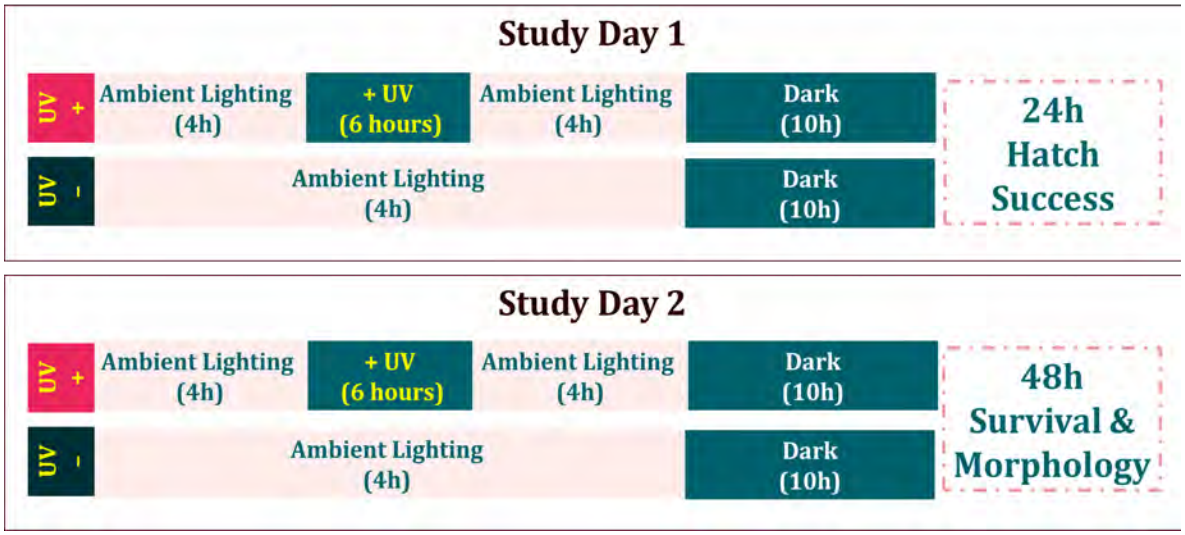
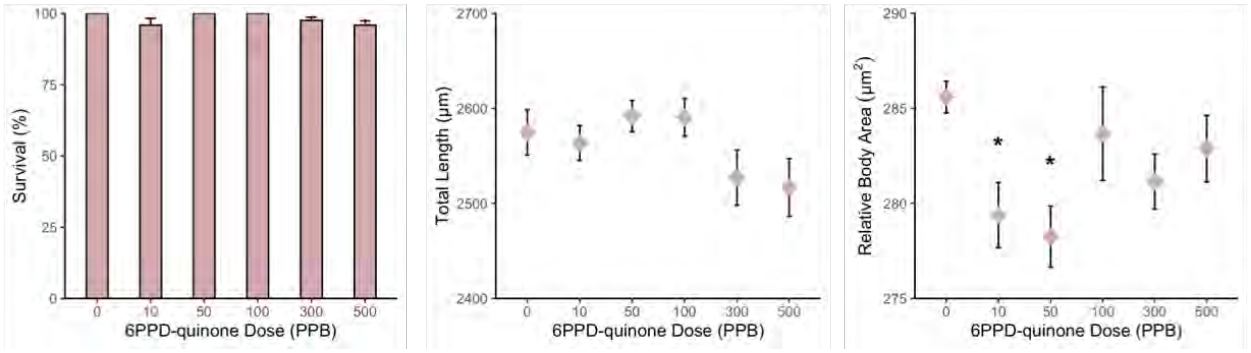


Figure 10. Timing of photoperiods & endpoint measurement for TWP leachate photo-induced toxicity testing with ELS red drum and Southern flounder.

(A)



(B)

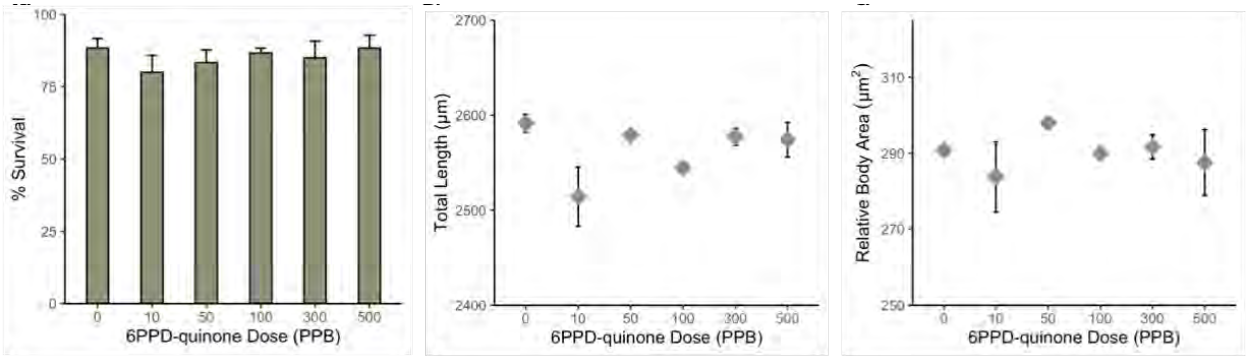
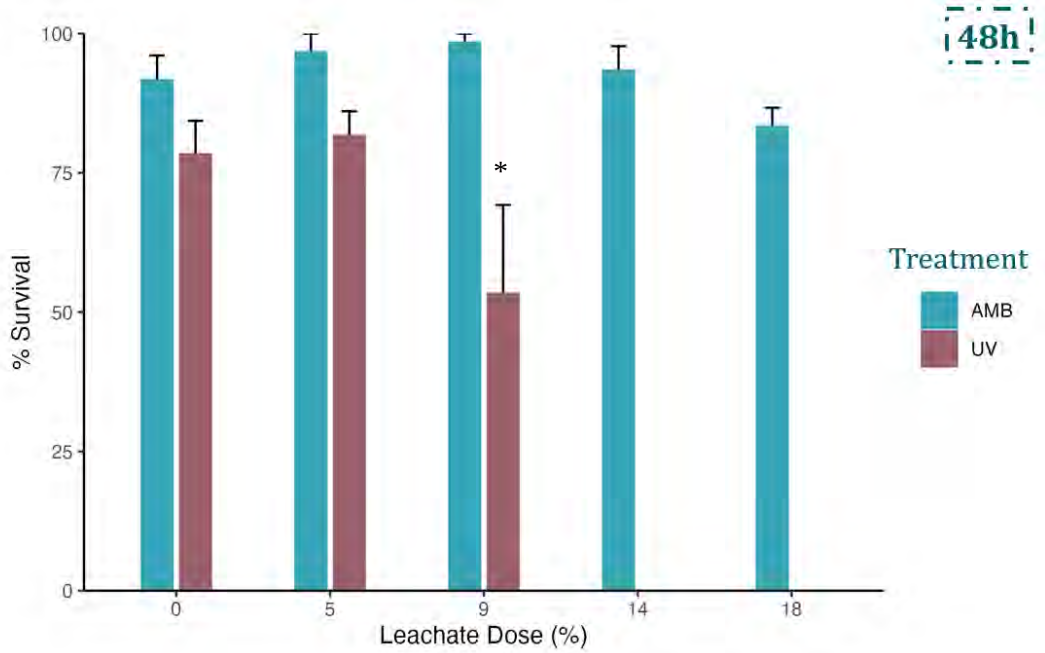


Figure 11. Developmental exposure to 6PPD-quinone did not significantly impact the survival or morphology of ELS **(A)** red drum or **(B)** Southern flounder.

(A) Results of acute TWP leachate testing



(B) Results of sub-lethal TWP leachate testing

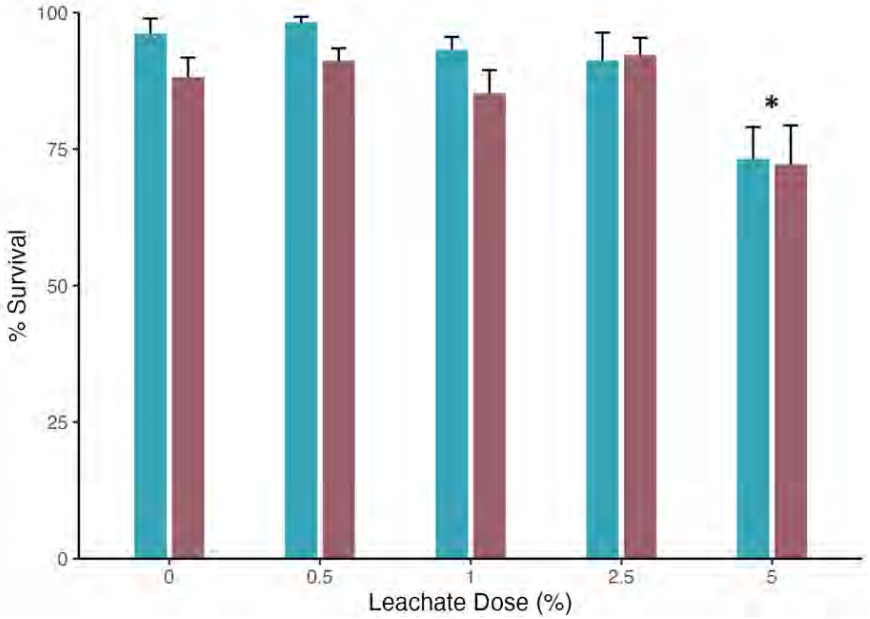


Figure 12. (A) Acute survival of ELS red drum at 48 HPF was significantly reduced by co-exposure to TWP leachate and UV in nominal treatments ≥ 9% leachate, yielding an LC50 of 12.41% leachate for UV co-exposed fish. **(B)** During subsequent sub-lethal toxicity testing, survival of both cohorts was significantly reduced by exposure to lower nominal concentrations of TPW leachate (5%); however, this effect was not exacerbated by UV in the 5% leachate group.

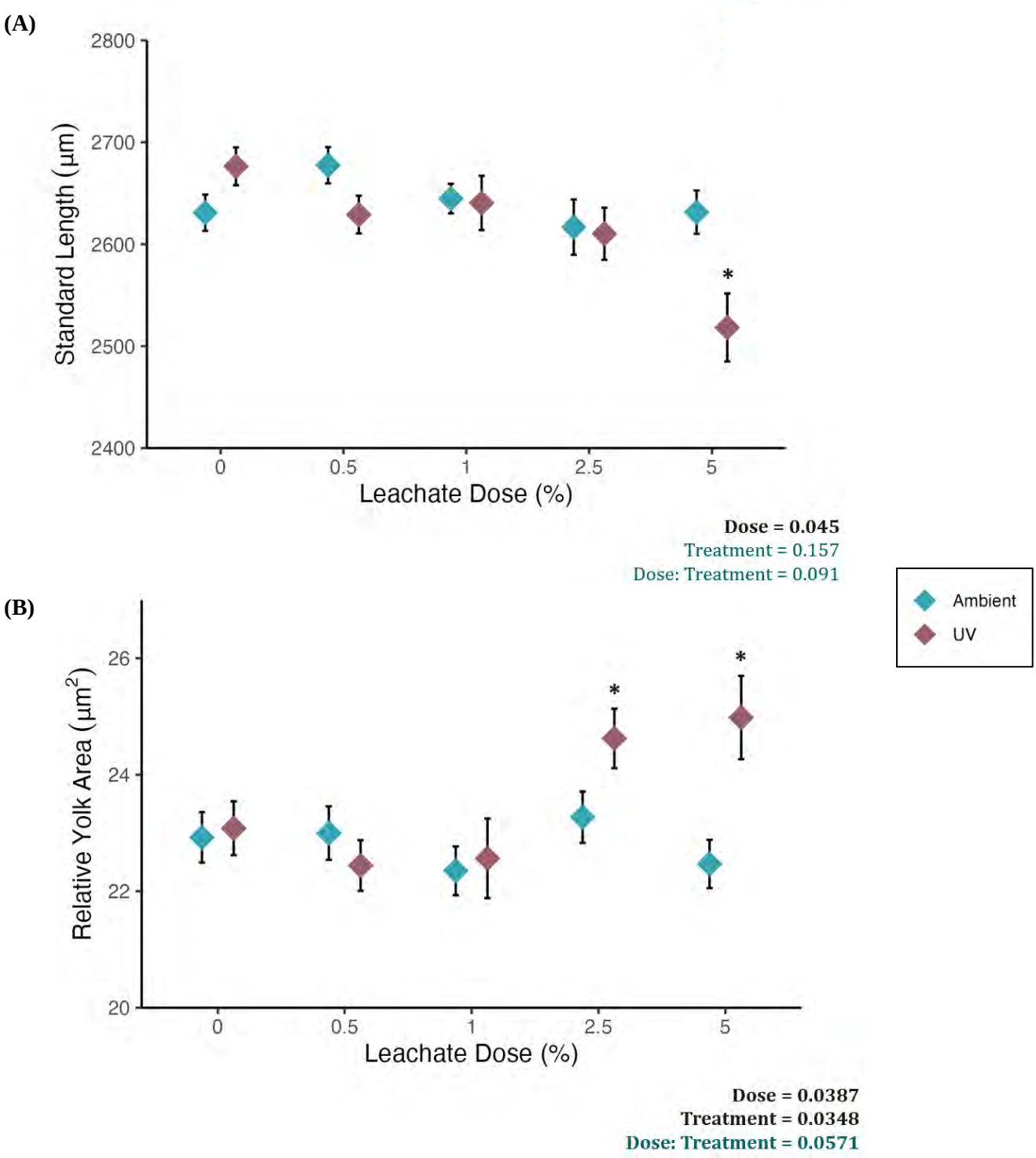


Figure 13. Surviving red drum larvae also demonstrated significant effects of TWP leachate exposure on morphological parameters. **(A)** The average standard length of larvae was significantly reduced in the 5% leachate group, but only in the UV+ cohort. **(B)** Similarly, the relative yolk area was significantly larger among UV co-exposed larvae from the 2.5% and 5% treatments, but these effects were not present in fish from the UV- cohort.

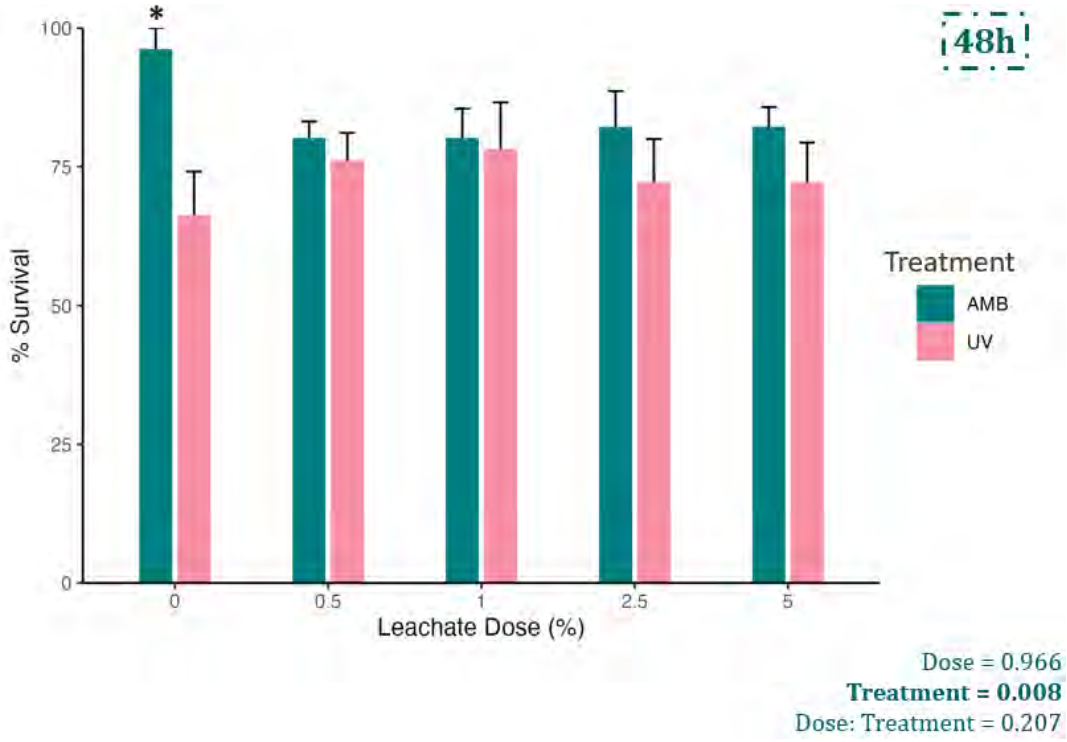


Figure 14. Survival of ELS flounder at 48 HPF was significantly reduced by exposure to TWP leachate and UV, both together and separately.

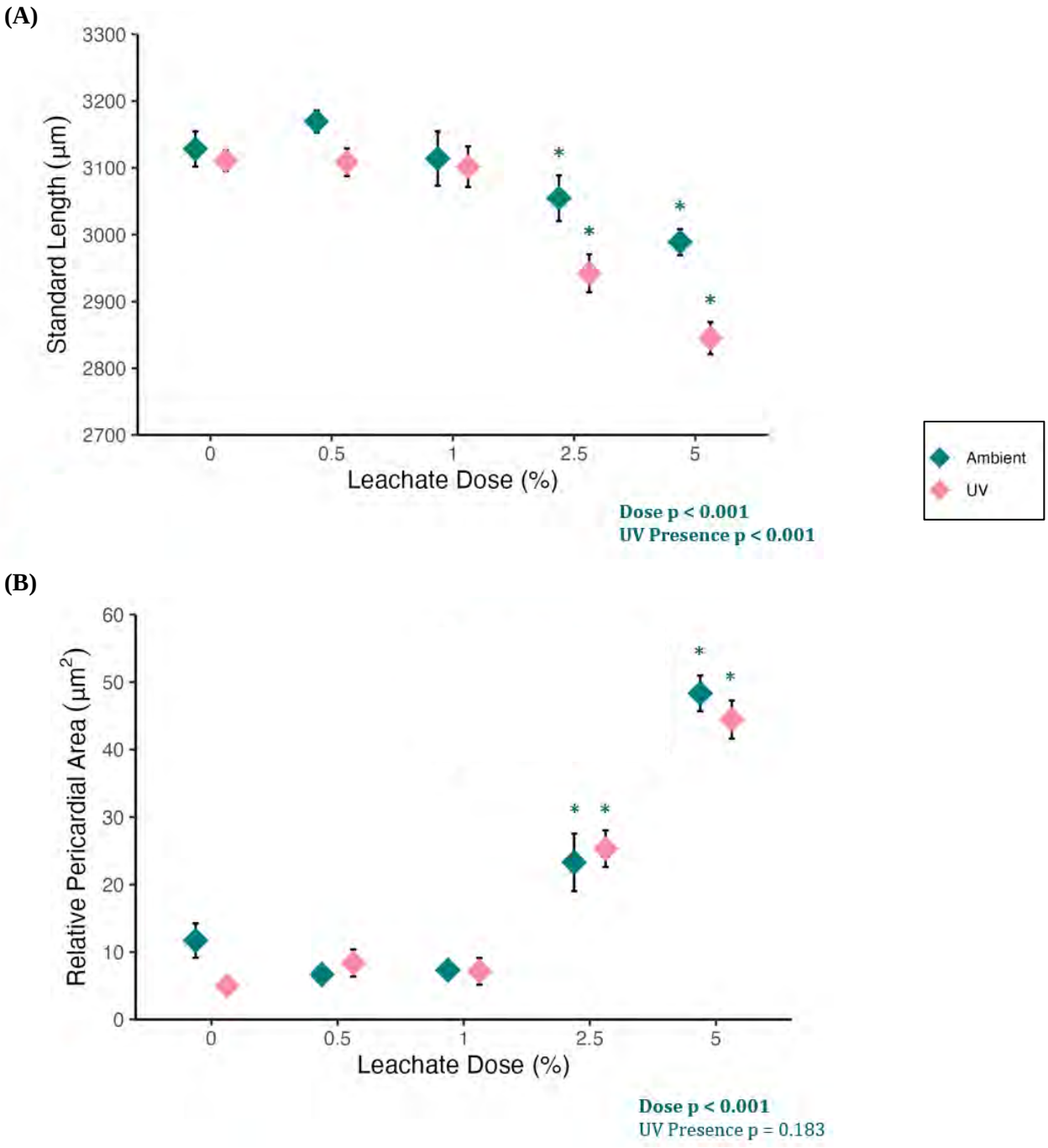


Figure 15. Southern flounder larvae also demonstrated significant effects of TWP leachate exposure on morphological parameters. (A) The average standard length of larvae was significantly reduced by exposure to nominal leachate concentrations ≥ 2.5%, with even greater effects observed in the UV+ cohort. (B) The incidence of pericardial edema was also significantly increased in larvae from the 2.5% and 5% treatments; however, UV did not appear to potentiate these effects.

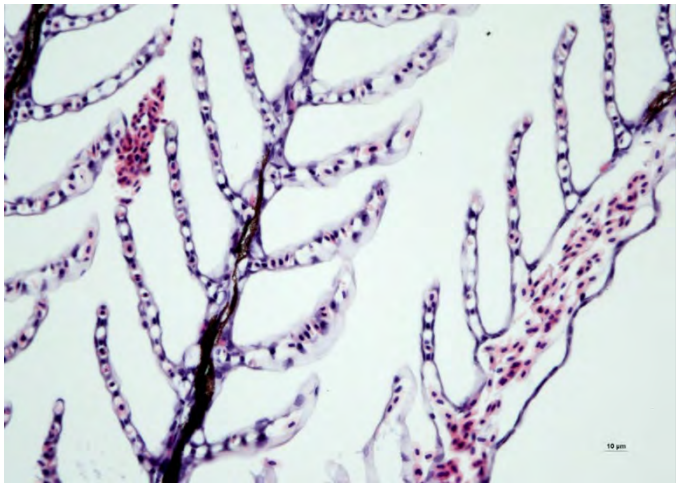


Image 1. Example of epithelial pavement hypertrophy in Southern flounder exposed to 50 µg/L 6PPD-quinone.

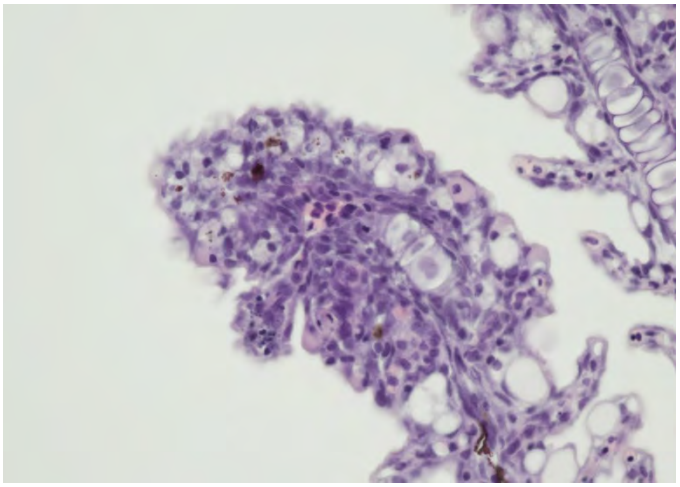


Image 2. Example of focal cell death with nuclear pyknosis and cytoplasmic fragmentation in Southern flounder exposed to 50 µg/L 6PPD-quinone.

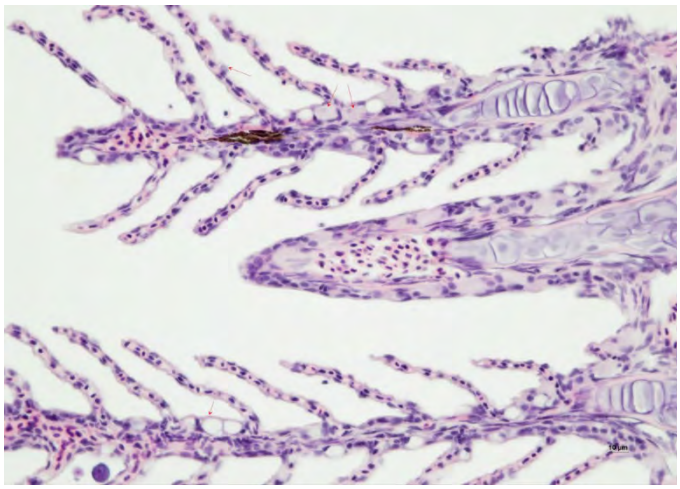


Image 3. Example of cellular hypertrophy + and increased cellularity of various cell types in Southern flounder exposed to 100 µg/L 6PPD-quinone.

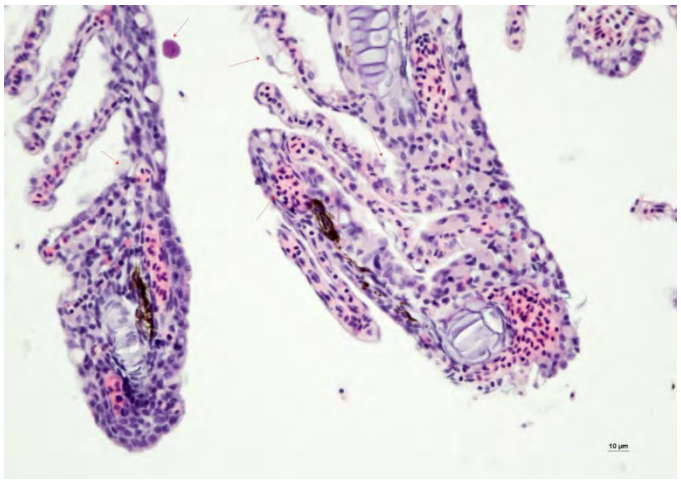
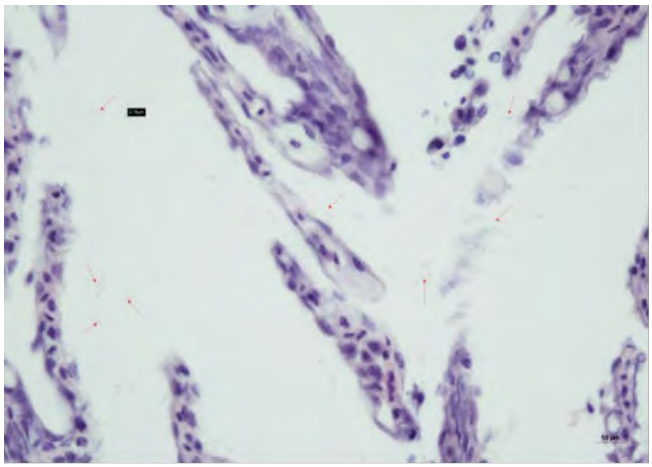
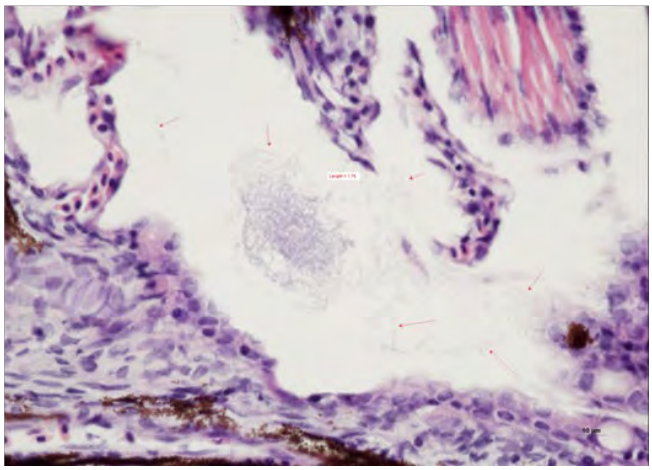
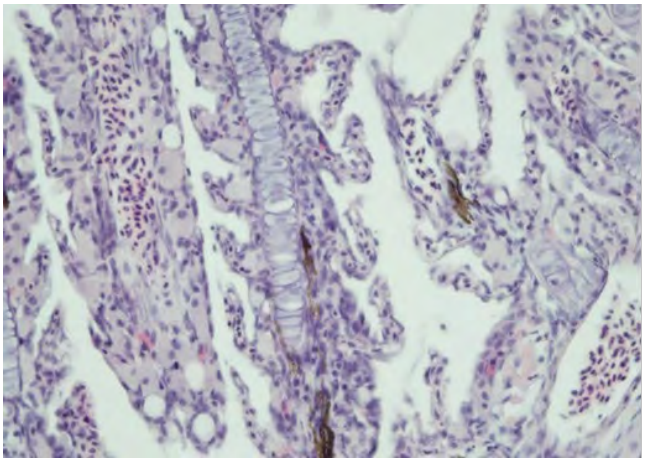


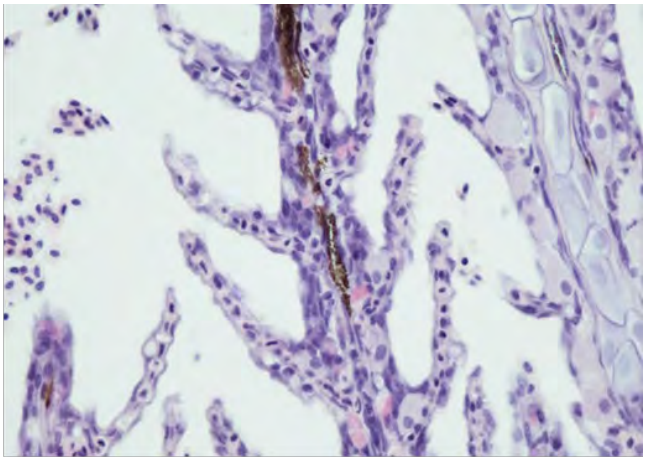
Image 4. Example of epithelial pavement cell hypertrophy and lifting (as well as presence of parasitic organism) in Southern flounder exposed to 100 µg/L 6PPD-quinone.





Images 5 & 6. Examples of increased presence of eosinophilic material in the gill sections of Southern flounder exposed to 100 µg/L 6PPD-quinone. Hyperplasia and altered epithelial pavement cell membrane integrity also observable in both sections.





Images 7 & 8. Rod-shaped bacteria were present in the gill sections of some Southern flounder exposed to 100 µg/L 6PPD-quinone.

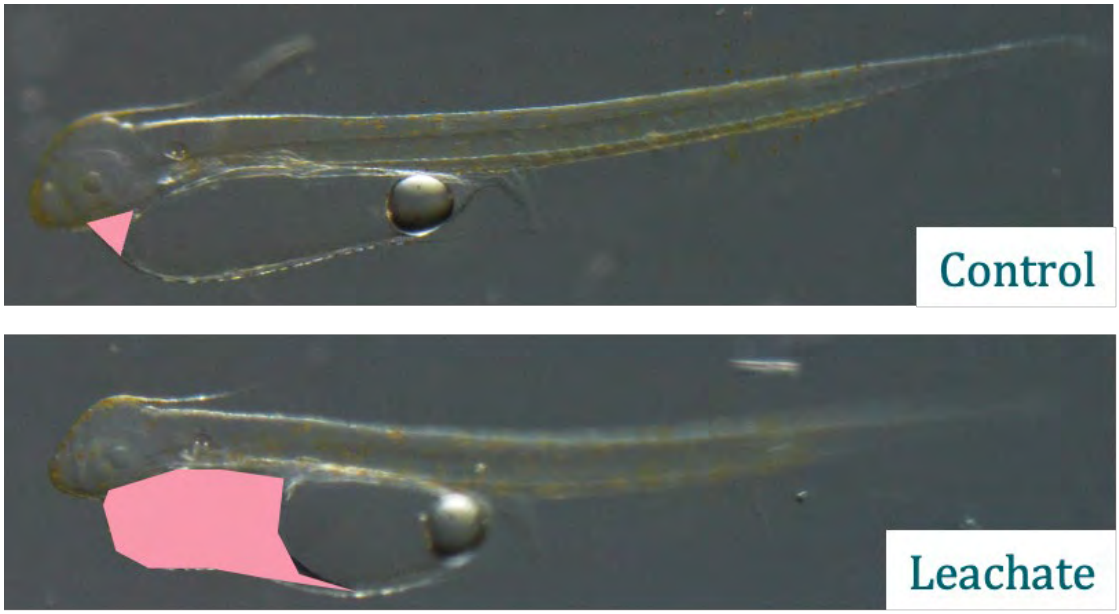


Image 9. Example of pericardial edema in ELS Southern flounder exposed to 2.5% TWP (relative to larvae from the control group).