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A Multiple Endpoint Analysis of the Effects of Chronic Exposure to Sediment Contaminated with Deepwater Horizon Oil On Juvenile Southern Flounder and Their Associated Microbiomes

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Short Title: Oiled sediments impact juvenile flounder

Abstract

Exposure to oiled sediments can negatively impact the health of fish species. Here, we examine the effects of chronic exposure of juvenile southern flounder, *Paralichthys lethostigma,* to a sediment-oil mixture. Oil:sediment mixtures are persistent over time and can become bioavailable following sediment perturbation or resuspension. Juvenile flounder were exposed for 32 days under controlled laboratory conditions to five concentrations of naturally weathered Macondo MC252 oil mixed into uncontaminated, field-collected sediments. The percent composition of individual polycyclic aromatic hydrocarbons (PAHs) of the weathered oil did not change after mixing with the sediment. Spiked exposure sediments contained 0.04-395 mg/kg tPAH50 (sum of 50 individual PAH concentration measurements). Mortality increased with both exposure duration and concentration of sediment-associated PAHs, and flounder exposed to concentrations above 8 mg/kg tPAH50 showed significantly reduced growth over the course of the experiment. Evident histopathologic changes were observed in liver and gill tissues of fish exposed to more than 8 mg/kg tPAH50. All fish at these concentrations showed hepatic intravascular congestion, macrovesicular hepatic vacoulation, telangiectasia of secondary lamellae, and lamellar epithelial proliferation in gill tissues. Dose-dependent upregulation of CYP1A expression in liver tissues was observed. Taxonomic analysis of gill and intestinal commensal bacterial assemblages showed that exposure to oiled sediments led to distinct shifts in commensal bacterial population structures. These data show that chronic exposure to environmentally-relevant concentrations of oiled sediments produces adverse effects in flounder at multiple biological levels.

Key Words: PAH, ecotoxicology, histopathology, gene expression, microbiome, *Paralichthys*

1.0 Introduction

The BP Deepwater Horizon (DWH) accident released an estimated 4.9 million barrels of Louisiana sweet crude oil into the northern Gulf of Mexico (GOM) over an 87 day period between 20 April and 15 July 2010. While some of this oil was recovered and removed, much was evident as surface slicks that washed onshore into marshes and onto beaches of Louisiana, Mississippi and Alabama (Michel *et al.*, 2013) throughout the duration of the spill; additionally, an unknown, but certainly large amount settled into the sediments of the northern GOM (Wang and Roberts, 2013). While measured PAH concentrations of surface waters showed negligible oil contamination one year after the spill (Allan *et al.*, 2012), crude oil remains in sediments for a much longer time and exhibits slower weathering (Liu *et al.*, 2012). Thus, benthic organisms may be susceptible to impacts from the DWH oil several years after the spill and associated clean-up, particularly as oiled sediments are mixed or disturbed by storms, currents and dredging activities. For instance, species number, density and biomass of macrobenthic communities remained depressed two years after the 2007 *Hebei Spirit* oil spill in Korea (Hong *et al.*, 2014), and pink salmon embryos spawned in sediments contaminated by the *Exxon Valdez* spill exhibited elevated mortality five years after the spill and associated clean-up (Ballachey *et al.*, 2014).

The effects of the DWH oil spill have impacted flora and fauna at multiple biological levels, including marine mammals (Schwacke *et al.*, 2013; Wise and and J. P. Wise, 2014), fishes (Brewton *et al.*, 2013; Brown-Peterson, 2014; Dubansky *et al.*, 2013; Echols *et al.*, 2015), invertebrates (Carmichael *et al.*, 2012; Echols *et al.*, 2015; Goodbody-Gringley *et al.*, 2013; McCall and Pennings, 2012), and gelatinous zooplankton (Almeda *et al.*, 2013) to microbial food webs (Ortmann *et al.*, 2012) and salt marsh vegetation (Silliman *et al.*, 2012). Many of these

studies have focused on exposure to oil in the water column, either through collections of specimens directly from the environment or in experimental exposures to a water accommodated fraction (WAF) of oil. Exposure of embryos of Gulf killifish (*Fundulus grandis*) and zebrafish (*Danio rerio*) to oiled sediments resulted in reduced hatch and developmental anomalies (Dubansky *et al.*, 2013; Raimondo *et al.*, 2014), suggesting oiled sediments can significantly impact young fishes. However, there is little information to date on the impact of oiled sediments from the DWH spill on juvenile or adult benthic fish species. Additionally, while many studies have examined physiological or molecular impacts of DWH oil exposure on fishes (Brette *et al.*, 2014; Crowe *et al.*, 2014; Dubansky *et al.*, 2013; Incardona *et al.*, 2014; Pilcher *et al.*, 2014; Whitehead *et al.*, 2011), relatively few reports combine whole animal and molecular measurements from the same individuals (e.g. (Brewton *et al.*, 2013)) to allow an overall assessment of the impacts of DWH oil on multiple aspects of fish condition.

Here, we report the effects of sediment mixed with naturally weathered DWH oil on juvenile southern flounder (*Paralichthys lethostigma*), a common ecologically and recreationally important benthic fish in the northern GOM. We hypothesized that oiled sediments would impact juvenile flounder at a variety of levels, including organismal (survival, growth), tissue (histopathology of liver and gill), molecular (gene expression), and holobiotic (gut and gill commensal microbiome) endpoints. By investigating effects at multiple biological levels, we are able to obtain a more complete and thorough understanding of the mechanisms by which oiled sediments affect benthic organisms.

2.0 Materials and Methods

2.1 Oil Samples and Sediment Preparation

Reference, non-oiled sediments were collected from Alabama marshes (30°22'45.72"N; 88°18'24.40"W) on 27 Nov 2012 and frozen for transport to the laboratory. Weathered Louisiana MC252 crude oil samples (BP code 00003277) used in this experiment (hereafter referred to as Slick B) were collected under chain of custody from the northern GOM on 19 July 2010 during DWH response efforts. Sediments were weighed, thawed and mixed with varying amounts of Slick B oil for 30 min using a KitchenAid stand mixer on medium speed (amounts and concentrations reported in Table S1) to achieve targeted concentrations of sediment-associated PAHs. The mixed sediments were distributed into individual numbered cages (10 cm glass petri dish with a 20 cm column of 2 mm nylon mesh; 75g sediment/cage) and placed into 20 L treatment aquaria. The sediments were allowed to settle overnight following addition of 10L of seawater to each aquarium. The exposures were performed in quadruplicate with 8 individual cages per replicate tank (32 total fish per treatment level), with a control and five concentrations of Slick B oil (measured concentrations of 0, 0.7, 8, 54, 127, 395 mg tPAH50/kg sediment). These concentrations are consistent with measured oil range organics from sediments of estuarine areas collected from June through October 2010 (30 – 200 mg/kg; (Floyd *et al.*, 2012), and thus represent environmentally realistic exposure levels.

2.2 Fish Exposures

Post metamorphosis juvenile southern flounder (53 days post hatch, 7-18 mm SL, spawned and raised in captive, uncontaminated conditions) were obtained from the University of Texas Marine Science Institute mariculture facility. Upon receipt, flounder were acclimated to culture conditions (salinity 15 psu, 22°C, pH 8.0, 12L:12D photoperiod) over a period of 25 days and maintained in these conditions for an additional 5 days prior to experimentation. Fish were fed

commercial pellet food (Ottohime B2) twice daily during acclimation and exposure. At test initiation, fish ranged from 18-35 mm SL.

Clean artificial seawater prepared from Fritz Super Salt, diluted to 15 psu salinity, filtered and UV-sterilized was used in a flow-through experimental system (Manning *et al.*, 1999). System cycling ensured addition of 500 ml of fresh seawater into each treatment aquarium every 15 min, resulting in daily water renewal of 48L/aquaria. Thus, this experimental design consisted of contaminated sediments with uncontaminated water flowing into each tank. Treatment aquaria were maintained at 22±2°C and a 12L:12D photoperiod throughout the experiment. Compressed oxygen was bubbled into each aquarium such that D.O. was maintained at $\geq$ 4 mg/L.

At test initiation, flounder were individually weighed (0.1 mg), measured (mm standard length (SL)), and added to 1 cage/ aquarium in random order in 8 rounds of additions. Aquaria were covered with foil lids to prevent evaporation and cross-tank contamination. The flounder were inspected daily for the 32-d duration of the experiment and dead fish were removed and recorded. We digitally recorded video footage of each flounder twice weekly for additional growth measurements before the end of the experiment. A 25-mm Teflon coated stir bar was placed into each cage and used as a size reference to facilitate SL measurements of each fish using the digital imagery. Still images were captured from videos in which both the entire fish and the entire inert bar were present in the frame. Both the inert bar and the fish were measured using digital measurement estimation in Microsoft Paint. Water quality (temperature, salinity, pH, D.O., and ammonia) was measured daily in each aquarium. At test termination, flounder were euthanized in MS-222, weighed and measured. Liver, intestine, and top and bottom gill tissues from 4 fish/replicate aquarium were removed and individually stored in 1 mL RNALater (Ambion) for subsequent analyses. Livers were weighed (LW, mg) following storage in

RNALater. The tails were removed from 4 fish/replicate aquarium and the anterior carcass was preserved in 10% neutral buffered formalin for histopathologic analyses.

2.3 Analysis of Sediment and Water Samples

Grain size was analyzed from sediments at the time of initial collection following standard ASTM methodology (ASTM, 2007). Total Organic Carbon (TOC, %) was analyzed in control sediments at experiment initiation and termination following EPA method 9060A (http://www.epa.gov/epawaste/hazard/testmethods/sw846/online/index.htm); TOC was not analyzed in the oil-mixed sediments to avoid interference from the signature of the oil added intentionally. Composite sediments from each treatment and the control and were analyzed for a suite of 50 PAH analytes (tPAH50) at experiment initiation and termination by gas chromatography with low resolution mass spectrometry using selected ion monitoring (GC/MS-SIM) . The analytical procedure is based on EPA Method 8270D (Kimbrough *et al.*, 2006; Murphy and Morrison, 2007). The PAH composition of the Slick B oil was analyzed following the same procedures. Filtered and unfiltered composite water samples were collected from each treatment at experiment initiation and at days 2, 4, 6, 8, 12, 19, 26, and 32 of exposure and analyzed for tPAH50 concentration following the same procedure referenced above. All chemical analyses were performed by ALS Environmental (Kelso, WA). Additionally, duplicate unfiltered water samples from each replicate aquarium were collected at the same 9 time points, mixed with an equal volume of absolute ethanol, centrifuged, and scanned at 380 nm emission and 270 nm excitation following (Greer *et al.*, 2012) to assess inter-replicate variability and changes in PAH in overlying water in treatments over the course of the experiment.

2.4 Histopathologic Procedures

Whole, preserved flounder were decalcified, processed and embedded in paraffin following standard histological techniques; tissue was sectioned at 4µm at two levels and stained with hematoxylin and eosin. Three separate readers independently evaluated liver, gill, heart and kidney tissues at both levels for all specimens and diagnoses were based on both levels combined; identity of treatments was blinded for initial readings. Histopathologic observations of top and bottom gills were combined for the analyses; while the bottom gill is in more direct contact with the contaminated sediments, water is pumped directly through both top and bottom gills, likely resulting in little difference in exposure. Histopathologic conditions in all evaluated tissues were rated as 0 (condition not observed) or 1 (condition observed), and the percentage of fish in each treatment presenting an observed histopathologic anomaly was calculated and presented as consensus scores. Photographs of all liver and gill tissue were taken of each specimen for quantification of treatment-related effects. The percentage of liver tissue exhibiting intravascular congestion and macro- or micro-vesicular vacuolation was quantified in three randomly chosen photos/specimen using the grid plug-in for Image J analysis based on methodology described by Tomkiewicz et al. (Tomkiewicz *et al.*, 2011). The total number of gill lamellae were counted in three randomly chosen photos/specimen; the number of those lamellae exhibiting epithelial proliferation (defined as proliferation on the lamellae at the base of the secondary lamellae) and the number containing secondary lamellae with telangiectasis were counted using Image J. Finally, a rank of 0-4 (none, <25%, 25-50%, 51-75%, >75%) was assigned for each lamellae based on the percentage of secondary lamellae per lamellae that exhibited telangiectasis, and a mean rank of telangiectasis was calculated for each specimen.

2.5 Gene Expression

Expression of Cytochrome p450 1A (Cyp1a) was assessed in the livers of eight flounder randomly selected from each treatment. Total RNA was isolated following standard protocols. Total RNA was assessed via NanoDrop spectrophotometer for quality and concentration, and acceptable samples were reverse transcribed to cDNA using a Revertaid kit. Species-specific primers for Cyp1a were used for qPCR analysis, and gene induction was expressed as fold change from control values (normalized to 18S expression levels) using the Sybr Select Master Mix and an ABI 7500 Fast Real Time PCR instrument.

2.6 DNA Extraction

Tissue samples from the top gill, bottom gill, and intestine of flounder from the 54 mg/kg treatment (N = 8) and control treatment (N = 8) were analyzed for variation in the organ-specific microbial communities associated with oil exposure. DNA was extracted from gill and liver tissues using a PowerSoil DNA Isolation Kit (MoBio Laboratories). A thoroughly homogenized aliquot of each tissue sample was added to the PowerSoil bead tube. Extraction proceeded per the directions in the kit resulting in 100 µl of DNA in elution buffer (10 mM Tris). Concentrations of DNA in each sample were measured and recorded using a NanoDrop Spectrophotometer (Thermo) to account for total DNA used in qPCR.

2.7 Microbial Diversity Analysis

The relationship between microbial communities in intestine and gill tissues of oil vs. non-oil exposed fish was determined by 16S rRNA gene amplification and sequencing as described by Dowd et al. (Dowd *et al.*, 2008), targeting the V1–V3 region. Sequences that failed to return at least half the expected amplicon length (or 250 bp, whichever was shortest) were removed from the data pool. All sequences were then denoised using an algorithm based on USEARCH

(Edgar, 2010) and checked for chimeras using UCHIME (Edgar *et al.*, 2011). After denoising and chimera checking, sequence data were separated into operational taxonomic units (OTUs) and annotated using the RDP classifier (Wang *et al.*, 2007) with GreenGenes v. 12.10 (McDonald *et al.*, 2012) used as a reference. Finally, relative abundances of taxa at each hierarchical taxonomic level were calculated using the summarize_taxa.py QIIME script.

Of 952 OTUs, 590 mapped to known taxonomic assignments (minimum 97% similarity). Those OTUs that failed to match were excluded from further analyses. Negative binomial generalized linear models were performed with the Deseq2 package within R (Anders and Huber, 2010; Love *et al.*, 2014; R Core Team, 2013). Oiling treatment effects were statistically analyzed with a Wald test with Benjamini-Hochberg correction. Alpha values for the Benjamini-Hochberg adjusted p-values were set at 0.1. Community composition analysis was performed via a distance-based redundancy analysis (db-RDA) with oil treatment and tissue (upper gill, lower gill, and intestine) as constraining factors.

2.8 Predictive Metagenomic Analysis

Closed-reference OTUs were picked from the Green Genes database (v13.5) with QIIME (v1.8.0) at 97% identity (Caporaso *et al.*, 2010). Metagenome predictions were calculated with PiCRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, v1.0.0 (Langille *et al.*, 2013)). From 890 OTUs, 6,909 pathways were calculated with 21,940,124 individual predicted gene counts. The average Nearest Sequenced Taxon Index (NSTI) for oiled and non-oiled samples for the metagenomic predictions was 0.14± 0.05. Lower NSTI values indicate that microbes in a given sample are more closely related to sequenced genomes (Langille *et al.*, 2013). Our NSTI values were consistent with previous work (Langille *et al.*, 2013) which revealed higher NSTI values in mammalian guts (0.14 ± 0.06) and environmental

communities (0.17 ± 0.02). The predicted Kyoto Encyclopedia of Genes (KEGG) pathways were collapsed down to level 3 KEGG Orthology groups (KOs) with the PiCRUSt script categorize_by_function.py. Negative binomial generalized linear models were fit to the predicted KOs of the microbiota samples. Alpha values for Benjamini-Hochberg adjusted p-values were set at 0.1.

2.9 Statistical Analysis

Log-logistic dose-response curves were fitted for mortality, growth, and histopathologic effects using the *drc* package (Ritz, 2010; Ritz and Streibig, 2005). Effect concentrations (LC and EC) and confidence intervals were estimated based on the profile-likelihood (Faraggi *et al.*, 2003; Venzon and Moolgavkar, 1988) using the *bbmle* package (Bolker, 2013; R Development Core Team, 2013). The LC estimates reported account for control mortality. For growth endpoints, reported EC_{20} and EC_{50} represent the exposure concentrations associated with 20% and 50% decreases relative to control growth, respectively. For histology endpoints, EC_{20} and EC_{50} represent the concentrations associated with 20% and 50%, respectively, of the difference between the control and the maximum response. We report LOECs for reduction in macrovesicular vacuolation and hepatosomatic indices because intermediate responses were not observed for these endpoints. These analyses were all performed in R using version 3.1.1 (R Core Team, 2013).

Differences in the percentage of swollen lamellae and the rank value of telangiectasia among treatments were tested using ANOVA with Bonferroni post-hoc analysis. Percentage values were arcsine squareroot transformed prior to analysis, and homogeneity of variance was tested using Levene's Test. These analyses were performed in SPSS, version 18. Gene expression of Cyp1a between treatments was expressed as fold-change from control values as Relative Quantitation

implemented in the ABI Data Assist v. 3.01 software. In all cases, differences were considered significant if $p < 0.05$.

3.0 Results and Discussion

3.1 Sediment and PAH Characterizations and Concentrations

The sediments used in this study were dominated by sands and silts with grain size < 0.5 mm (Table S2). There was a slight increase in TOC of control sediments from experiment initiation (0.505%) until experiment termination (0.594% TOC).

The process of mixing Slick B oil into sediment did not change the composition of the PAHs (Figure S1A). Phenanthrenes/anthracenes remained the most common component of the complex oil mixture, with flourenes, dibenzothiophenes, chrysenes and pyrenes each contributing >2% to the PAH composition. This PAH distribution is similar to that seen in sediments collected near the DWH wellhead one year after the DWH blow-out as well as in estuarine sediments several months following the blowout (Liu *et al.*, 2012), suggesting that the mixed sediments used for this experiment are therefore a valid proxy for estuarine sediments contaminated with oil from the DWH incident. Furthermore, the similarity between the PAH profiles in Slick B samples and the PAH profiles from the sediment mixed with different amounts of Slick B demonstrate that the mixing process did not alter the relative proportion of PAHs. Nominal and measured PAH concentrations were similar (Figure S1B), and show that the mixing process was also successful in producing the targeted sediment concentrations. The sum of 50 individual PAH concentration measurements (tPAH50) in the sediments of the different treatments were 0.03, 0.7, 8, 52, 127, and 395 mg/kg sediment at experiment initiation. The tPAH50 concentration of

the sediments decreased little over the 32 day experiment (Table S1) despite constant renewal of clean water into the system.

The tPAH50 concentration of the unfiltered water overlying the sediments was < 5 µg/L in all but the highest treatment throughout the study (Figure S1C), confirming that the majority of the oil remained in the sediments for the duration of the study. Furthermore, tPAH50 composition in the water column and the sediments were similar, suggesting any PAHs in the water column likely came from oil-bound suspended fine grain material and not dissolved constituents. Measured tPAH50 of water overlying the highest treatment (395 mg/kg in sediment) peaked at 45 µg/L at day 2 and declined to 10 µg/L by day 10 of the experiment. No further measurements were taken from this treatment, as 94% of the flounder in this treatment died by day 11. Fluorescence measurements showed that variability in PAH concentrations of replicate aquaria within treatments was minimal, and also showed little decrease in PAH concentrations of overlying water across treatments during the course of the experiment (Figure S1D). As there was little release of PAHs into the water column throughout the 32 day experiment, the observed effects on exposed flounder are likely due to sediment-associated PAHs, rather than aqueous PAHs. These data suggest that sediment-associated PAHs are likely to remain a contaminant of concern for benthic organisms in the area affected by DWH for years after the spill. Recent analyses of DWH-oiled sediments from Louisiana marshes confirms minimal degradation of PAHs three years post-spill (Turner *et al.*, 2014). Long-term persistence of oil in intertidal sediments following the 1989 *Exxon Valdez* oil spill has resulted in delayed recovery of benthic marine communities, which can have ecosystem-wide implications (Ballachey *et al.*, 2014; Peterson *et al.*, 2003).

3.2 Mortality and Growth

Flounder mortality increased with increasing concentrations of oil in sediments, and no mortality was observed in control treatments (Figure 1A). Some fish in the highest concentration (395 mg/kg tPAH50) died within 24 h of exposure or appeared lethargic, and by day 18 of the experiment all fish in this treatment were dead. The LC_{50} for the 32 day exposure was 78.1 mg/kg tPAH50 (95% confidence intervals = 56.6, 104 mg/kg), and the associated LC_{20} was 36.3 mg/kg tPAH50 (95% CI = 20.2, 54.7). These calculated LC values are within the range of concentrations of oil range organics found in sediments along coastal Mississippi and Alabama 3 months after capping the flow from the DWH spill (55-75 mg/kg; (Floyd *et al.*, 2012)), suggesting benthic fishes along the north central GOM coast in summer and fall 2010 were likely impacted by the DWH oil spill.

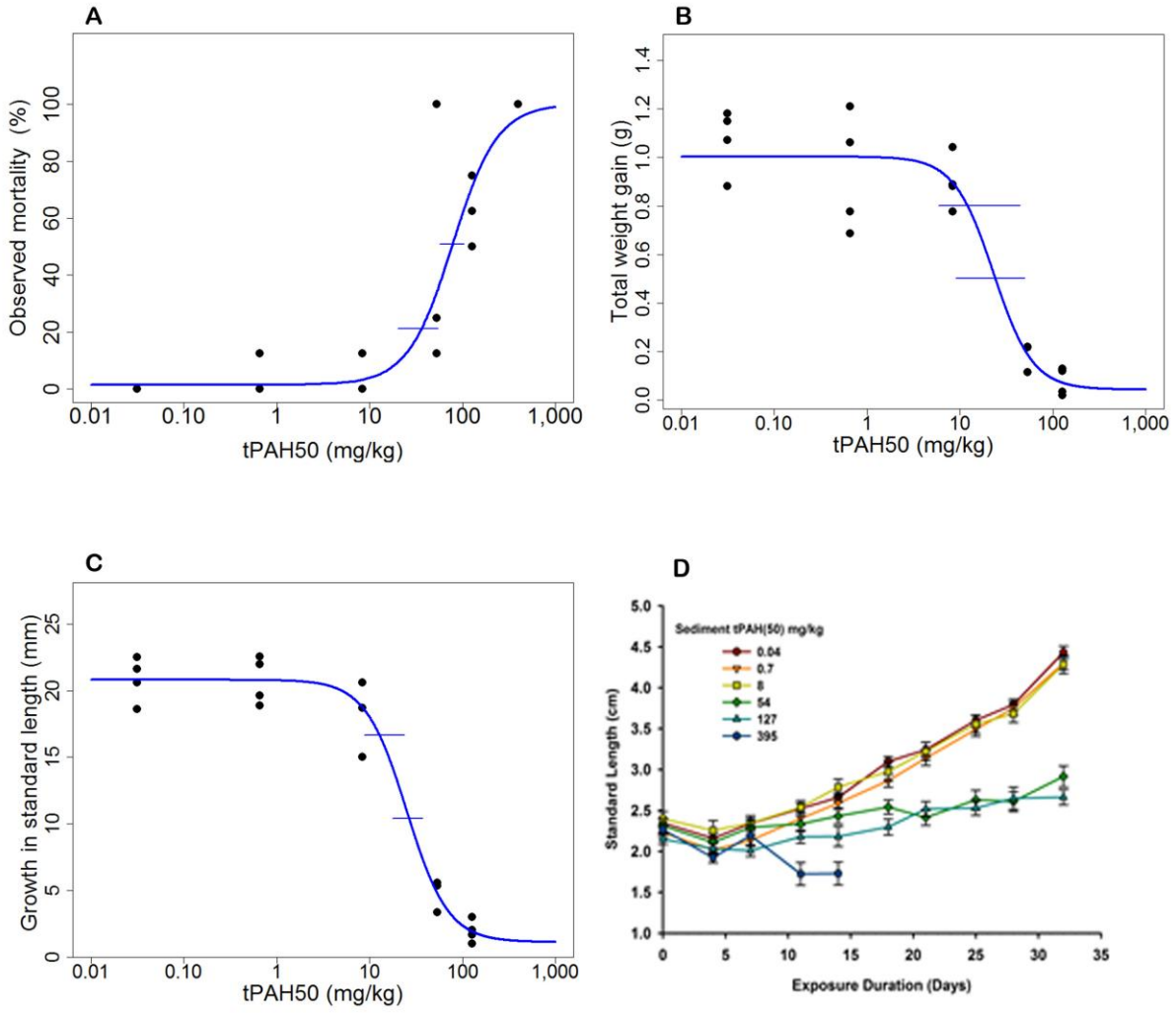

Figure 1. Mortality and growth of southern flounder exposed to various concentrations of oiled sediments for 32 d. Dose-response relationship between exposure concentration and mortality (A), total weight in grams (B) and change in standard length (SL) in mm (C). Points indicate mean values per replicate tank. Upper and lower horizontal lines indicate the 95% confidence intervals on estimated LC/EC_{20} and LC/EC_{50}, respectively. EC values are based on the percent reduction from control mortality or growth. D. Mean (±SE) length (cm SL) of flounder in each treatment. Measurements for Day 0 and Day 32 were taken directly on the fish with a ruler; other measurements were taken from video images.

There was wide variability in fish growth within all replicate aquaria (differences of 3 to 29 mm SL among individual fish in a single aquarium at the end of the study), but there was no trend in this variability associated with treatment. Increasing sediment concentrations of tPAH50

reduced growth in both weight (Figure 1B) and length (Figure 1C). The effective concentrations (EC) impacting growth in weight were calculated as EC_{20} = 12.0 mg/kg tPAH50 (95% CI = 5.9, 44.3) and EC_{50} = 23.9 mg/kg tPAH50 (95% CI = 9.0, 49.6) (Figure 1B). Similarly, concentrations impacting growth in length were EC_{20} =12.8 mg/kg tPAH50 (95% CI = 8.8, 23.7) and EC_{50} = 26.7 mg/kg tPAH50 (95% CI = 17.6, 37.4) (Figure 1C). The contaminant-related reduction in growth was evident early in the experiment; after 10 d exposure to 395 mg/kg tPAH50 and after 14 days of exposure to concentrations > 8 mg/kg (Figure 1D). Similar results have been shown for other benthic species; four species of juvenile flatfishes showed significantly reduced growth after 90 days exposure to oiled sediments (Moles and Norcross, 1998), and juvenile brown shrimp (*Farfantepenaeus aztecus*) grew slower after seven days exposure to heavily DWH-oiled sediments (Rozas *et al.*, 2014).

3.3 Histopathologic Analyses

Histopathologic examination revealed only occasional mild evidence of background disease in both control and treated flounder indicating that the histopathologic changes reported were a result of exposure to oil-contaminated. Histopathologic conditions and consensus scores for kidney, heart, liver and gill tissues from controls and four treatments are listed in Table S3; fish from the highest treatment were not analyzed due to mortality prior to experiment termination. Kidney tissue showed mild tubule proliferation and/or granuloma in some specimens from every oiled sediment treatment, but these observations showed no strong relationship to increasing sediment tPAH50 concentration and can be considered background lesions present in all fish. Atrophy of myocardial trabeculae was observed in heart tissue from one specimen each in the 0.7, 8 and 54 mg/kg tPAH50 treatments, and increased edema was noted in heart tissue in some specimens from all oiled sediment treatments. Recent evidence shows disruption of

cardiomyocyte repolarization in heart tissue (Brette *et al.*, 2014) and defects in heart development of larval marine fishes exposed to DWH oil (Incardona *et al.*, 2014). Furthermore, embryonic zebrafish exposed for 48 h to oiled water exhibited changes in heart shape and reduced cardiac output as adults (Hicken *et al.*, 2011). Therefore, in light of this information, the mild histopathologic changes in heart tissue of juvenile flounder exposed to oiled sediments reported here warrants further investigation.

Distinct histopathologic effects of oiled sediment exposure in flounder were seen in liver and gill tissues. Flounder exposed to non-oiled sediments exhibited healthy hepatocytes and exocrine pancreatic tissue (Figure 2A). There was a reduction in exocrine pancreatic tissue in fish exposed to 54 and 127 mg/kg tPAH50 oiled sediments (Table S3), although diseased or atrophied exocrine pancreatic tissue was not observed in fish exposed to oiled sediments > 8 mg/kg tPAH50. A loss of zymogen granules in the cytoplasm of acinar cells in the pancreas, which can result in reduction of pancreatic tissue, is often observed during starvation (Zachary and McGavin, 2012). In addition, some exocrine pancreatic tissue in flounder from 8 mg/kg and 127 mg/kg exhibited large eosinophilic inclusions in the duct lumens/cells (Figure S2), likely containing cellular debris and pancreatic enzymes (Zachary and McGavin, 2012). Hepatic edema, characterized by curved and branching interlobular vascular profiles and perivascular sinusoidal ectasia, was observed in flounder from all treatments including control, but hepatic edema prevalence was higher in flounder exposed to oiled sediments containing $\geq$ 1 mg/kg tPAH50 (Table S3).

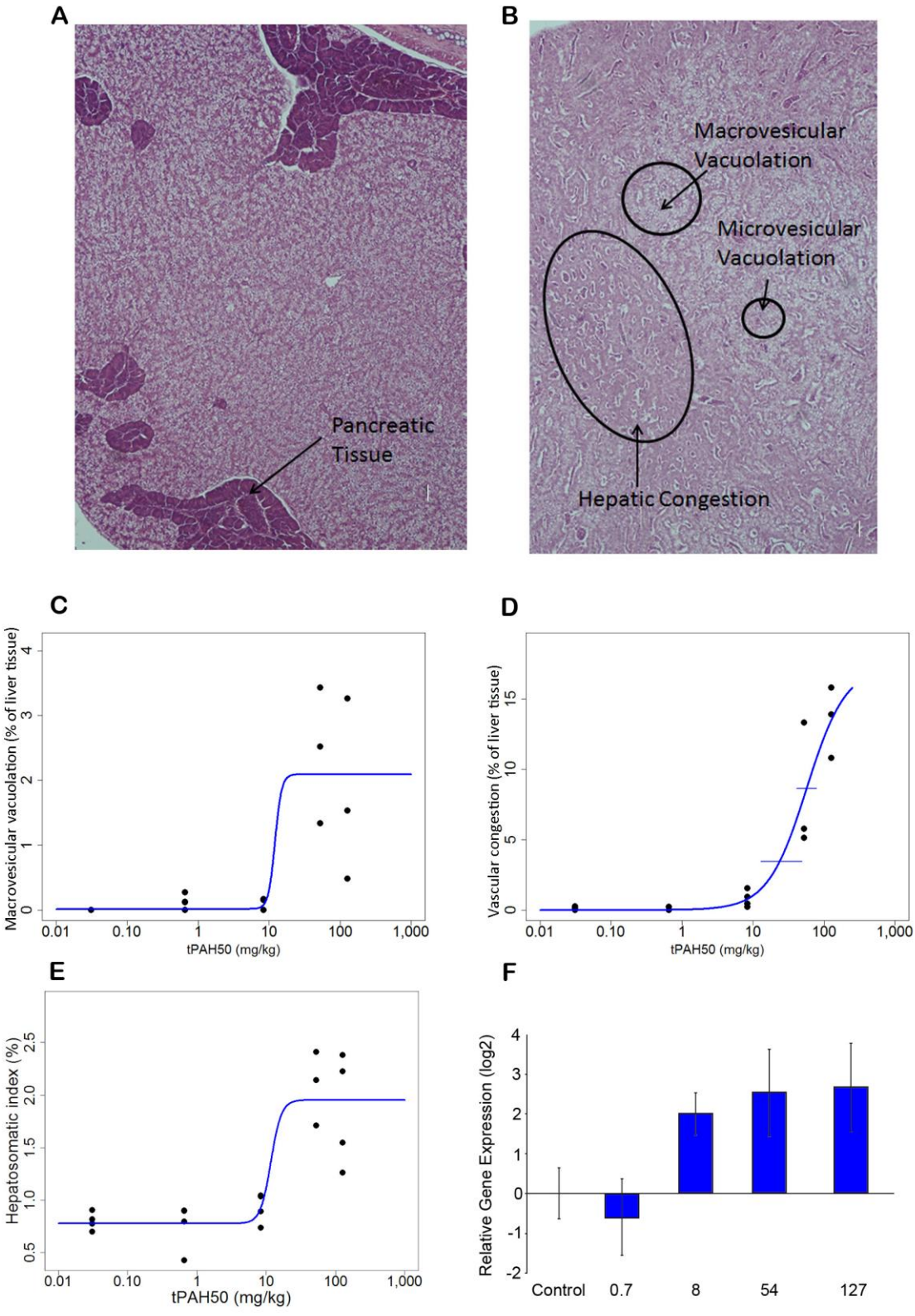

Figure 2. Effects of exposure to oiled sediment for 32 days on hepatic tissue of southern flounder. A. Histological section of normal liver tissue from control fish. Bar = 100 µm. B.

Histological section of liver tissue from flounder exposed to oiled sediment (54 mg/kg tPAH50). Bar = 100 µm. Pathological responses include macrovesicular vacuolation, microvesicular vacuolation and hepatic vascular congestion. C. Relationship between sediment tPAH50 concentration and the extent of hepatic tissue showing macrovesicular vacuolation. D. Relationship between sediment tPAH50 concentration and the extent of hepatic tissue affected by vascular congestion. Upper and lower horizontal lines indicate the 95% confidence intervals on estimated LC/EC_{20} and LC/EC_{50}, respectively. E. Relationship between sediment tPAH50 concentration and Hepatosomatic Index (HSI) of flounder. F. Relative expression of Cyp1a in liver tissues of flounder exposed to various concentrations of oiled sediments for 32 days. For all dose response curves, EC values are based on the range of response.

Overall, changes in hepatic structure were the most marked histopathologic indicators of oiled sediment exposure. Hepatocellular vacuolation was evident in fish exposed to all oiled sediment treatments but was not evident in control fish (Figure 2B). All fish exposed to oiled sediment concentrations > 8 mg/kg tPAH50 exhibited some micro- and macro-vesicular vacuolation; dose response curves show the EC_{50} for this lesion is 12.09 mg/kg tPAH50, although intermediate responses were not observed for this endpoint and thus 95% CI could not be calculated (Figure 2C). In addition, increasing tPAH50 concentration resulted in increasing occurrence of hepatic vascular congestion (EC_{20} = 23.76 mg/kg tPAH50, 95% CI = 12.85, 48.71; EC_{50} = 57.03 mg/kg tPAH50, 95% CI = 40.65, 78.58; Figure 2D). At the highest exposure concentration (127 mg/kg tPAH50), 13.04 ± 2.43% of the liver tissue in all fish exhibited hepatic vascular congestion.

Increases in hepatic congestion and macro- and micro-vesicular hepatocellular vacuolation are recognized responses to PAH-contaminated sediment exposure in benthic fishes (Cachot *et al.*, 2013; Costa *et al.*, 2009; Moles and Norcross, 1998; Myers *et al.*, 2003). Increases in both hepatic glycogen content (microvesicular vacuolation, most common in flounder exposed to 8 mg/kg tPAH50) and hepatic lipid content (macrovesicular vacuolation, most common in flounder exposed to 52 and 127 mg/kg tPAH50) are reported to be inducible by exposure to toxins in

several fish species (Wolf and Wolfe, 2005), consistent with our findings. In addition, the observed changes in liver histopathology suggest malabsorption and a negative energy balance in flounder exposed to higher oiled sediment concentrations during the 32 day experiment, as macrovesicular vacuolation is symptomatic of sick, stressed or malnourished fish (Wolf *et al.*, 2014a). Similar histopathologic changes were shown in liver of fasting European sea bass (*Dicentrarchus labrax*; (Gambardella *et al.*, 2012); upon refeeding, liver histopathology returned to normal. The reduced growth observed in flounder at concentrations >8 mg/kg tPAH50 (Figure 1C-D) was in conjunction with evidence from histopathologic examination of stomachs and intestines of reduced feeding at the higher concentrations. Food was observed to be present in 100% of the individuals exposed to <8 mg/kg tPAH50, while some fish at higher concentrations had no food in the stomach (Table S4). Furthermore, control fish and those exposed to 0.7 mg/kg tPAH50 had large amounts of food present in the intestine, while there was no food in the intestine of 90% of the flounder exposed to 54 and 127 mg/kg tPAH50. While the oiled sediments may not directly cause the observed hepatic histopathologic changes, flounder exposed to > 8 mg/kg tPAH50 appear to have decreased feeding during the course of the experiment, consistent with the histopathologic changes observed in the liver.

The Hepatosomatic Index (HSI = [LW/W]*100) values of flounders also showed a dose-response relationship to oiled sediment concentration (EC_{50} = 10.66 mg/kg tPAH50; Figure 2E), although intermediate responses were not observed for this endpoint and 95% CI could not be calculated. Elevated HSI values have been reported previously in four species of field-collected flounders exposed to petroleum or PAHs compared to control areas (Kahn, 1995; Kleinkauf *et al.*, 2004), suggesting this is a common response. Indeed, histopathologic observations in this study suggest an accumulation of glycogen and/or lipids at the highest treatment concentrations;

lipid or glycogen vacuolization has been shown to increase the size of hepatocytes (Wolf and Wolfe, 2005) which would therefore increase the HSI value.

Flounder exposed to non-oiled sediments exhibited low occurrence of gill abnormalities such as swollen or fused lamellae and epithelial proliferation on gill lamellae (Figure 3A). A much higher percentage of flounder exhibited swollen or fused gill lamellae at the highest oiled-sediment concentrations (Table S2, Figure 3B), and this lesion is significantly dose dependent (ANOVA, $F_{4,51} = 19.39$, $p < 0.001$, Figure S3A) . Similar results have been reported for six other species of juvenile and adult flatfish exposed to oiled sediments (Costa *et al.*, 2009; Kahn, 1995; Moles and Norcross, 1998). Epithelial cell proliferation of gill lamellae (Figure 3B) appears to be very sensitive to oiled sediments, with dose-response curves showing EC_{20} = 0.29 mg/kg tPAH50 (95% CI = 0.0, 7.2) and EC_{50} = 3.07 mg/kg tPAH50 (95% CI = 1.04, 8.64; Figure 3C). Similar proliferation was observed in the gills of plaice (*Pleuronectes platessa)* (Haensly *et al.*, 1982) as well as juvenile and adult winter founder (*P. americanus)* exposed to oiled sediments (Kahn, 1995). A similar increase in the percentage of secondary lamellae showing telangiectasia (Figure 3B) with exposure to oiled sediments was observed (Figure 3D), and this endpoint was also sensitive to oiled sediment concentrations (EC_{20} = 1.34 mg/kg tPAH50, 95% CI = 0.15, 6.35; EC_{50} = 6.30 mg/kg tPAH50, 95% CI = 2.35, 16.09). Telangiectasia is characterized by club shaped expansion of the secondary lamellae caused by microaneurysms in the distal gill capillaries. There was also a significant (ANOVA, $F_{4,51} = 16.56$, $p < 0.001$) difference in the rank value of telangiectasia (from 0.40 ± 0.04 to 1.40 ± 0.26; Figure S3B) as oil concentrations increased. These results indicate that not only did a greater percentage of lamellae have secondary lamellae exhibiting telangiectasia in the higher oil concentrations, but the severity of the condition also increased in the higher oil concentrations, from a mean of ~12.5% of the

secondary lamellae exhibiting telangiectasia in controls to a mean of ~37.5% of the secondary lamellae exhibiting the condition in the highest oil concentration. Euthanasia using MS222 in ice water can impact the secondary lamellae of gill tissue resulting in telangiectasia (Wolf *et al.*, 2014a; Wolf *et al.*, 2014b), and this may explain the relatively high percentage of control fish with telangiectasia in this study; however, the marked increase in the percentage of gill tissue displaying telangiectasia in the higher treatments suggests a treatment effect above background levels. Telangiectasia has also been reported in secondary lamellae of adult plaice and winter flounder exposed to oil contaminated sediments (Haensly *et al.*, 1982; Khan, 2003). Overall, the marked changes in the gills at the highest oiled sediment concentrations suggest a profound impairment of gas exchange, resulting in increased energy expenses to support oxygen demand with damaged gills (Ferguson, 2006). Since morphological change lags far behind functional impairment (Ferguson, 2006), even subtle changes in gill tissue may impact gas exchange, contributing to the increased mortality seen at the higher oiled sediment concentrations.

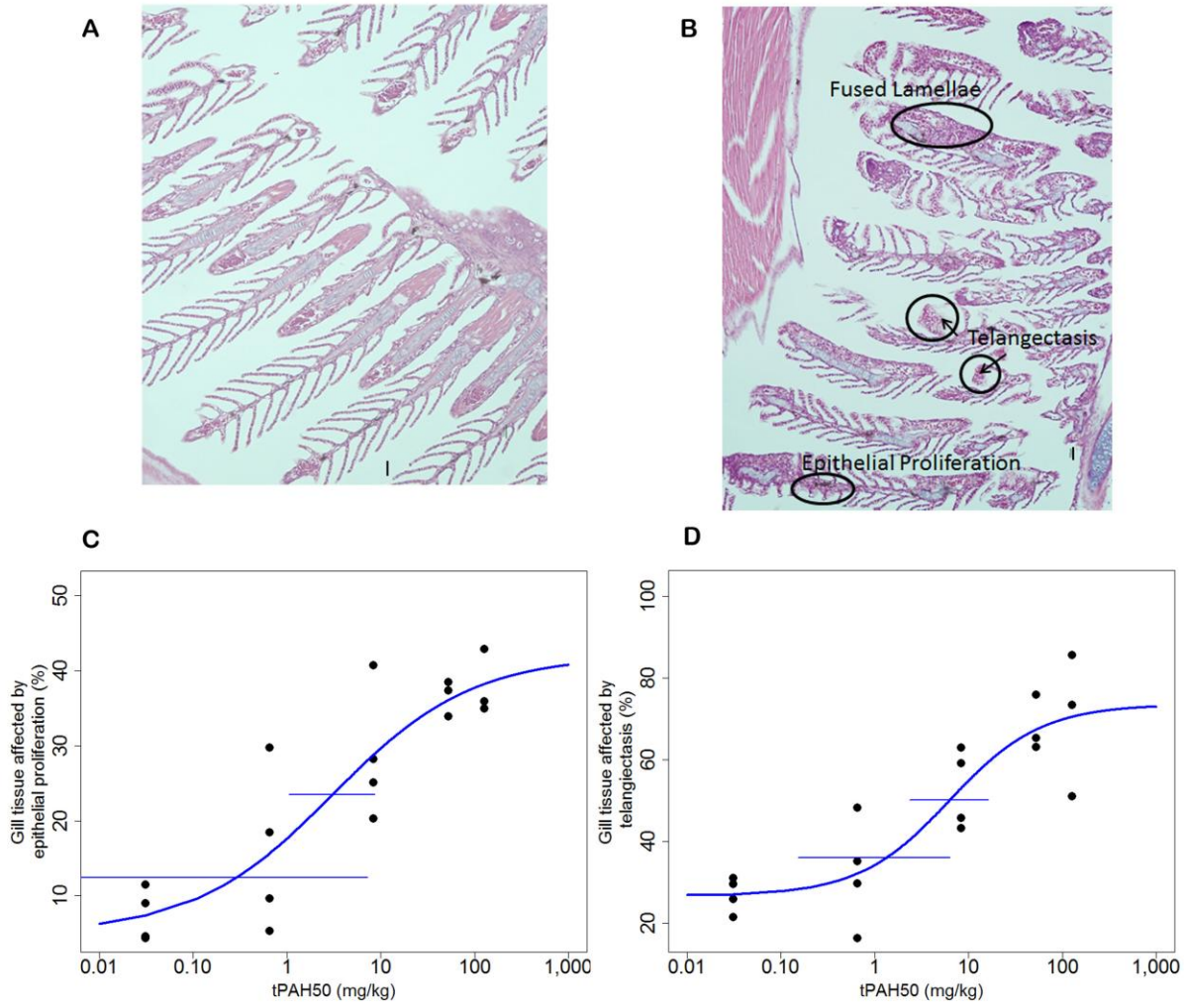

Figure 3. Histopathologic effects on southern flounder gill tissue after 32 days of exposure to oiled sediment. A. Photomicrograph of normal gill tissue from control fish. Bar = 100 µm. B. Photomicrograph of gill tissue from flounder exposed to oiled sediments (127 mg/kg tPAH50). Bar = 100 µm. Pathological responses include fused secondary lamellae, telangiectasis of secondary lamellae, and epithelial proliferation along the lamellae. C. Relationship between sediment tPAH50 concentration and the extent of gill tissue affected by epithelial proliferation D. Relationship between sediment tPAH50 concentration and the extent of gill tissue exhibiting telangiectasis. For dose-response curves, points indicate mean values per replicate tank. Lower and upper horizontal lines indicate the 95% confidence intervals on estimated EC_{20} and EC_{50}, respectively. EC values are based on the range of response.

3.4 Gene Expression

Expression of Cyp1a in hepatic tissue was dose-dependent following 32 days exposure to oiled sediments. Control flounder, as well as those exposed to 0.7 mg/kg tPAH50, showed no expression of Cyp1a. Exposure to oiled sediments > 8 mg/kg tPAH resulted in a dose-dependent increase in expression of CYP1A, with greater expression evident at higher concentrations (Figure 2F). Increased expression of Cyp1a is a common biomarker for exposure to hydrocarbons (Hylland, 2006; Sarasquete and Segner, 2000), and provides evidence that the adverse effects reported here are a function of oil exposure. Induced Cyp1a expression has also been documented in other flatfish species exposed to oiled sediments (Myers *et al.*, 2003; Roy *et al.*, 2003).

3.5 Commensal Microbial Population Analysis

In addition to the primary effects of sediment associated PAHs on flounder, we observed significant shifts in commensal assemblages of gill and intestinal bacteria in flounder exposed to oil-contaminated sediment. Distance-based redundancy (db-RDA) analysis indicated a strong and statistically significant shift in microbial communities in both organs following oil exposure (Figure 4). Oiling treatment and host tissue type were significant drivers in differentiating microbiota composition but not the interaction of tissue type and oil treatment (Permutation ANOVA on db-RDA constraints; Host tissue type: df = 2, F = 4.513, p = 0.01, Oil: df = 1, F = 3.921, p e= 0.02, Oil*x*Tissue: df = 2, F = 1.031, p =0.38).

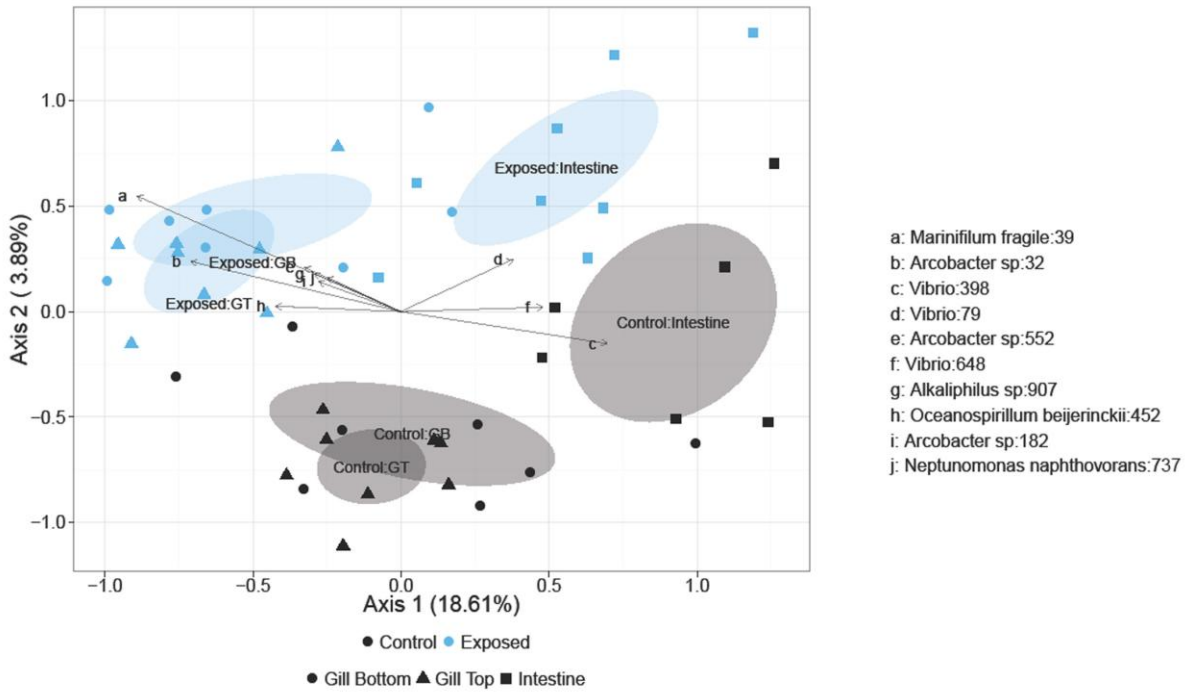

Figure 4. Principal Coordinate Analysis plot of bacterial communities in top gill (GT), bottom gill (GB) and intestinal tissues of southern flounder exposed to control and oiled sediments (54 mg tPAH50/kg). Vector length and direction are indicative of the relative contribution of bacterial species to the ordination.

Overall, *Gammaproteobacteria* (47.06 ± 8.87%), *Sphingobacteria* (9.03 ± 2.16%), *Deltaproteobacteria* (8.05 ± 2.94%), and *Epsilonproteobacteria* (9.77 ± 2.86%) increased in intestinal microbiota of host fish exposed to oil, while *Alphaproteobacteria* (13.72 ± 3.96%) and *Clostridia* (5.08 ± 1.73%) decreased. A total of 37 Operational Taxonomic Units (OTUs) had altered abundances in oil-exposed hosts. Of those 37 OTUs, 12 increased in abundance (Figure 5A). The 12 over abundant OTUs included members from *Alcanivorax*, *Arcobacter*, unidentified members of *Rhodovacteraceae*, *Pseudoaltermonas*, *Oceanospirillales*, and *Donghicola*. *Owenweeksia hongkongensis* was reduced greatly (-24.98 ± 2.875 $\log_2$ fold change).

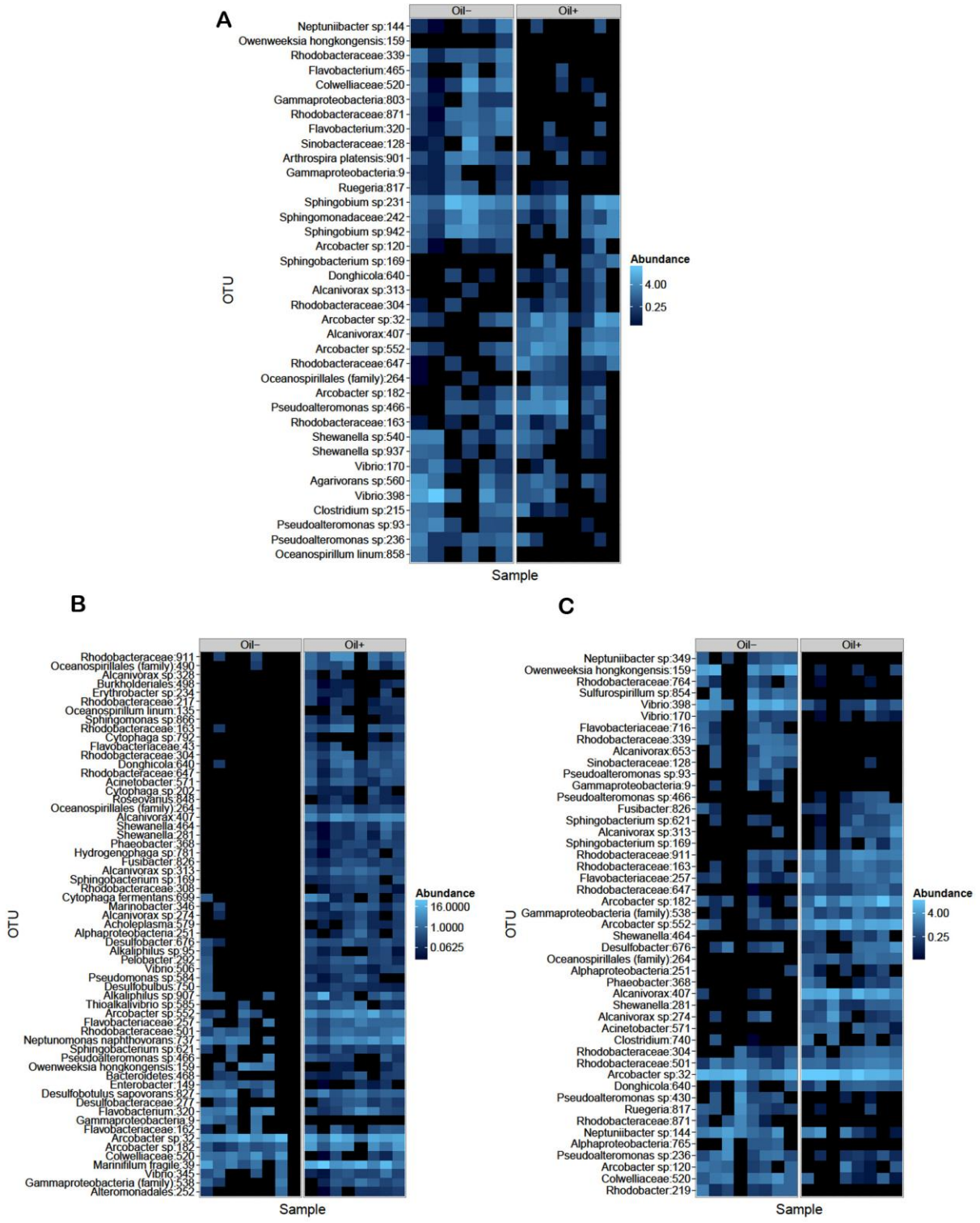

Figure 5. Microbial communities significantly affected by oil exposure. Color scale indicates Operational Taxonomic Unit (OTU) abundance. A. Intestinal OTUs affected. B. Lower gill OTUs affected. C. Upper gill OTUs affected.

Oil exposure affected the microbial assemblages on lower gills differently than upper gills. Sixty-one OTUs in lower gills changed in abundance compared to 47 in the upper gills. Both top and bottom gill microbial assemblages were more sensitive to oiling exposure than the intestinal microbial communities. In microbiota associated with lower gill tissue, only four OTUs were reduced in abundance, including *Owenweeksia hongkongensis* (-7.061 ± 2.010 $\log_2$ fold change), which also was reduced in upper gills (-12.047 ± 1.996 $\log_2$ fold change). *Alcanivorax*, *Cytophaga*, *Acinetobacter*, *Rhodobacteraceae* and *Oceanospirillum linum* were among the OTUs that had the highest $\log_2$ fold change due to oiling in lower gill microbiota (Figure 5B). OTUs that had the highest increase in $\log_2$ fold change abundance in upper gills also included *Alcanivorax*, *Acinetobacter*, *Shewanella*, *Oceanospirillales*, and *Phaeobacter* (Figure 5C).

3.6 Predictive Metagonomics Analysis

The slight differences between upper and lower gills were echoed in the predictive metagenomic analysis. Bottom gills had 95 level-3 KEGG pathways that were differentially abundant, and upper gills had 41. There were no significant changes in the abundance of level-3 KEGG pathways between control and oiled exposed gut microbiomes.

Of the 14 pathways that were overly abundant in upper gill-associated microbiota, 11 were involved in metabolism, one in environmental information processing, one in human diseases (distant enzymatic homolog), and one unclassified. Xylene, atrazine, dioxin, and bisphenol degradation had the greatest increase in $\log_2$ fold change (Figure 6A). Pathways that were reduced in abundance due to oiling also included metabolism (n = 15), environmental information processing (n = 2), human diseases (n = 3), unclassified (n = 5), and organismal systems (n = 2). Caffeine metabolism, enzymatic pathways associated with *Staphylococcus aureus* infection, and secondary bile acid biosynthesis were some of the most reduced pathways.

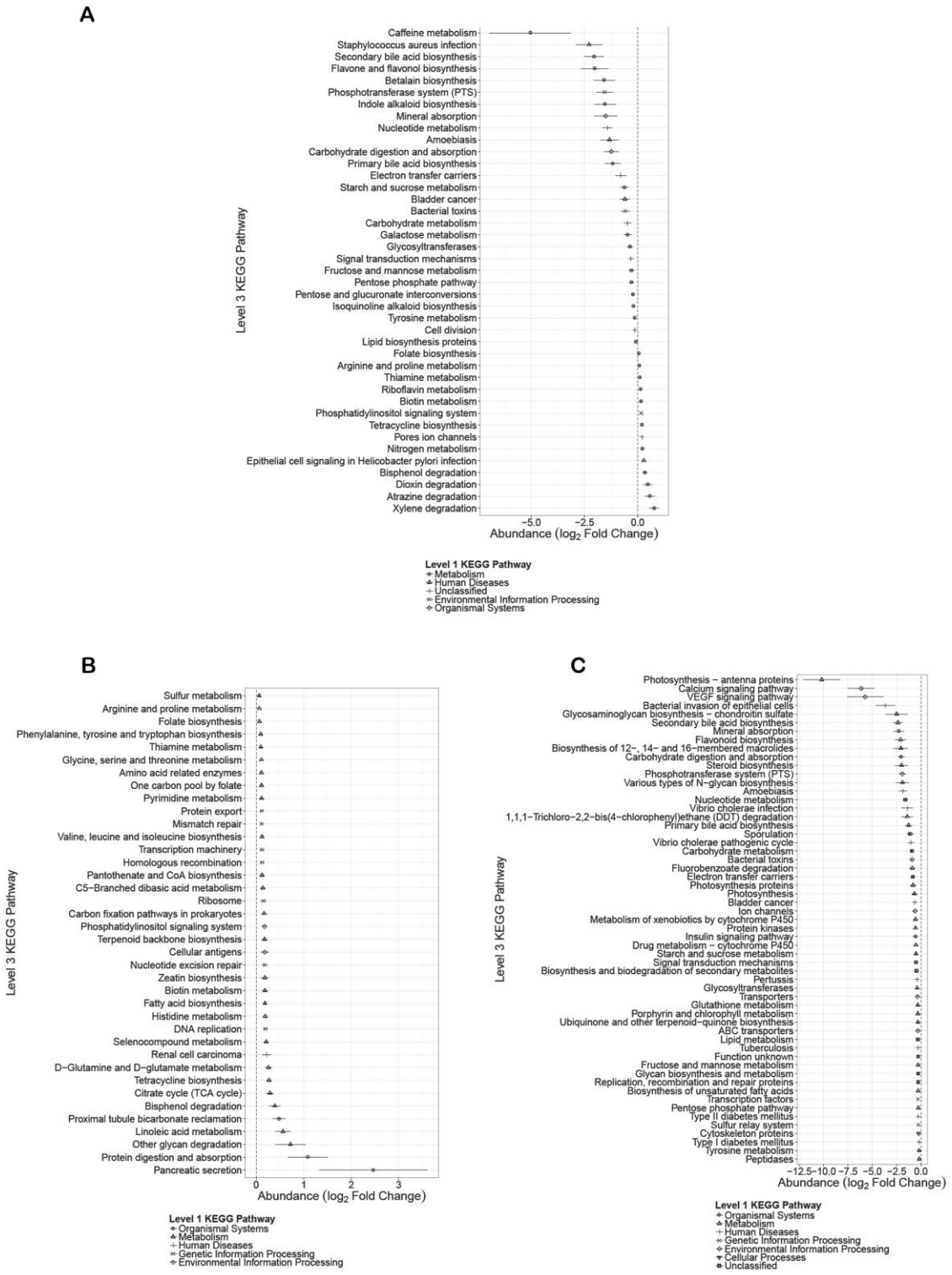

Figure 6. Predictive metagenomics analysis of the bacterial taxa affected by oil exposure. A. Pathways differentially affected by oil exposure in upper gill. B. Pathways overrepresented in lower gills exposed to oil. C. Pathways underrepresented in lower gills exposed to oil.

Of the 95 predicted differentially abundant pathways found in lower gill microbiota, 38 were overly abundant. The 38 level-1 KEGG pathways that were overly abundant were represented under metabolism (n = 25), genetic information processing (n = 7), organismal systems (n = 3), environmental information processing (n = 2) and human diseases (n = 1). Level-1 KEGG pathways that were reduced in abundance included metabolism (n = 25), unclassified (n = 10), human diseases (n = 9), environmental information processing (n = 7), organismal systems (n = 3), genetic information processing (n = 2), and cellular processes (n = 1). At level-3, the bisphenol degradation pathways again had one of the highest $\log_2$ fold increases, with distant homolog enzymatic pathways to pancreatic secretion, DNA replication, nucleotide excision repair, and transcription machinery (Figure 6B). Pathways that had the greatest reduction in $\log_2$ abundance included photosythesis, calcium signaling and vascular endothelial growth factor (VEGF) signaling pathways (Figure 6C).

Very little is known about the functions of bacteria that were affected in this study, and even less about their role in marine fish. It is therefore difficult to state the definite effects of the observed oil-induced microbiome shifts on fish health with any certainty. However, in both gills and intestines, several of the bacterial taxa that increased the most (*Alcanivorax*, *Arcobacter*, *Donghicola*, and *Acinetobacter*) have all been shown to be involved in degradation of hydrocarbons (Harayama *et al.*, 2004; Liu *et al.*, 2009; Liu and Liu, 2013; Sabirova *et al.*, 2006; Schneiker *et al.*, 2006; Tan *et al.*, 2009). This is an obvious response to the hydrocarbon-contaminated sediment exposure, and suggests that the contaminant-induced response is best viewed in terms of the host:bacteria system, rather than that of the host organism alone.

There is increasing evidence that microbial communities play an important role in mediating or influencing organism:chemical interactions. Changing the microbial community structure results

in altered acetaminophen sulfonation kinetics in Sprague-Dawley rats (Clayton *et al.*, 2009). Exposure to L-arginine has been shown to result in an increase in excretion of the gut microbial metabolites, suggesting an interaction between pancreatic function and microbiome activity in Spague-Dawley rats (Bohus *et al.*, 2008), and exposure to TNBS has been shown to result in a microbiome-dependent shift in immune activity in zebrafish (Oehlers *et al.*, 2011). Therefore, the observed oil-induced alterations in the microbial population structures of exposed flounder may produce a secondary adverse effect that is distinct from the primary effects of PAHs to the host organism itself, but also important to the health of the fish. This has important implications for aquatic toxicology, implying that examining the response of the fish alone may lead to an incomplete understanding of the effects of contaminants on aquatic organisms.

3.7 Conclusion

These results from a 32 day chronic exposure of juvenile southern flounder to environmentally relevant concentrations of sediment contaminated with naturally weathered oil show that oil-contaminated sediment is capable of causing severe adverse effects across multiple biological endpoints. The combination of increased mortality, decreased growth, histopathologic gill and liver changes indicating negative energy balance, and altered gill and intestinal microbiome structures with increasing PAH concentration in the sediment suggests system-wide impairment of important biological processes and alteration of energy budgets. Upregulation of CYP1A gene expression provides strong evidence that the oil was responsible for the observed effects. These data suggest that oil-contaminated sediments in the northern GOM are capable of causing adverse effects to exposed species.

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Supplemental Figures and Tables.

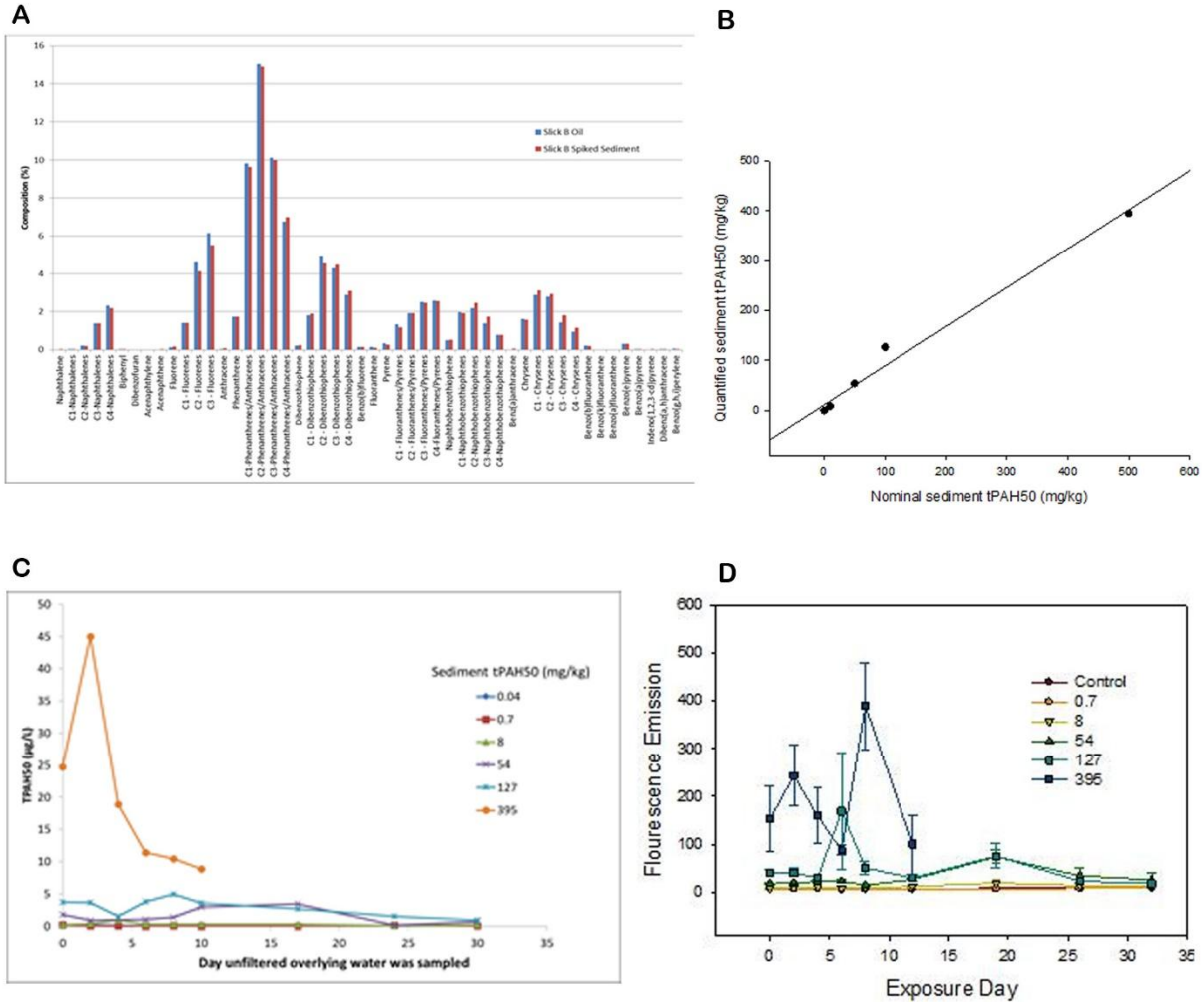

Figure S1. Quantification of oiled sediments and overlying water. A. Composition of 50 measured PAH analytes in Slick B weathered oil and uncontaminated sediment spiked with Slick B weathered oil. B. Relationship between the amount of Slick B oil added to sediments (nominal sediment tPAH50) and quantified concentration C. Concentration (µg/L) of tPAH50 in unfiltered water overlying oil-spiked sediments during the 32-day study. Values represent a composite of replicate aquaria for each treatment. Water samples not collected from the 395 mg/kg sediment treatment after day 11 due to 100% mortality in that treatment. D. Mean (±SE) fluorescence values of unfiltered water overlying sediments demonstrating variability among replicate aquaria.

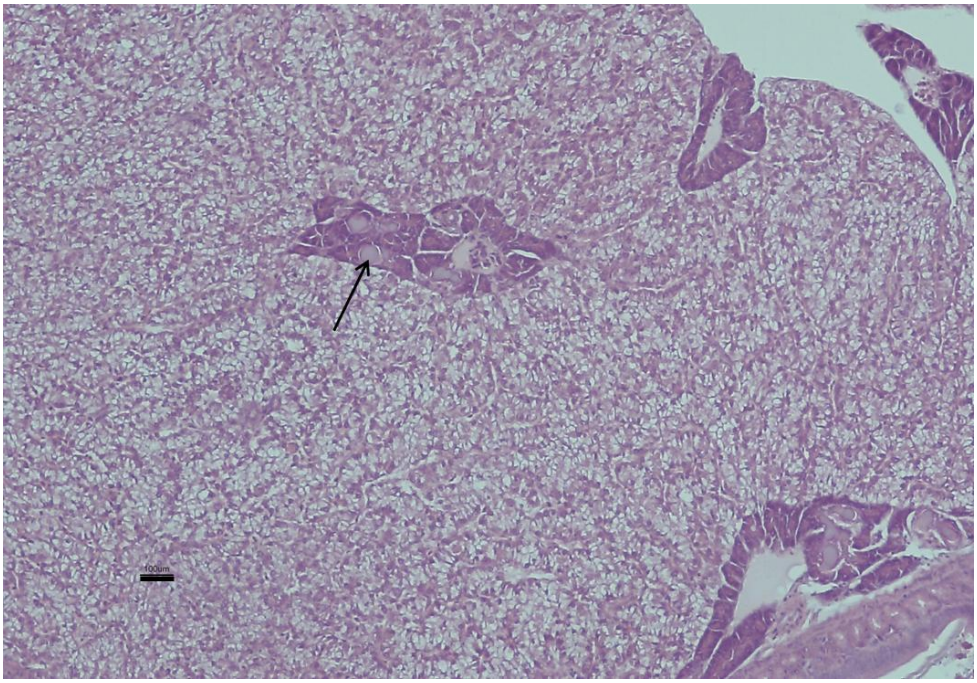

Figure S2. Histological section of liver tissue from flounder exposed to oiled sediment (127 mg/kg tPAH50). Bar = 100 µm. Arrow indicates large eosinophilic inclusions in the duct lumens/cells.

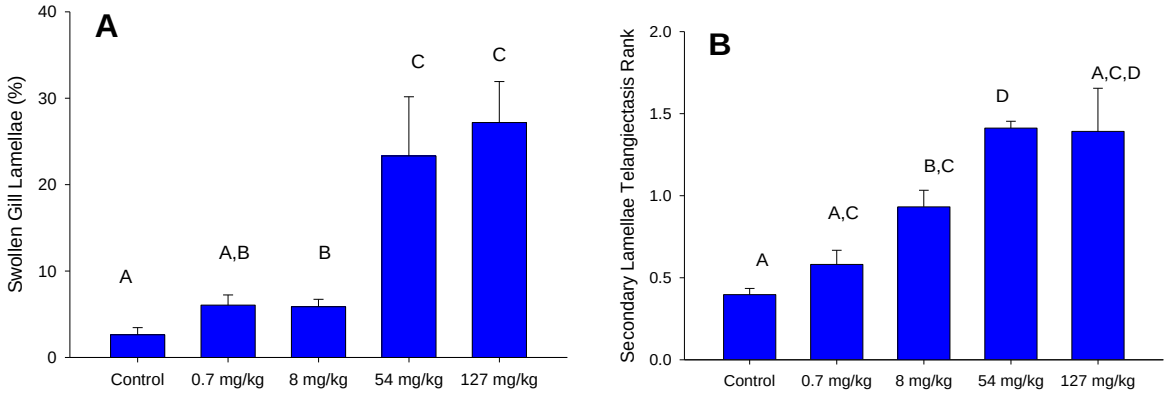

Figure S3. Histopathologic effects on southern flounder gill tissue after 32 days of exposure to oiled sediment. A. Mean (± S.E.) percentage of fused gill lamellae observed in five oiled sediment treatments. B. Rank values of secondary lamellae exhibiting telangiectasis in five oiled sediment treatments. Letters indicate significant differences among treatments (ANOVA, Bonferroni post-hoc test).

Table S1. Amount of sediment, loading rate of Slick B oil, and initial and final concentrations of treatments. Initial measurements taken at experiment initiation, final measurements taken after 32-d of flow-through clean water at experiment termination.

Treatment	Sediment Mass (kg)	Nominal loading concentration (g/kg)	Measured concentration at initiation (mg/kg tPAH50)	Measured concentration at termination (mg/kg tPAH50)
1	3.207	0	0.031	0.023
2	3.221	0.499	0.656	0.573
3	3.702	4.51	8.4	6.36
4	3.216	25.1	53.9	50.09
5	3.202	56.2	126.6	188.7
6	3.204	270.2	394.9	425.2

Table S2. Grain size characteristics of the sediment used in this experiment. Grain size was analyzed from a composite sample taken from the field-collected sediment prior to spiking with oil, and therefore is representative of all treatments.

Grain Type	Grain Size (mm)	Composition (%)
Gravel	>2.00	0.04
Sand, very coarse	1.00 – 2.00	0.06
Sand, coarse	0.50 – 1.00	0.25
Sand, medium	0.250 – 0.0500	10.29
Sand, fine	0.125 – 0.250	44.51
Sand, very fine	0.0625 – 0.125	15.04
Silt	0.0039 – 0.0625	19.63
Clay	<0.0039	6.97

Table S3. Summary of consensus opinion of histological observations on four tissues of juvenile southern flounder exposed to oiled sediments for 32 days. Score is ranked as 0 (condition not present) and 1 (condition present). N indicates total number of specimens containing each tissue type on the slide; score numbers indicate total number of specimens with that score for each histological observation at each treatment concentration. Values in parentheses indicate the percentage of fish exhibiting each condition per treatment.

		Concentration of oil in sediment (mg/kg tPAH50)									
Histological Observation	N	Control		0.7		8		54		127	
Score		0	1	0	1	0	1	0	1	0	1
Kidney											
Tubular Proliferation	49	12(100)	0(0)	15(100)	0(0)	11(85)	2(15)	4 (100)	0(0)	5(100)	0(0)
Granuloma	47	12(100)	0(0)	10(77)	3(23)	12(92)	1(8)	3(75)	1(25)	3(60)	2(40)
Heart											
Mycardial Trabiculae Atrophy	41	11(100)	0(0)	11(92)	1(8)	9(90)	1(10)	3(75)	1(25)	4(100)	0(0)
Pooled Plasma	41	11(100)	0(0)	10(83)	2(17)	7(70)	3(30)	3(75)	1(25)	3(75)	1(25)
Liver											
Macro/micro vesicular vacoulation	57	16 (100)	0(0)	12 (75)	4 (25)	7 (47)	8 (53)	0 (0)	5 (100)	0 (0)	5 (100)
Congestion	57	15(94)	1(6)	15 (94)	1 (6)	12 (80)	3 (20)	0 (0)	5 (100)	0 (0)	5 (100)
Edema	57	12 (75)	4 (25)	10 (63)	6(38)	5 (33)	10 (67)	0 (0)	5 (100)	1 (20)	4 (80)
Eosinophilic Inclusions in Exocrine Pancreas	57	16(100)	0 (0)	16(100)	0(0)	11(73)	4(27)	5 (100)	0(0)	2(40)	3(60)
Reduced Amount of Exocrine Pancreas	57	16(100)	0(0)	16(100)	0(0)	15(100)	0(0)	0(0)	5(100)	1(20)	4(80)
Gill											
Telangiectasis in Secondary Lamellae	57	9(56)	7(44)	8(50)	8(50)	3(20)	12 (80)	0(0)	5 (100)	0(0)	5(100)
Epithelial Proliferation	57	15(94)	1 (6)	13(81)	3(19)	9(60)	6(40)	0(0)	5 (100)	0 (0)	5 (100)
Swollen/fused Lamellae	57	16(100)	0(0)	16(100)	0(0)	15(100)	0(0)	0(0)	5(100)	1(20)	4(80)

Table S4. Presence of food in stomachs and intestines of juvenile southern flounder exposed to oiled sediments for 32 days. Data expressed as percentage of total number of flounder in each treatment

Treatment (mg/kg tPAH50)	N	Stomach			Intestine	
		Full	Part full	Empty	Food present	Empty
Control	16	100	0	0	100	0
0.7	15	100	0	0	100	0
8	15	93	0	7	100	0
54	5	0	20	80	0	100
127	5	60	0	40	20	80