2025 Q1 & Q2 Progress Report: *Assessing the threat of tire leachate and urban runoff on Matagorda Bay fish populations*

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Toxicology.

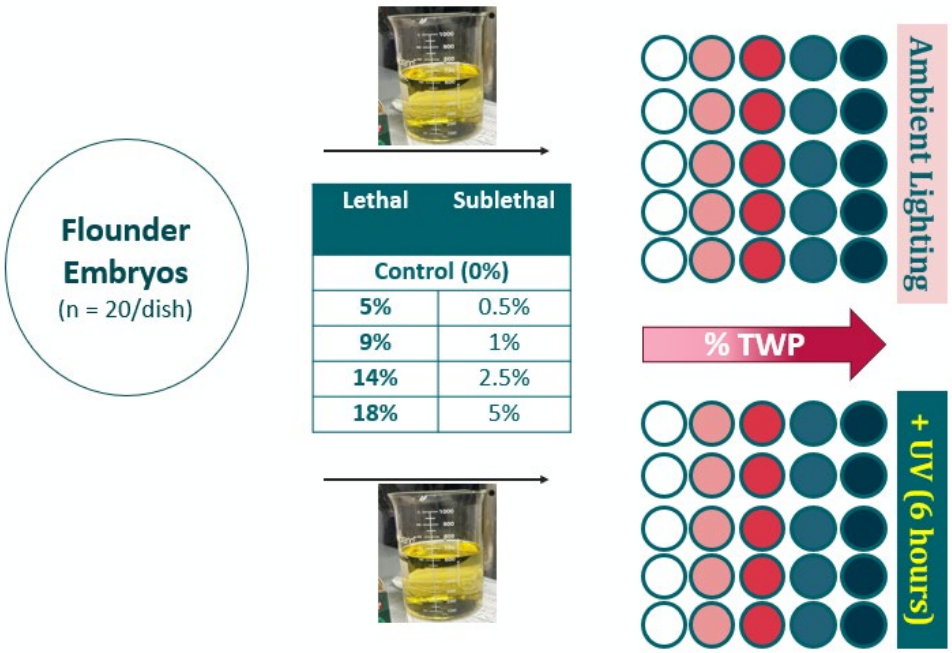
All initially proposed activities involving toxicity testing of 6PPD-quinone and TWP leachate were completed and included in previous reporting, as well as in a peer reviewed publication that was submitted and accepted by MBMT. However, we conducted additional testing (i.e., not included in the originally proposed scope of work) during Q1 and Q2 of 2025, including acute/lethal and sub-lethal toxicity testing with Southern flounder *(Paralichthys lethostigma)* exposed to a range of TWP leachate dilutions, both with and without UV. The goals, approaches, and results of those studies are reviewed below.

Acute Toxicity Testing Study Goals: Acute toxicity testing with ELS Southern flounder was conducted in order to (1) develop of a 48-hour median lethal concentration (LC50) for an additional species of estuarine-dependent sportfish exposed to TWP leachate under ambient lighting conditions, (2) to determine whether photodynamic compounds present in leachate may potentiate toxicity to ELS Southern flounder via a phototoxic mechanism, and (3) determine whether Southern flounder demonstrated a different relative susceptibility to TWP leachate exposure.

Sub-Lethal Toxicity Testing Study Goals: Similarly, the goals of sublethal toxicity testing with Southern flounder included (1) development of a 48-hour median effect concentration (EC50) for reduced fitness caused by changes in larval flounder morphology, (2) to determine whether sub-lethal effects occur at lower relative exposure concentrations when UV is also present, and (3) to evaluate the sensitivity of Southern flounder to TWP leachate relative to other estuarine fishes included in previous toxicity testing.

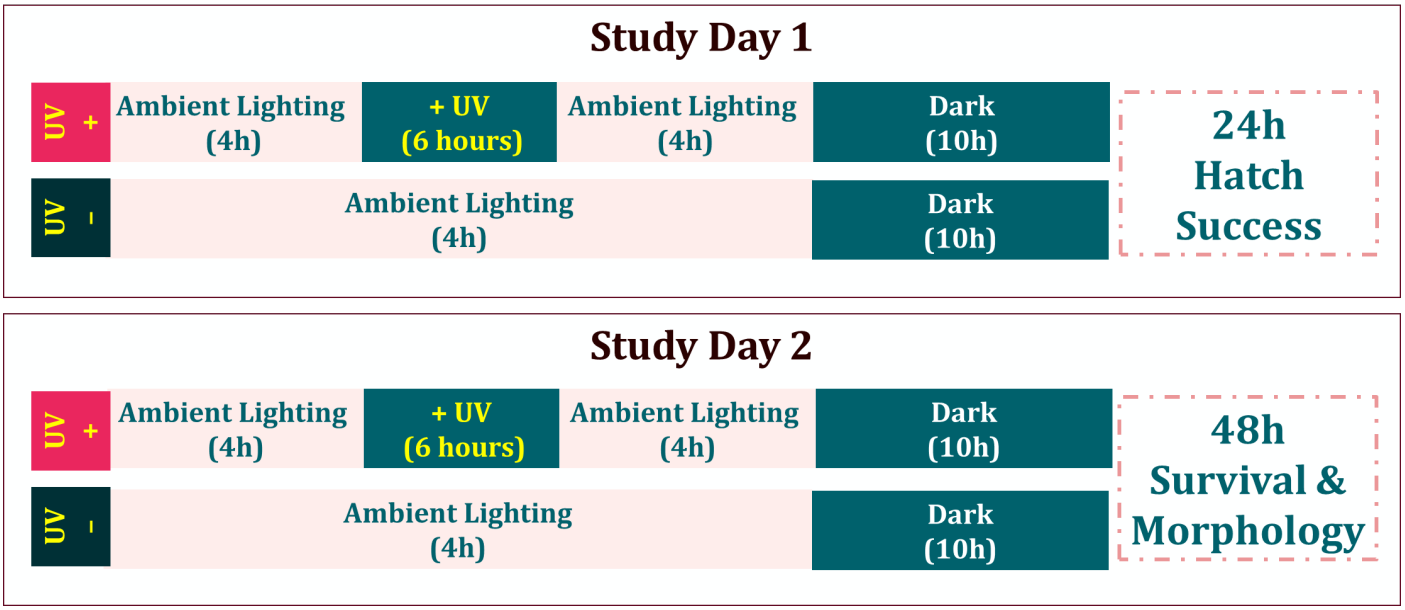
Study Design & General Methods: TWP leachate stock was generated by adding 100-g of cryo-milled TWPs to 1-L of saltwater, which was agitated for 10-days at 22°C. The resulting mixture (i.e., our 100% TWP leachate stock) was further diluted in seawater to nominal concentrations of 0 (control seawater), 5, 9, 14, and 18% leachate for acute toxicity tests, and 0, 0.5, 1, 2.5 and 5% leachate for sub-lethal testing. Testing was conducted in a fully factorial manner for both lethal and sublethal testing, with and without UV co-exposure (Figure 1).

Figure 1: General study design for Southern flounder toxicity testing with TWP leachate



The photoperiod for toxicity testing was designed to represent intermittent exposure to solar radiation over the course of two days (Figure 2). The UV intensity used in the study was monitored at the 380 nm wavelength and was approximately equivalent to 50% of the intensity of incident radiation measured in the Gulf of Mexico during the *Deepwater Horizon* oil spill (UV_{380} = 0.017 mW/cm^2/s).[1, 2] For both acute and sublethal testing, red drum and flounder embryos were exposed to leachate under their assigned light treatment (i.e., UV positive, or ambient lighting) for a total of 48-hours. Successful hatch was evaluated at test hour 24 and larval survival was assessed at 48 hours (i.e., test termination).

Figure 2. Timing of photoperiods & endpoint measurement for photo-induced toxicity testing with Southern flounder exposed to TWP leachate.



At the conclusion of sublethal tests, endogenously feeding larvae were imaged (n = 10 fish 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) and evaluated for treatment effects using JMP Pro V 18.0.

Results

Acute Toxicity Testing. Survival of Southern flounder was significantly impacted in by TWP leachate exposure after 48-hours, with a nominal LC50 of 17.6% leachate in the absence of UV. In the presence of UV, leachate became more toxic to ELS flounder included in the acute toxicity test, resulting in 50% mortality at nominal leachate concentrations nearly 6% lower than those observed in the UV – treatment. This indicates that ELS flounder exposed to TWP contamination in the wild are likely to experience lethality at lower exposure concentrations than would otherwise be indicated by traditional laboratory toxicity testing conducted under laboratory lighting conditions. It is also important to note that these exposures were conducted using un-weathered 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.

Chronic Toxicity Testing. Survival of ELS flounder included in chronic toxicity testing was again significantly impacted by exposure to TWP leachate at test hour 48; however, it was also impacted by UV exposure, even in control dishes (Figure 3). This indicates that ELS flounder are sensitive to damage from UV radiation, as well as to TWP contamination. Surviving fish also demonstrated significant effects of TWP leachate exposure on morphological parameters, including length and pericardial edema. Length was significantly reduced in all TWP leachate exposure concentrations > 1% (Mixed Model $p < 0.001$), with even more significant reductions observed in the UV+ group (UV Present $p < 0.001$; Figure 4). 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 $p < 0.001$) in ELS flounder exposed to TWP leachate at exposure concentrations > 1% (Figure 5). While this effect occurred in both UV + and UV – treatments, the effect was not significantly greater in the UV co-exposed group. Thus, effects on pericardial edema appear to be driven by non-phototoxic mechanisms.

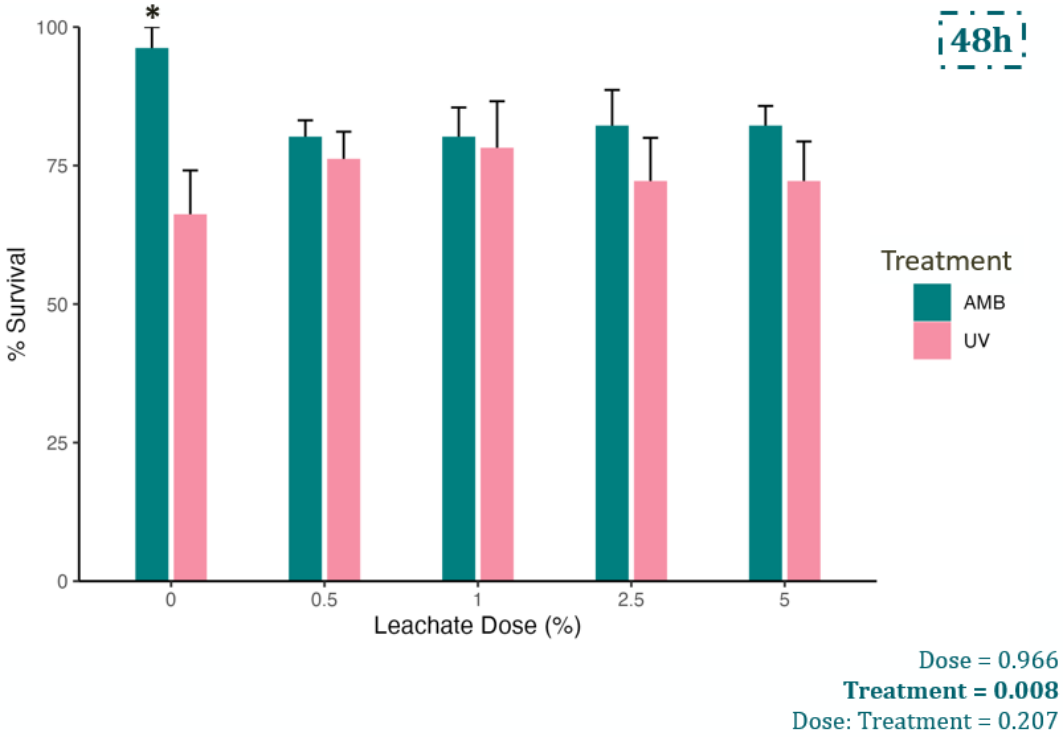


Figure 3. Survival of ELS flounder was significantly reduced by exposure to TWP leachate, and UV (together and separately).

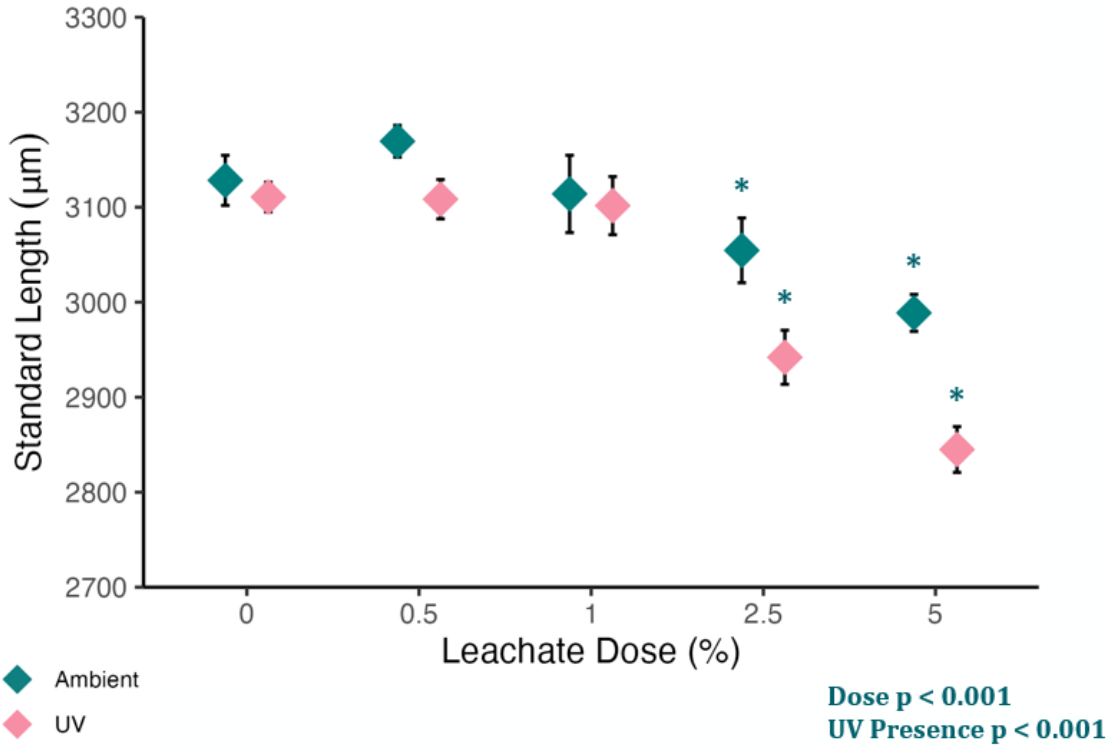


Figure 4. TWP exposure significantly reduced the standard length of ELS flounder, with even greater effects observed in the presence of UV.

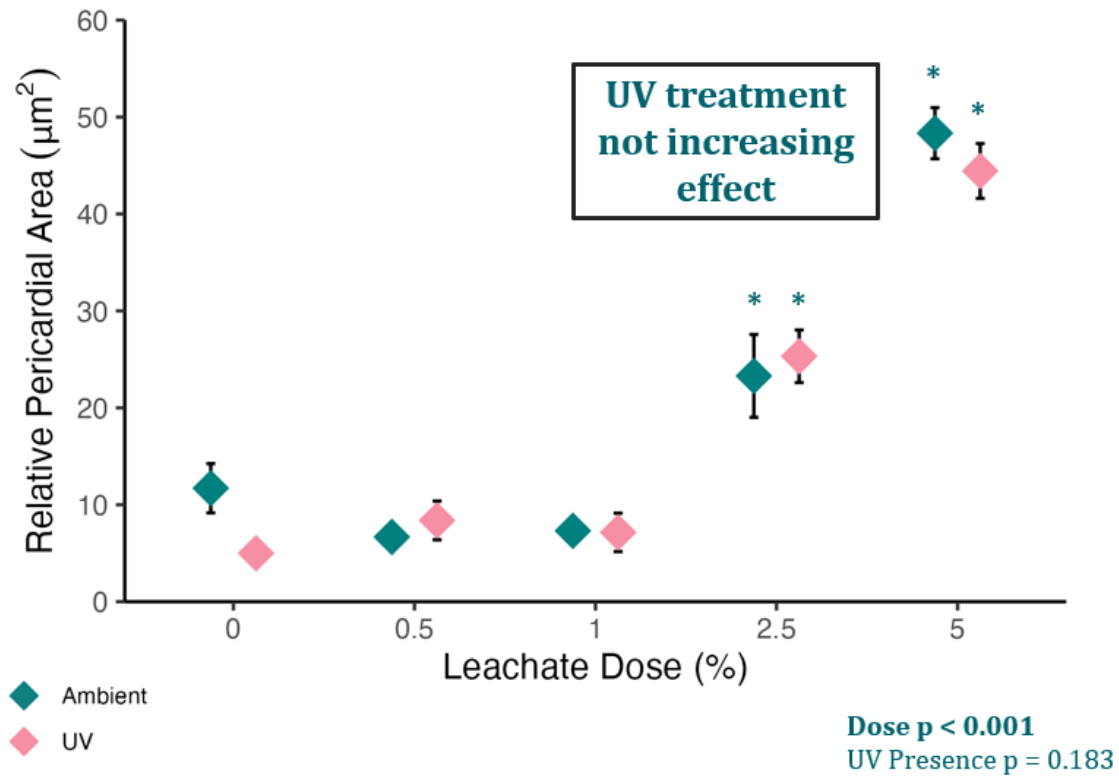


Figure 5. TWP exposure, but not UV, significantly increased the incidence of pericardial edema in ELS flounder.

Analytical Environmental Chemistry.

Environmental sampling

Water samples were collected from 4 sites (Mission River, Aransas River, Nueces River, Cole Park) during our June 2024 sampling trip (Figure 6). All of these water samples have been extracted via solid phase extraction (SPE) with HLB Prime cartridge (spiked with surrogate standard D5-6PPD-Q), eluted with methanol, and analyzed via LC-MS. The observed concentrations of 6PPD-Q in these environmental samples are listed in Table 1 and Figure 6 below. These results indicate that 6PPD-Q is present at relatively low concentrations in the areas sampled.

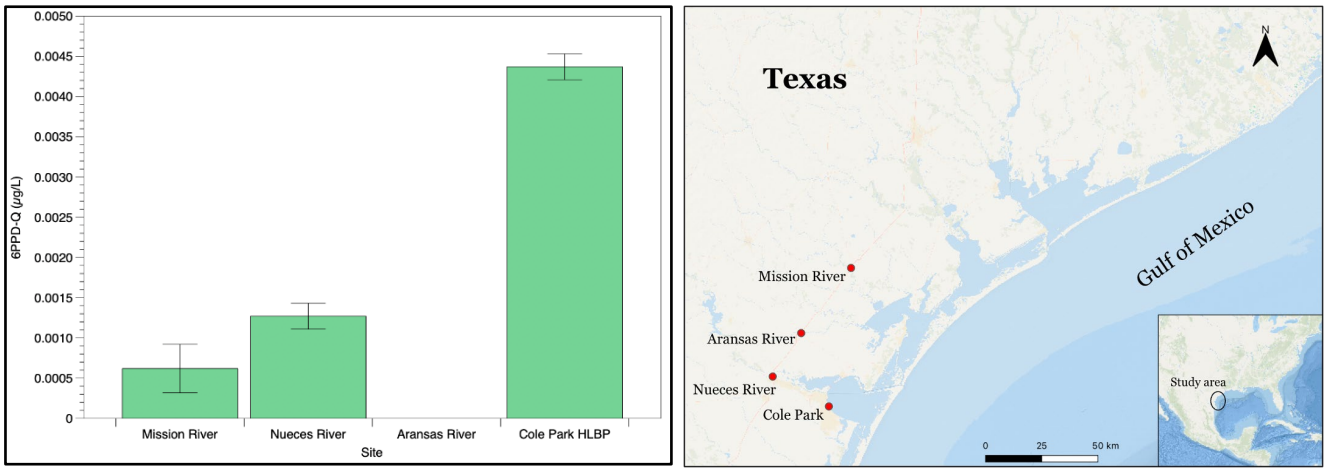


Figure 6. Concentrations of 6PPD-Q (µg/L) in south Texas rivers and Cole Park, Corpus Christi Bay.

Table 1. Concentrations of 6PPD-Q (µg/L) in south Texas rivers and Cole Park, Corpus Christi Bay.

Site	Concentration (µg/L)
Mission River	0.00062 ± 0.0
Aransas River	0.00 ± 0.0
Nueces River	0.00127 ± 0.0
Cole Park	0.00437 ± 0.0

Previously extracted water samples from our September 2023 sampling trip were eluted and are awaiting analysis via LC-MS. These include water samples from 5 sites (Grab 50, Grab 33, Grab 17, Grab 13, and Grab 12) in San Antonio Bay and Matagorda Bay, as well as 2 sites from Guadalupe River and Lavaca River respectively (Figure 7).

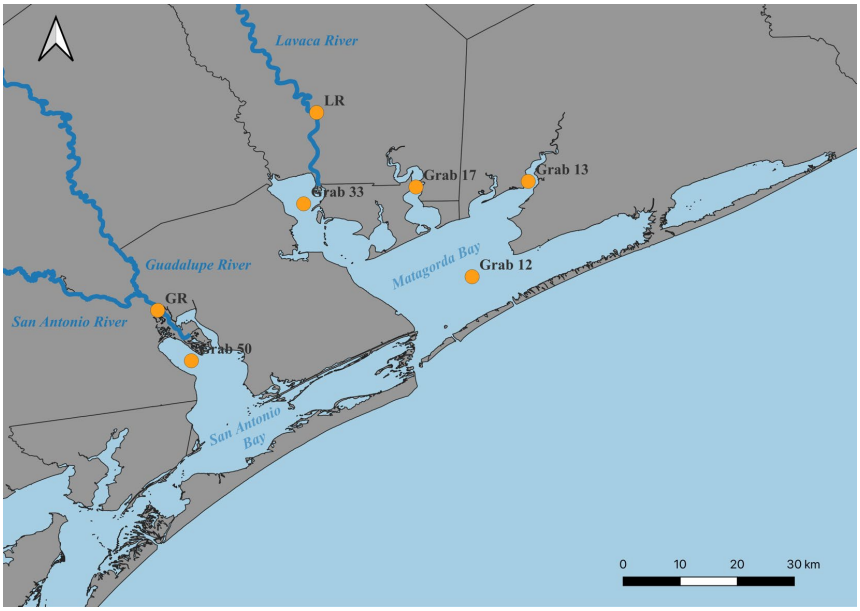


Figure 7. September 2023 sampling locations.

Cartridge resin tests

Cartridge resin tests were performed to determine which SPE cartridge yielded the highest recovery rate of 6PPD-Q upon analysis via LC-MS. For the cartridge tests, 4L of seawater was collected from the Port Aransas ship channel and filtered through a 0.2 µm filter. The filtered seawater was then subdivided into six different 200mL aliquots, which were then spiked with D5-6PPD-Q surrogate standard. Each aliquot was then extracted using a different SPE cartridge (Bond Elut PPL, Bond Elut C8, Bond Elut C18, Oasis HLB, Oasis Prime MCX, and Oasis Prime HLB); cartridge types were selected based on previous use in the literature. After initial extraction, each bottle was rinsed with 20 mL LC-MS water 3x to remove any 6PPD-Q that potentially had adsorbed to the glass, then flushed each cartridge with two cartridge volumes of LC-MS water to remove any residual salts from the cartridge. Each cartridge was then eluted with 16 mL methanol and concentrated with a stream of N_2 gas to a final concentration of 20 ug/L before being analyzed on the LC-MS. QA/QC was ensured by running a standard curve of the D5 stock solution dissolved in methanol to concentrations of 0.0 µg/L, 0.1 µg/L, 0.5 µg/L, 1 µg/L, 5 µg/L, 10 µg/L, 20 µg/L, 50 µg/L, and 100 µg/L prior to sample analysis.

We found that HLB Prime had the greatest recovery rate of the D5-6PPD-Q at 80%, followed by MCX Prime at 78.1%, and Bond Elut C18 at 70.3% (Figure 8). After running an ANOVA it was found that there were no significant differences among the different cartridge types with the exception of Bond Elut PPL, which had a recovery rate of 26.9%. This may be because Bond Elut PPL is meant for very polar analytes, which may not capture the amphiphilic 6PPD-Q as well. HLB Prime performed the best likely due to its stationary phase structure, which enabled the different polar and nonpolar regions of D5-6PPD-Q to effectively adsorb to.

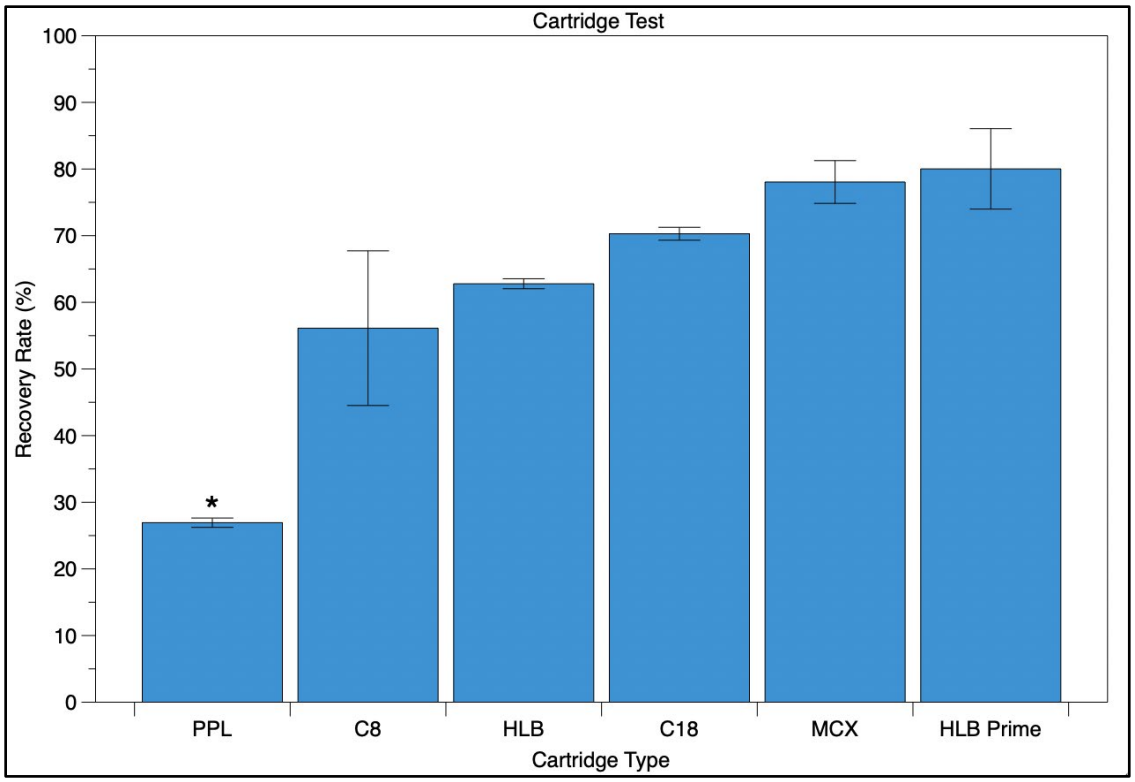


Figure 8. Recovery rate according to cartridge type.

Filtration test

The three best performing cartridges from the cartridge resin tests, C18, MCX, and HLB Prime, were selected for a filtration test to observe if filtration had any effect on 6PPD-Q recovery from seawater samples. To do this, another 4L seawater sample was collected from the Port Aransas ship channel and immediately spiked with D5-6PPD-Q to a final concentration of 20 µg/L. This spiked seawater sample was then filtered through a 0.2 µm filter and subdivided into three different 200mL aliquots which were analyzed in duplicates using the aforementioned technique.

It was found that recovery rates ranged from 60.6 to 78.6% (Figure 9), indicating that filtration does not have a significant impact on recovery rates. Statistically, there were no significant differences between obtained filtered recovery rates. These results suggest that it is fine to filter environmental water samples when analyzing 6PPD-Q without fear of losing too much of the compound.

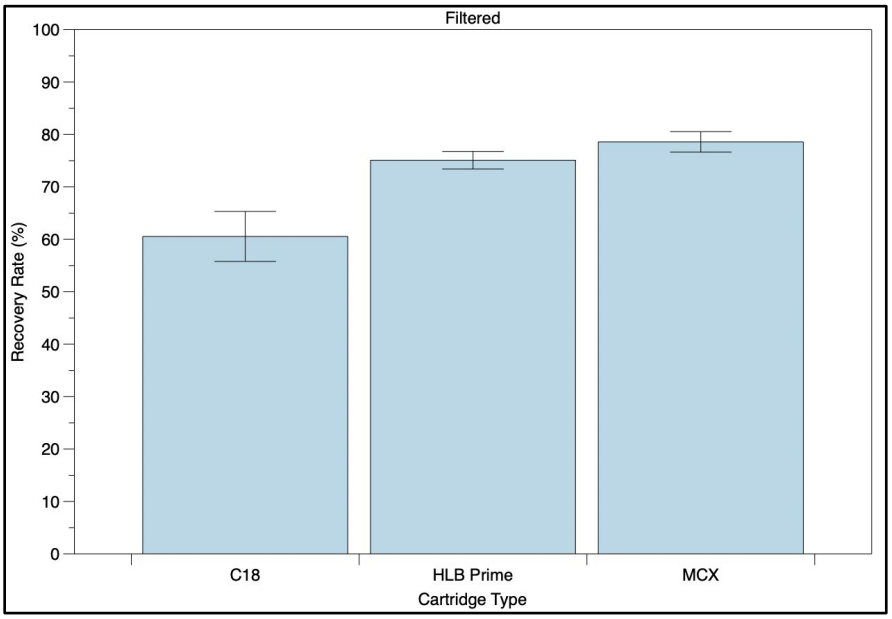


Figure 9. Filter test recovery rate.

Biodegradation test

We then tested the stability of 6PPD-Q in natural seawater samples over a two-week incubation period. For the biodegradation experiment, 4-L of seawater from the Port Aransas ship channel was collected and filtered through a 5.0 µm filter. This ensured that only larger particulates, and not the microbial community, were removed. The filtered seawater was then subdivided into seven different 100mL aliquots, which were then spiked with D5-6PPD-Q surrogate standard at an environmentally relevant initial concentration of 1-µg/L. Each aliquot corresponded to a different time point (t = day 0, 0.5, 1, 2, 5, 8, 14) that was analyzed in triplicate. Filtered seawater controls were also analyzed in triplicates on days 0, 8, and 14. Samples were stored at room temperature in the dark and then frozen at -20 ˚C at their corresponding time point. Prior to extraction, each sample was thawed and spiked with surrogate standard D5-6PPD-Q to a final concentration of 20-µg/L. Each aliquot was then extracted using Oasis Prime HLB cartridges and analyzed via LC-MS following the same protocols as outlined in the cartridge resin test section.

It was observed that concentrations of 6PPD-Q remained constant and did not degrade significantly over the incubation period as concentrations remained close to 1.0-µg/L (Figure 10), indicating that 6PPD-Q is relatively stable in natural seawater over the time period observed.

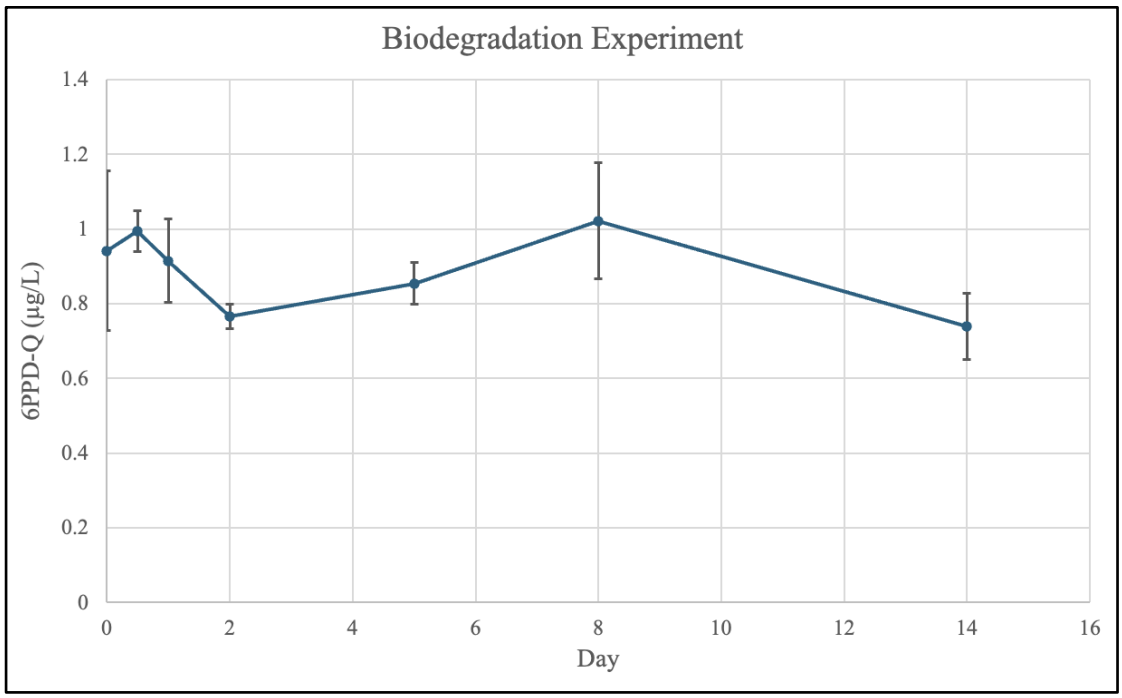


Figure 10. Concentration of 6PPD-Q (µg/L) over the 14-day incubation period.

Future Steps

We are planning to work with the oil fingerprinting lab at Louisiana State University to conduct non-targeted analysis of leachate to identify additional photodynamic compounds that may be inducing phototoxic effects on flounder. We are repeating exposures when flounder begin spawning to confirm findings and assess additional endpoints, which will be included in a second peer reviewed manuscript. In terms of environmental chemistry, we are currently measuring 6PPD-Q in the field samples, and all the 6PPD-Q data will be finalized and written into a manuscript for submission.

References

(1) Nielsen, K.; Krasnec, M.; Magnuson, J.; Morris, J.; Gielazyn, M.; Chavez, J.; Roberts, A. Influence of variable ultraviolet radiation and oil exposure duration on survival of red drum (Sciaenops ocellated) larvae. *Environmental Toxicology and Chemistry* **2018**, *37* (9), 2372-2379.

(2) Nielsen, K.; Lay, C.; Alloy, M.; Gielazyn, M.; Morris, J.; Forth, H.; Takeshita, R.; Travers, C.; Oris, J.; Roberts, A. Estimating incident ultraviolet (UV) radiation exposure in the Northern Gulf of Mexico during the *Deepwater Horizon* oil

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