Year 1 Progress Report

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

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Red drum *(Sciaenops ocellatus)* Toxicity Testing.

Due to the lack of LC50 data for 6PPD-quinone, we first conducted a rangefinder study to determine the median lethal concentration (LC50) for our chosen species. An LC50 is the concentration that is expected to lead to mortality in 50% of exposed organisms within a pre-selected test duration (e.g., 24-hours). As LC50 values can vary considerably by species, life stage, or populations, derivation of an LC50 is a necessary first step that informs the experimental design of subsequent toxicity studies.

Concentrations of 6PPD-quinone selected for the rangefinder spanned from 0 to 500 µg/L. The selected concentration range exceeds the maximum reported environmental concentrations present in multilane roadway runoff [1] (19 µg/L) and receiving waters in the Pacific Northwest [1] (3.5 µg/L) as well as in nearby freshwater sources that supply local bay systems (up to 2.6 µg/L; unpublished data generated by Co-PI Liu). 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 encompassed the range of potentially relevant exposure scenarios for wild fish populations.

Red drum embryos and newly hatched larvae (yolk sac larvae) were used to conduct LC50 testing over the course of 72-hours. Each borosilicate exposure vessel contained 20 embryos or larvae per dish, with 3 replicate dishes per concentration (0, 10, 50, 100, 300, or 500 µg/L). This process was repeated a second time to ensure a sufficient sample size, while also accounting for potential genetic variability between spawns that may impact the reliability of LC50 values.

Results of the initial 72-hour rangefinder studies revealed that red drum embryos and larvae are tolerant of 6PPD-quinone exposure, even at concentrations as high as 500 µg/L (i.e., well above the concentrations that are considered environmentally relevant; Figure 1)

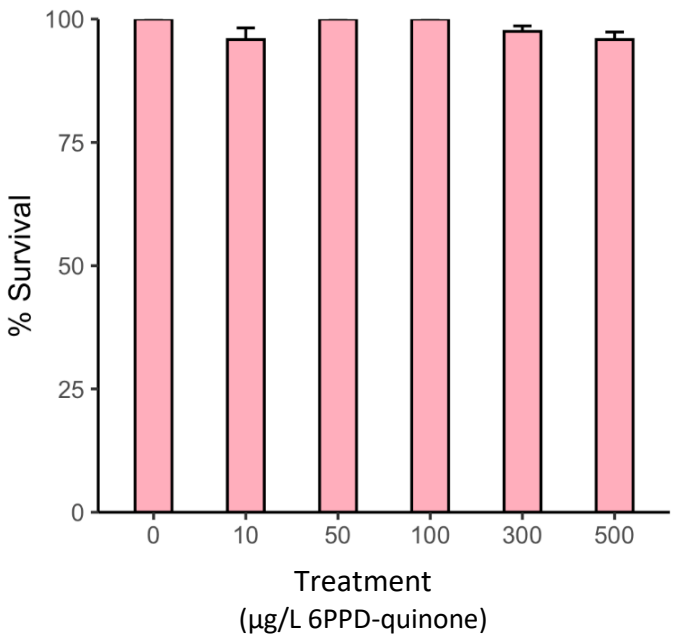

Figure 1. Exposure to concentrations of 6PPD-quinone up to 500 µg/L did not significantly impact survival of red drum larvae after 72 hours.

It is widely accepted that non-lethal exposures to environmental contaminants can still lead to adverse effects on ELS fish populations via indirect mechanisms and latent mortality.[2] Thus, we also evaluated a suite of sub-lethal endpoints that are known

to correspond with reduced fitness in wild ELS fish, including overall fish size (evaluated as both length and body area; Figure 2), as well as morphological changes in the size of the pericardium, brain, and eye.[3, 4]

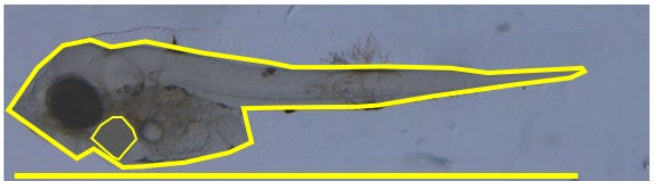

Figure 2. Example image showing total length, body area, and pericardium measurements in red drum larvae.

Briefly, 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 each of the aforementioned parameters. No significant sublethal impacts were indicated for any of the investigated parameters (Figure 3).

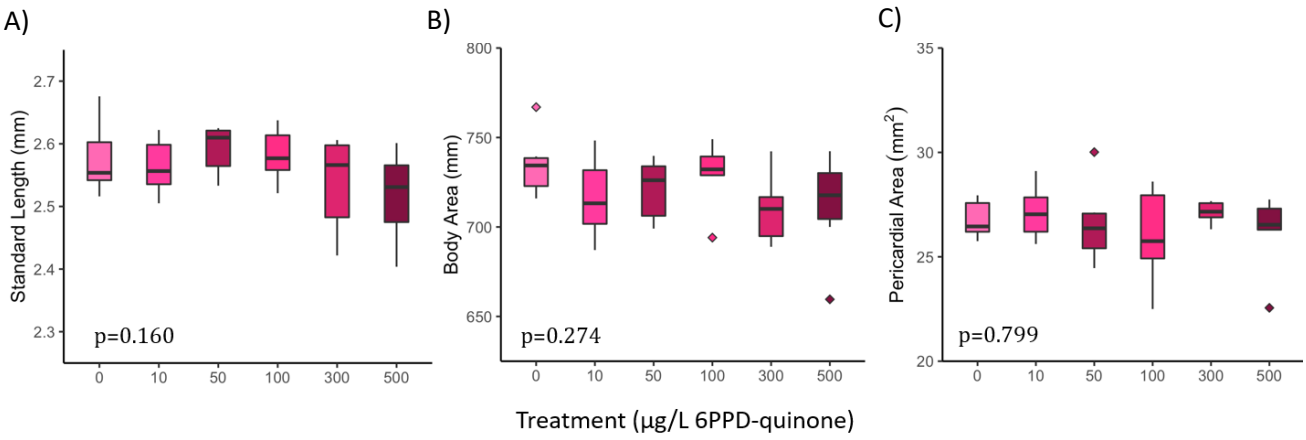

Figure 3. No significant treatment effects on sublethal endpoints were indicated, including for A) standard length (mm), B) body surface area (mm^2), or C) pericardium size (mm^2). Brain and eye size (not included in figure) were also found to be similar between treatments.

Since Tian et al[1] first implicated 6PPD-quinone in urban runoff mortality syndrome among Pacific northwest coho salmon *(Oncorhynchus kisutch)* populations in 2021, toxicity testing has been conducted on a handful of other freshwater and anadromous species. Results of these studies indicate that species sensitivity is remarkably variable. For example, the 24-hour LC50 values derived for rainbow trout *(Oncorhynchus mykiss)* and brook trout *(Salvelinus fontinalis)* are similar to those of coho salmon, at 1 and 0.59 µg/L, respectively [5]. Conversely, Arctic char *(Salvelinus alpinus)*[5], white sturgeon *(Acipenser transmontanus)*[5], white-spotted char *(Salvelinus leucomaenis pluvius)*[6], Southern Asian dolly varden *(Salvelinus curilus)*[6], landlocked masu salmon *(Oncorhynchus masou maso)*[6], zebrafish (*Danio rerio)*[7], and fathead minnow (*Pimephales promelas; Nielsen et al., In preparation*).

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.[8] Because red drum utilize cutaneous respiration prior to settlement (rather than relying on gas exchange at the

gills), 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 conducted additional rounds of testing with slightly older red drum. These included studies with 14-day post hatch (DPH) fish and 50 - 80 DPH fish.

While the study design remained generally the same, the maximum tested 6PPD-quinone concentration for tests conducted with older red drum was reduced to 100 µg/L and the test duration was shortened to 48-hours. These changes were made based on the availability and distribution of newly published LC50 values in the literature, and findings of our ELS red drum studies. Results of all testing with older red drum indicated that tolerance to 6PPD-quinone is conserved post-settlement in this species at exposure concentrations up to 100 µg/L (Figure 4).

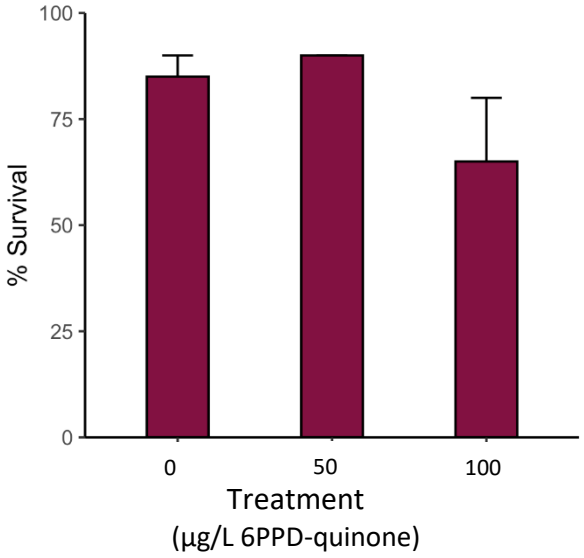

Figure 4. Results of toxicity testing conducted with 14-DPH red drum indicated no significant treatment effects on survival after 48-hours, at exposure concentrations up to 100 µg/L 6PPD-quinone.

To our knowledge, the present study is the first to investigate the potential toxicity of 6PPD-quinone to estuarine dependent species. As relatively few toxicity studies use marine or estuarine models (with far fewer utilizing large-bodied sport fish species), toxicity values generated based on these data may be relied upon disproportionately by risk assessors. Due to the apparent lack of sensitivity demonstrated by red drum, combined with the urgent need to consider potential sensitivity differences across a broader range of life stages for such species, we elected to conduct testing using a second estuarine-dependent species. Thus, all previously described tests were repeated using Southern flounder *(Paralichthys lethostigma)*, another regionally important estuarine-dependent fish species.

Southern Flounder *(Paralichthys lethostigma)* Toxicity Testing.

Methods utilized for Southern flounder tests are consistent with those described previously for red drum. Results of toxicity testing with embryo-larval stages of Southern flounder also indicate that

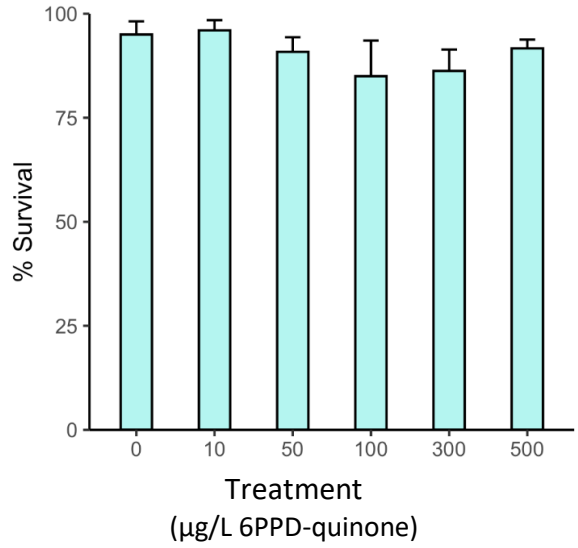

Figure 5. Exposure to concentrations of 6PPD-quinone up to 500 µg/L did not significantly impact survival of Southern flounder larvae after 72 hours.

embryo-larval stages are tolerant to exposure to 6PPD-quinone (up to 500 µg/L with no significant effects on survival indicated after 72 hours of exposure (Figure 5).

The sublethal morphological parameters evaluated for red drum were also evaluated for this species, using the approach previously described. Consistent with results observed in red drum, no significant effects on the morphology of ELS Southern flounder were observed (Figures 6 -7).

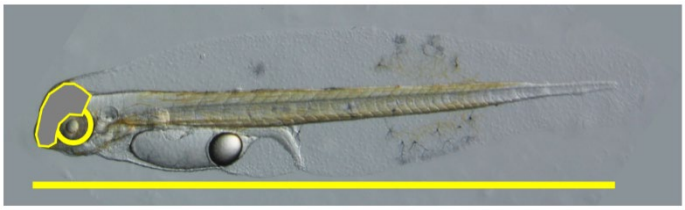

Figure 6. Example image showing measurements of total length, brain area, and eye size in larval Southern flounder.

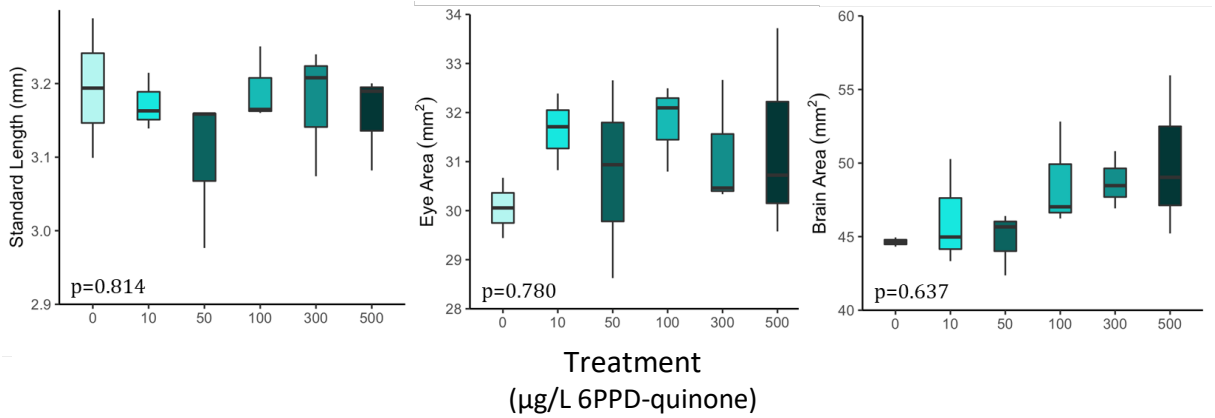

Figure 7. No significant treatment effects on sublethal endpoints were indicated for ELS Southern flounder, including for A) standard length (mm), B) eye size (mm^2), or C) brain size (mm^2). Body surface area and pericardium size (not included in figure) were also found to be similar between treatments.

Around 30 – 40 DPH, pelagic Southern flounder larvae begin to metamorphose into the familiar "flatfish" juvenile/adult phase.[9] This process, which proceeds over the course of 2 to 3 weeks, is characterized by a series of dramatic structural rearrangements that are energetically costly. Thus, it was deemed necessary to consider potential sensitivity differences that may exist between pre- and post-metamorphosis fish exposed to 6PPD-quinone. Toxicity testing with post-metamorphosis Southern flounder was conducted over the course of 96-hours, using concentrations of 6PPD-quinone that ranged from 0 to 100 µg/L. Results of testing revealed that the tolerance demonstrated by pelagic ELS flounder is conserved in the post-metamorphosis life stage (Figure 8).

Based on early findings that indicate potential impacts to the respiratory capacity of sensitive species and cultured cell lines, we elected to dissect gill baskets from fingerling flounder exposed to 6PPD-quinone for further evaluation. Following removal, gills were fixed in buffered 10% formalin and sent to the Fish Health and Pathology Lab at Texas A&M for further processing. The finished slides were recently sent to our collaborator at the University of Alaska Fairbanks, Dr. Morag Clinton (a veterinary surgeon who specializes in fish gill histopathology) for further evaluation.

A)

B)

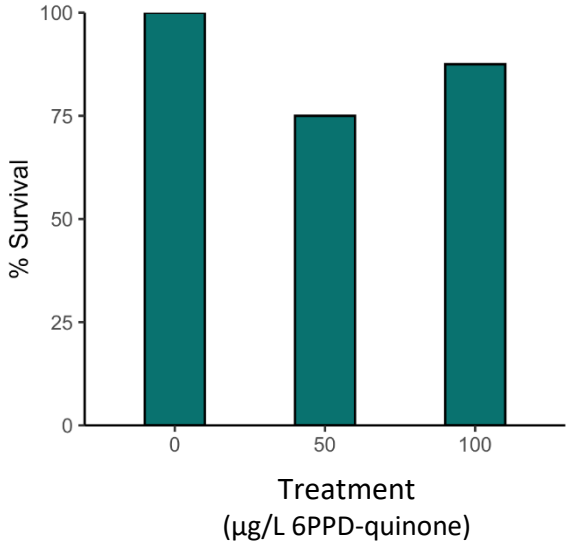

Figure 8. A) Example of a metamorphosed (84-DPH) Southern flounder used for toxicity testing. B) Results of LC50 testing indicated no significant treatment effects on survival of metamorphosed fish after 96-hours, at exposure concentrations up to 100 µg/L 6PPD-quinone.

Sheepshead Minnow *(Cyprinodon variegatus)* Toxicity Testing.

Comparative toxicity studies have demonstrated that important sensitivity differences may exist between wild caught and lab cultured fish populations within the same species.[3] Thus, in an effort to generate representative toxicity values for sheepshead minnow *(Cyprinodon variegatus)* – a notoriously tolerant model species used in toxicity studies – wild caught adult sheepshead minnow were used to conduct preliminary exposures to evaluate sensitivity to 6PPD-quinone. Briefly, adult fish were exposed to either 0, 5, 10, 15, or 20 µg/L 6PPD-quinone (n = 10 per treatment) for 48-hours, with survival assessed at 24 and 48-hours. No significant treatment effects were indicated after 48 hours, with 100% survival in all treatments.

Completed Toxicity Studies and Immediate Next Steps.

A visual summary of all toxicity testing that has been conducted to date can be found below (Table 1).

Table 1. Summary of completed toxicity tests. Dashes (-) indicate study combinations that were not included in the proposed scope of work and are not forthcoming/not practical to conduct. Asterisks (*) indicate studies that were not originally included in the proposal but were found to be necessary to reduce uncertainty for risk assessment purposes (additional studies were performed at no cost to the Matagorda Bay Mitigation Trust).

Life stage	**Red drum** *Sciaenops ocellatus*	**Sheepshead minnow** *Cyprinodon variegatus*	**Southern flounder** *Paralichthys lethostigma*
Embryos	**Yes**	No	**Yes***
Yolk sac larvae	**Yes**	No	**Yes***
Post-settlement	**Yes***	No	**Yes***
Adults	-	**Yes**	-

In the coming months, we will begin performing toxicity tests with tire wear particle (TWP) leachate, as proposed. However, the bimodal and highly variable nature of documented species responses to 6PPD-quinone, combined with variations between study designs (e.g., indoor versus outdoor studies), has also redoubled our interest in exploring whether 6PPD-quinone may act through a phototoxic mechanism of action. Thus, our most immediate plans (i.e., in the next few weeks) involve preliminary photo-induced toxicity tests using Dr. Liu's solar simulator.

Analytical Method Development and Field Sampling.

We have developed a customized analytical protocol for analysis of 6PPD and 6PPD-quinone in seawater. Specifically, we conducted the following experiments: (1) determined whether pre-filtering of the samples is needed (samples are filtered through 0.2 um filter), (2) chose the solid phase extraction resin that can yield the highest recovery rate (Oasis HLB cartridge is used with a recovery rate of 60 – 70%), and (3) developed an HPLC and MS method to quantify the concentrations of 6PPD and 6PPD-quinone and their stable isotope-labeled counterparts (Figure 9).

With the developed protocol, we further measured the background level of 6PPD-

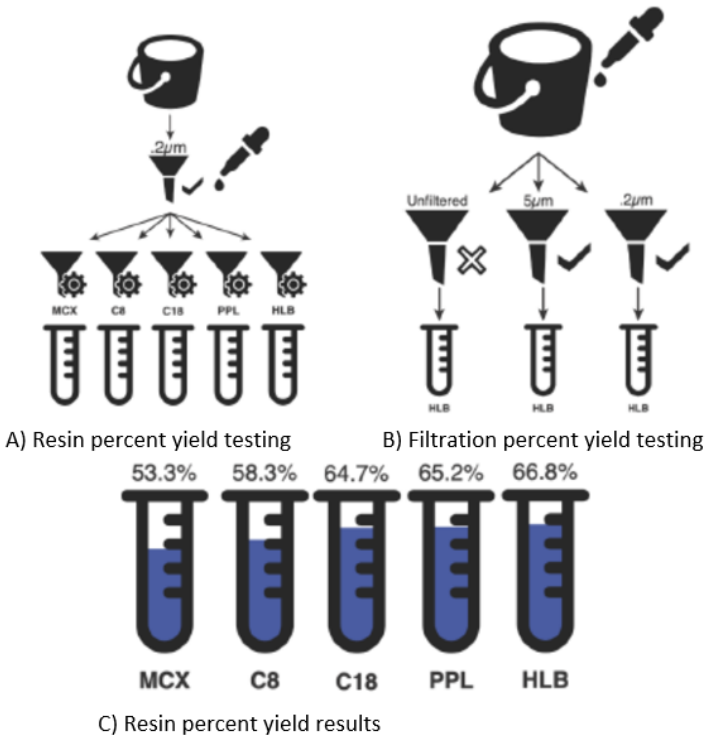

Figure 9. Study design used to conduct resin & filtration testing

quinone in several local water systems near the University of Texas Marine Science Institute (UTMSI), including adjacent rivers and bays. In the four local rivers analyzed, 6PPD-quinone concentrations range from below detection in the Aransas River and San Antonio Rivers to 0.400 µg/L in the Nueces River, and over 2.6 µg/L in the Mission River. Concentrations of 6PPD-quinone in bays are much lower compared with rivers. For example, 6PPD-quinone is below the method detection limit in Corpus Christi Bay and between 0.002 and 0.010 µg/L in the Mission-Aransas Bay (Figure 10).

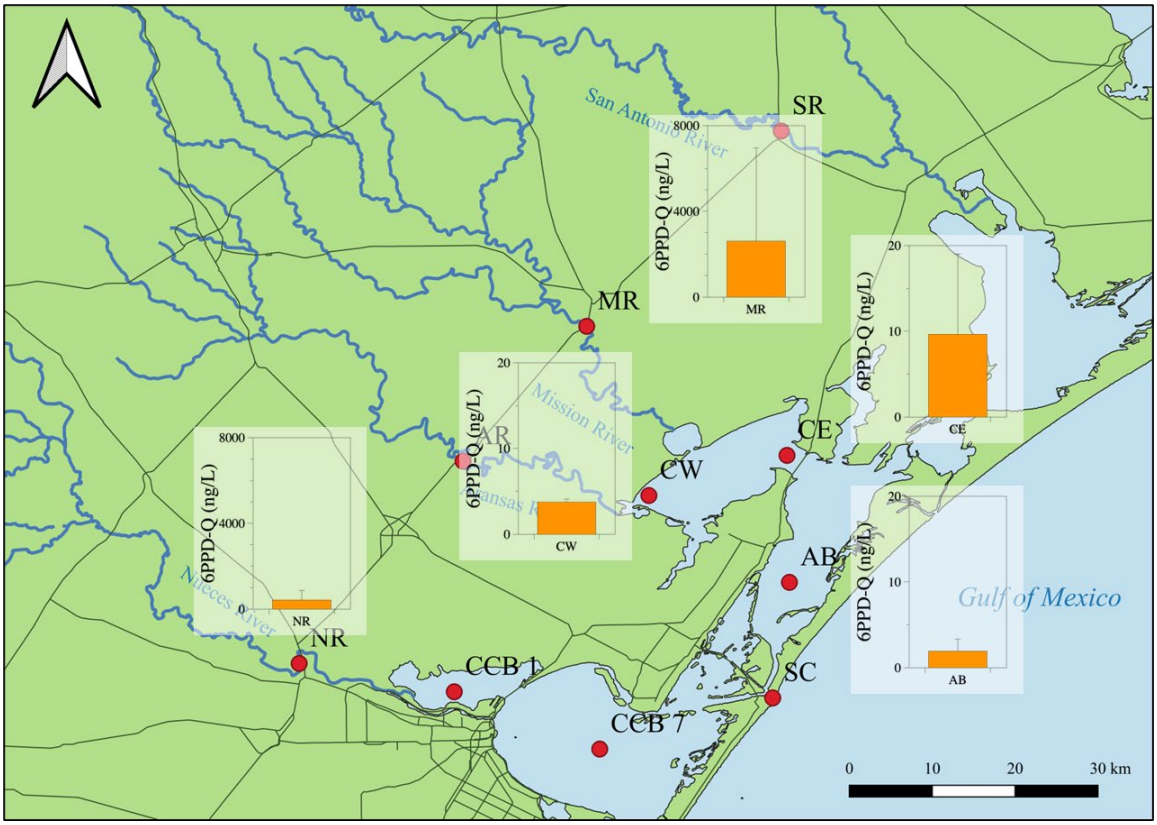

Figure 10. Concentrations of 6PPD-quinone in select rivers and bays of South Texas.

Moreover, we conducted a series of pilot incubations to investigate the biodegradation potential of 6PPD-quinone. After 5 days of incubation under dark conditions, we found very little change from our initial 6PPD-quinone concentration (10 µg/L). While a decline was observed by day 8, the variance between replicates was large enough to warrant further investigation. Nevertheless, our preliminary results suggest that 6PPD-quinone does not readily undergo biodegradation (Figure 11).

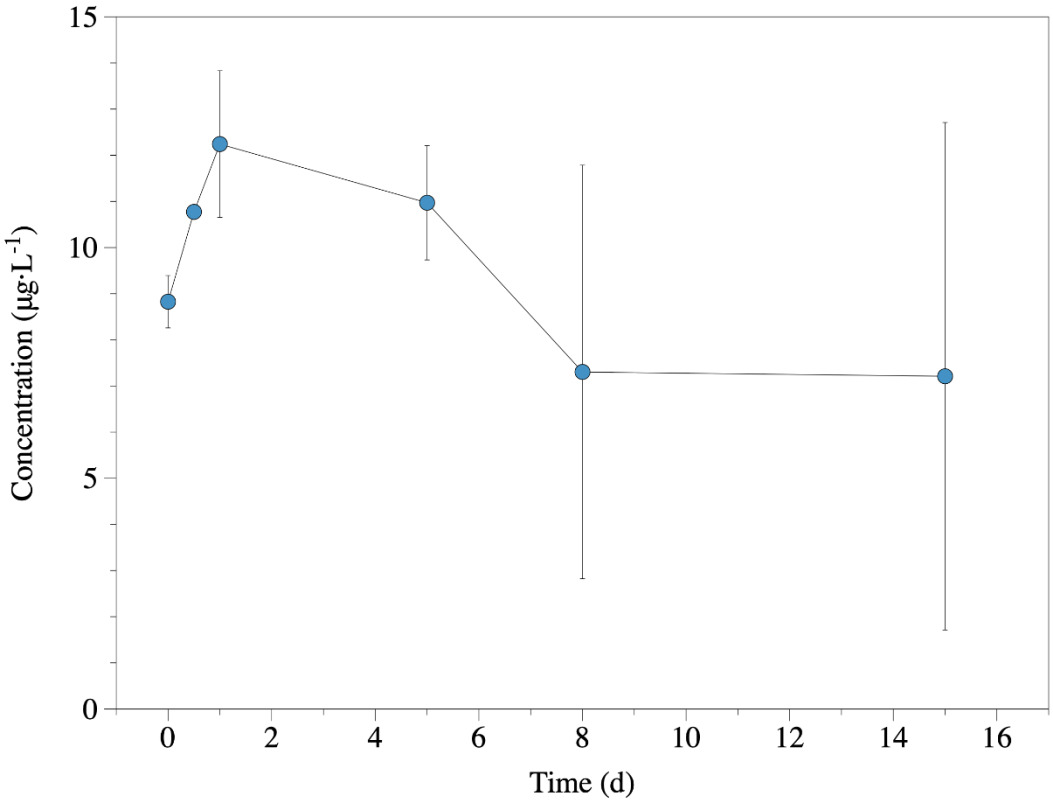

Figure 11. The biodegradation of 6PPD-quinone in amber glass bottles under dark conditions at room temperature. Concentrations did not change until day 8, although the variance between replicates was relatively large.

References.

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