Quarter 3 Progress Report

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

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Q3 Toxicity Testing Activities

Gill Histopathology. As stated in our second quarterly report, early studies investigating potential mechanisms of toxic action (MOAs) by which 6PPD-quinoneuinone causes urban runoff mortality syndrome (URMS) in select species of anadromous and freshwater fish point to potential impacts on gas exchange and/or oxygen transport. Although URMS (i.e., an acute response to exposure) did not manifest in Southern flounder *(Paralichthys lethostigma)* exposed to 0, 50 or 100 ug/L 6PPD-quinoneuinone in our initial studies, we elected to further analyze gill baskets dissected from fingerling to investigate potential chronic effects of this compound on respiratory processes.

Following dissection, 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. Dr. Clinton's initial evaluation was performed on blinded slides to avoid the potential introduction of unconscious bias. Upon concluding her blinded evaluation, Dr. Clinton was provided with a key that allowed her to aggregate results into treatment groups to identify potential impacts to gill health. A summary of Dr. Clinton's preliminary findings is included below, organized by ascending exposure concentration.

0 ug/L 6PPD-quinone Treatment (Controls) - Fish in group 0 represent a baseline and demonstrate evidence of tissue handling and histological artifacts informative in interpretation of treated groups.

- Fish in group 0 were noted to have evidence of small lamellar and filament tip bleeds, consistent with acute damage from handling or sample collection. No evidence of erythrocyte degradation or fibrinogen deposition was noted within these petechial bleeds (changes that would have indicated this damage occurred prior to immediately before or during euthanasia). Provided group 0 is indeed the untreated group, the presence of these bleeds indicates that observation of similar bleeds in fish from groups 50 and 100 are not of clinical significance.
- 'Starry' nuclei within cells (mainly erythrocytes, but a small number of pavement epithelial cells also) were also present within group 0 fish. The is an artifactual change associated with tissue embedding and sectioning, and not of significance to gill pathology. Additional erythrocyte (RBC) artefacts noted within this group included micronuclei, double nuclei, irregular shaped RBC nuclei, and nuclei of unusually granular appearance (all of unknown cause).
- The common artifact of rippling/irregularity in the arrangement of pavement epithelia cells was present within fish in this group. Despite this, cellular integrity was good (no membrane damage or lysis) and epithelial pavement cells were well attached to the underlying pillar cells of lamellae. Epithelial lifting artifacts were of low prevalence (indicating good fixation of gill tissue in this sample set). Gill sections were very small, a potential factor in the high level of structural disruption within filaments present. A small number of samples within this dataset did not contain gill filaments. Limited infectious pathologies were noted within this group. A single celled parasitic organism was noted in fish 0K.

Assuming the group 0 fish were untreated individuals, there appear to be some species-specific features that are of note in interpreting these sections. All filament tips (groups 0, 50 and 100) were densely cellular, with a regenerative appearance. Gill structure was generally delicate (no interlamellar cell mass noted), and a small number of eosinophils are present at the base of lamellae within gill filaments (much lowers numbers of eosinophils than would be present in carp species, for example).

50 ug/L 6PPD-quinone Treatment - Various pathological features are noted within group 50 fish of varying potential clinical significance.

- Hemorrhagic change was noted in fish 50K + 50B, at similar levels of severity to fish in group 0.
- Mild cellular hypertrophy was noted within gill sections (primarily with enlarged ionocyte/chloride cell types).
    - Hypertrophy of ionocyte/chloride cells was noted in fish 50K, 50L, + 50H
    - Hypertrophy of epithelial pavement cells was noted in fish 50F + 50B (small number of cells impacted). See image 1 for epithelial pavement cell hypertrophy in fish 50F.
    - Particularly large goblet cells were noted in fish 50K + 50L
- Increased cellularity (higher numbers overall) of goblet cells was noted within group 50 fish relative to group 0 fish. High numbers of goblet cells were noted in fish 50K + 50H.
- High cellularity of chloride cells was noted in fish 50B.
- An isolated foci of cell death (localized to a single lamellae) was noted in fish 50K, with pyknosis of epithelial pavement cell nuclei and basophilic fragmentation of cells (approx. 5-10 cells total impacted). See image 2. Basophilic granular material was also noted elsewhere in the tissue section of 50K, present between gill filaments. This material is of unknown origin, but potentially associated with foci of cell death noted above given its similarity in staining appearance.
- Increased presence of eosinophilic granular material was noted in the filaments of group 50 fish relative to group 0 fish. This granular material is often present around the central cartilaginous portion of gill filaments, however relative to fish in group 0, an increase in relative presence of this material was noted in fish 50M
- Similar artefactual change to group 0 was noted in this group ('starry' nuclei etc)
- Limited infectious pathologies were noted within this group. A single celled parasitic organism was noted in fish 50F.

100 ug/L 6PPD-quinone Treatment - Various pathological features are noted within group 100 fish of varying potential clinical significance.

- Hemorrhagic change was noted in fish of this group at similar levels of severity to fish in group 0 + 50. Fish 100F was noted to have greater evidence (more diffuse) hemorrhagic change, however overall, this was considered likely to be due to handling.
- Mild cellular hypertrophy was noted within gill sections (primarily with enlarged epithelial pavement cell types) in fish 100K, 100H, 100P, 100F. See image 3.
- Increased cellularity (higher numbers overall) of goblet cells was noted within group 100 fish relative to group 0 fish. High numbers of goblet cells were noted in fish 100K, 100G and 100F.
- Increased cellularity (higher numbers overall) of ionocyte/chloride cells was noted in fish 100F.
- Localized epithelial pavement cell disruption was noted in multiple fish. This includes fish 100L, 100P, 100G, and 100F. This change was in excess of subtle epithelial pavement cell alternations noted in group 0, however were similar in appearance to artifactual changes AND identified in association with infectious conditions within gills (see below). Epithelial cell lifting and loss of membrane structure was observed. See image 4.
- Lamellae were notably thickening in appearance in fish 100G (due to the combination of epithelial pavement cell hypertrophy and cell disruption in that region.
- Increased presence of eosinophilic granular material was noted in the filaments of group 100 fish. Fish 100H + 100F are noted as having high presence of eosinophilic granular material within gill filaments AND lamellar structures. See image 5.

- Similar artefactual change to group 0 was noted in this group ('starry' nuclei, etc.)
- Infectious pathologies were noted within this group.
    - Single celled parasitic organisms were noted in fish 100P (single observed parasite), 100G (single observed parasite), and 100F (multiple observed parasites).
    - Significant presence of basophilic stained, rod-shaped bacteria were noted in the gill sections of fish 100P, 100G + 100F. Bacteria were consistent in appearance between samples and although highest numbers were not in direct association with gill lamellae (located closer to filament bases + gill arches, mainly) high presence of these organisms is suggestive that their infection is the cause of noted epithelial pavement cell damage. See images 7 + 8.
    - Subtle increase in inflammatory cells (lymphocytic cell types) was noted in fish 100G.

Image 1: Fish 50F, epithelial pavement hypertrophy

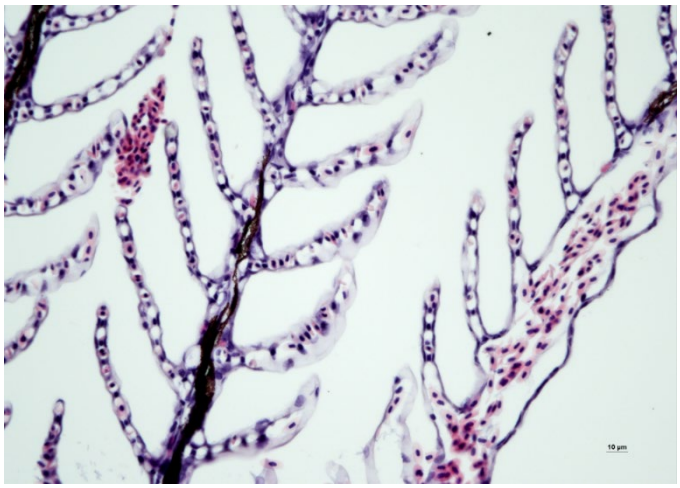


Image 2: Focal cell death with nuclear pyknosis and cytoplasmic fragmentation fish 50K.

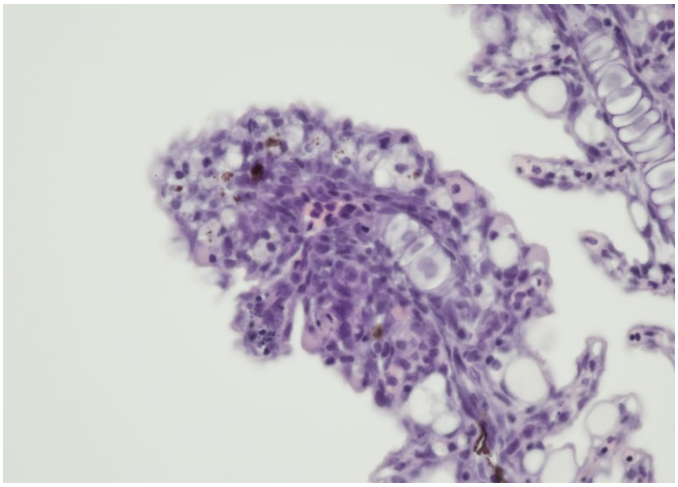


Image 3: Fish 100F, cellular hypertrophy + increase cell numbers of various cell types.

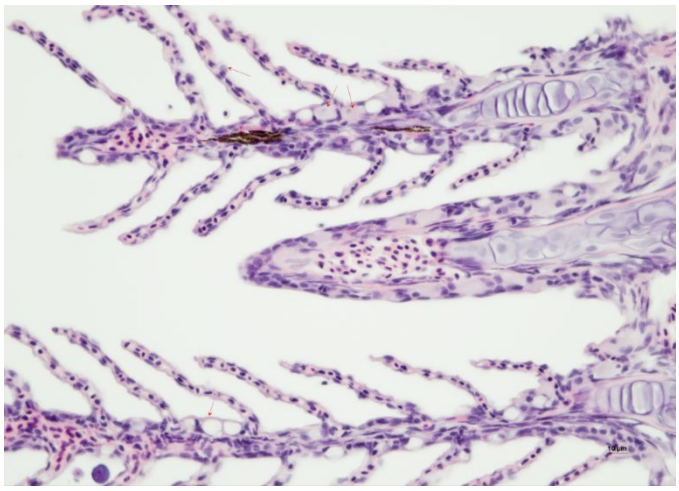
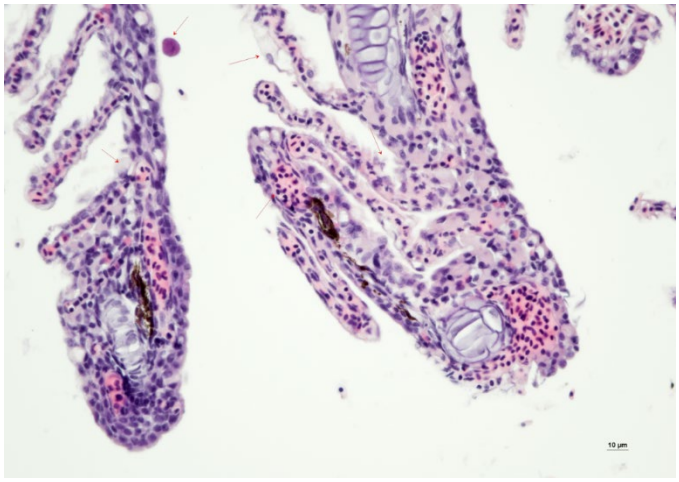
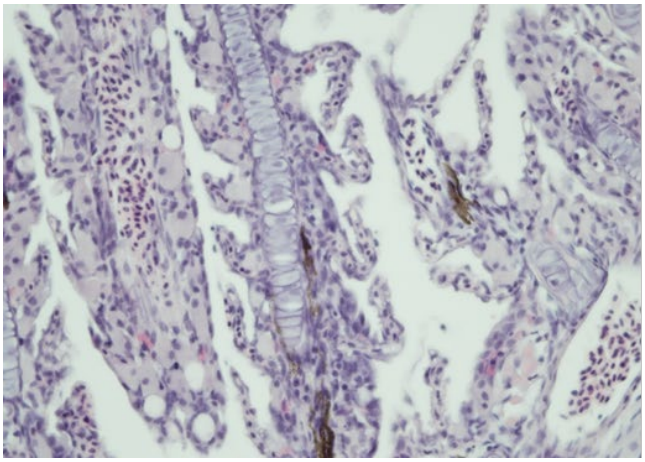
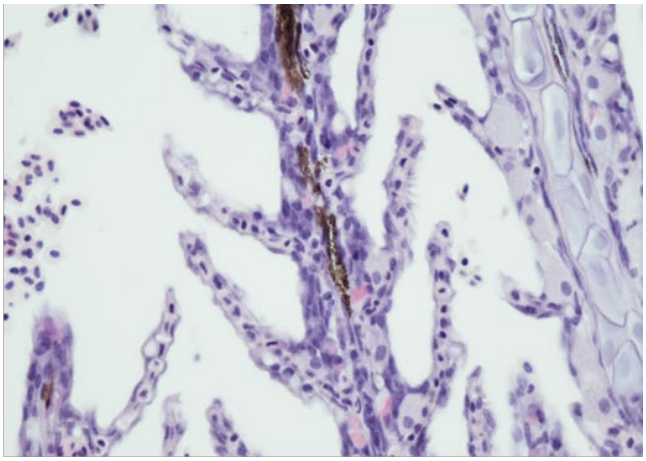


Image 4: Fish 100F. Epithelial pavement cell hypertrophy and lifting (as well as presence of parasitic organism)

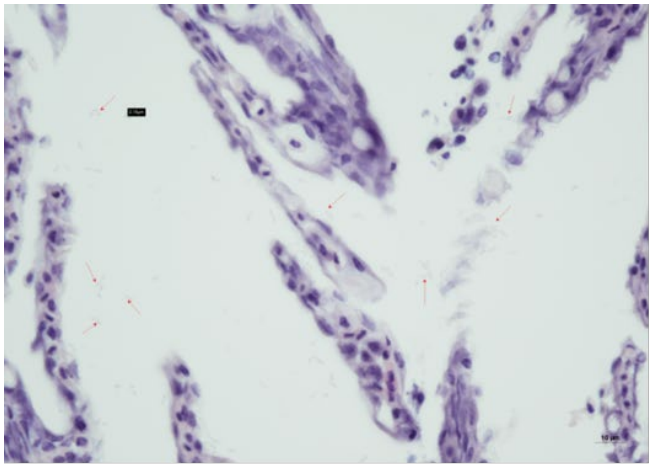


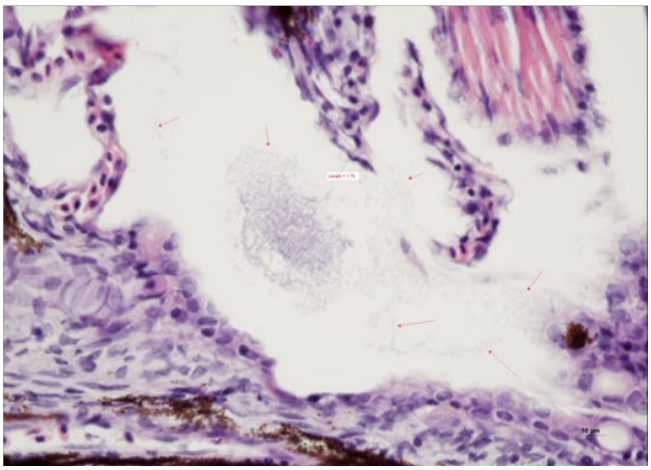
Images 5 + 6: Increased presence of eosinophilic material in gill sections. Fish 100G. Hyperplasia and altered epithelial pavement cell membrane integrity also observable in both sections.





Images 7 + 8: Rod-shaped bacteria. Fish 100G + 100P respectively.





Dr. Clinton's results suggest that 6PPD-quinone may lead to immunotoxic effects in Southern flounder. Environmental toxicants that act via immunotoxic MOAs can lead to higher level impacts on sensitive fish populations via increased susceptibility to pathogens that would not normally lead to infection in individuals with healthy immune function. Immunotoxic outcomes can also increase the adverse effects of exposure to other environmental contaminants, particularly those that also act through an immunotoxic MOA. More investigation is needed to determine whether immunotoxicity is a concern for Southern flounder chronically exposed to environmentally relevant concentrations of 6PPD-quinone. Dr. Nielsen is actively planning follow up studies for Q4 to further elucidate the potential, if any, ecological implications of these results.

Preliminary Photosensitization Study. During Q3, a preliminary study was conducted to investigate the potential for 6PPD-quinone to act via a photosensitizing MOA. Fertilized red drum *(Sciaenops ocellatus)* embryos (n = 20 embryos/dish) were exposed to either 0, 25, 50, or 100 ug/L 6PPD-quinone for 24-hours, either under ambient laboratory lighting (visible light spectrum only) or simulated full spectrum solar radiation. The intensity of simulated solar radiation for the test was designed to be representative of incident surface UV experienced by red drum embryos during the *Deepwater Horizon* oil spill (Nielsen et al., 2018). Moreover, exposure to similar doses of UV radiation are known to interact with photodynamic constituents of crude oil, resulting in widespread mortality through a photo-sensitization MOA.
Aside from 6PPD-quinone concentrations and previously described light regimes, all other environmental parameters were held constant between treatments, with successful hatch/survival assessed at 24h. Co-exposure to full spectrum solar radiation and 6PPD-quinone did not significantly increase mortality, indicating that photosensitization is unlikely to be an environmentally important MOA for Southern flounder exposed to this toxicant.

Confirmatory Chemical Analyses for 6PPD-quinoneuinone studies. Dr. Liu's lab has been performing confirmatory analyses for water samples collected during 6PPD-quinone toxicity testing. These results are needed to confirm nominal exposure concentrations prior to publication of results (manuscript preparation is underway).

Tire Wear Particle Studies.

Together with the Liu lab, we have been working to prepare and characterize whole tire wear particle (TWP) leachate for subsequent toxicity tests. After initially attempting to generate TWP ourselves (and encountering many difficulties), we decided to utilize cryomilled tire particles provided by the U.S. Tire Manufacturers Association, which we have begun to characterize. The use of tires from an external commercial source allows us to standardize leachates prepared for forthcoming toxicity tests, which is important as both the source and progression of weathering/aging are known to impact the composition and toxicity of leachates, resulting in highly variable toxicities.

With regard to characterization, we first determined the dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) present in leachate that was generated as part of a 10-day preliminary study. These parameters, particularly DOC,

are known to affect the bioavailability (and thus toxicity) of many environmental contaminants and will be necessary for manuscript publication. Results indicate that DOC is approximately 120 parts per million (ppm) and TDN is approximately 20 ppm. Characterization of whole leachate is ongoing, with toxicity tests to follow (to allow for normalization of leachate to known 6PPD-quinone concentrations).

References

Nielsen, K., Lay, C., Alloy, M., Gielazyn, M., Morris, J., Forth, H., . . . Roberts, A. (2018). Estimating incident ultraviolet (UV) radiation exposure in the Northern Gulf of Mexico during the *Deepwater Horizon* oil spill. *37*(6), 1679-1687. https://doi.org/10.1002/etc.4119