Genomic Expression Response to Experimentally-Weathered South Louisiana Crude Oil in Gulf Killifish Profiled Across Tissues, Doses and Time.

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GENOMIC EXPRESSION RESPONSE TO EXPERIMENTALLY-WEATHERED SOUTH LOUISIANA CRUDE OIL IN GULF KILLIFISH PROFILED ACROSS TISSUES, DOSES AND TIME

A Thesis
Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science

in

The Department of Biological Sciences

By Whitney Rose Pilcher
B.S., Louisiana State University, 2009
December 2012
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I thank Reid Brennan, Jeffrey Miller, Eve McCulloch and David Roberts for all their help with strange requests required to complete my project, spending hours collecting data in the lab alongside me and providing words of wisdom. They continue to be valuable although we have all gone separate ways. For their encouragement through my academic journey, I would like to thank my parents, Darin and Kimberly Pilcher. Lastly, I would like to thank my best friend, Hilary Glenn for helping me through this roller coaster by staying focused and supportive.

For the research reported here, Whitney Pilcher (WP) and Andrew Whitehead (AW) created the experimental design. WP collected fish, weathered oil, performed the pilot and definitive weathering studies, performed range finding and definitive exposure experiments, dissected tissues and blood, and collected WAF samples for chemical analysis. M. Scott Miles performed water chemistry analysis, provided text for methods, and generated Table 4 and Table 5. Greg Mayer’s lab collected the DNA strand break data. Dr. Mayer’s student Song Tang performed statistical analysis of DNA strand break data provided text for materials and methods, and generated Fig. 3. WP collected the genome expression profiling data. WP and AW analyzed the genome expression data. AW performed the network analysis. WP wrote this document with editorial contributions from AW. Committee members M. Hellberg, F. Galvez, and V. Wilson provided editorial suggestions for mature versions of this document.
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Abstract

Almost five million barrels of south Louisiana crude oil were released into the environment following the explosion of the Deepwater Horizon oil platform on April 20, 2010, however, little is known of the effects of the spill to native species of the northern Gulf of Mexico. Field studies provide a context for ecological realism but laboratory-based studies can connect the specific cause to a specific biological response. A previous field study, completed in 2010, tracked genome expression responses in native killifish resident in oiled and several non-oiled marshes in the northern Gulf of Mexico. Those data suggested significant biological effects from contaminating oil. As a post hoc companion to field studies, we sought to determine the genome expression response of the same native killifish to oil under controlled laboratory conditions to directly link a causal agent to a specific biological response. A concentration response experiment was conducted using experimentally-weathered surrogate oil to further characterize the genome transcriptional response, and to test for additional impacts on health. Transcriptome-wide gene expression responses were determined from the gill and liver tissues of fish exposed to experimentally weathered surrogate oil across a range of concentrations and throughout a time-course of exposure. Transcriptional responses to oil in the laboratory were predictive of the transcriptional response observed in the field study that coincided with the timing and location of oil contamination. These responses included increased expression in the genes activated by the aryl hydrocarbon receptor signaling pathway, including phase 1 and phase 2 metabolism genes, among others. Additionally, network and pathway analyses implicated the effects of transcription, centrosome, cell cycle progression, RNA processing, DNA damage, and apoptosis on gene regulation. These genome expression profiles offer additional scope for interpreting genome expression responses observed in the field, and offer additional insight into consequences of oil exposures in this important native Gulf of Mexico coast species.
Introduction

Following an environmental disaster, field studies are useful for determining an immediate biological effect in the natural habitat, but integration with laboratory studies adds scope for inferring the cause and effect relationship between contamination and the corresponding biological response. On April 20, 2010, the Deepwater Horizon (DWH) oil platform exploded and initiated the largest marine oil spill in history. South Louisiana crude oil was released into the Gulf of Mexico from the deep sea Macondo well and began to weather and contaminate marshlands in the northern Gulf of Mexico (primarily Louisiana)(http://gomex.erna.noaa.gov). Several field studies to date have investigated the direct impacts of crude oil on native wildlife species and the immediate impacts that followed the disaster [2-4]. While field studies are important for characterizing biological responses in nature, they share a common challenge of determining a direct cause and effect relationship from the oil exposure.

Field studies offer insights about risk to resident species within an ecologically realistic context. A general challenge with field studies is lack of power to directly link a causal agent to a specific biological response. This is often because temporal and spatial variation of ecological factors, such as hypoxia, salinity variation, temperature variation, and community interactions, can complicate interpretation of the cause-effect relationships [5]. In contrast, laboratory studies have greater power to determine cause and effect relationships through careful control of experimental, environmental, and biological variables [6]. However, laboratory-based studies lack ecological realism for various reasons sometimes including oversimplified exposure scenarios and mismatch between focal species and species at ecological risk [6, 7]. Strategically designed and integrated laboratory and field studies can improve environmental risk assessment [8], since complimentary data from both the field and the laboratory will strengthen causal relationships while linking important effects to those observed in the field [9]. A carefully chosen model organism is required to collect data for both a field and laboratory study. The best model species for integrative laboratory-field studies would be one that is easily manipulated in the laboratory setting and easily collected in the field, is resident and abundant in at-risk habitats, non-migratory with high site-fidelity, and sensitive to the stressor of concern.

Gulf killifish (*Fundulus grandis*) are a good model species for estimating ecological impacts of the DWH oil spill because they are the most abundant vertebrate in at-risk marsh habitats in the northern Gulf of Mexico. Much ecological and physiological data are available for *Fundulus heteroclitus*, the closely-related Atlantic-distributed sister species to *F. grandis*. Data indicate that *F. heteroclitus* have high site fidelity, are an ecologically important member of the marsh community [10-14] and are sensitive to organic pollutants in comparison to other fish species [15]. Because of their very recently shared evolutionary ancestry [16] and similar ecological niches in the Gulf of Mexico, we infer that these same traits are shared by *F. grandis*. For these reasons, *F. grandis* were used in a field study to determine the immediate effects of the oil spill on health and physiology [1]. Prior to the arrival of contaminating oil adult male *F. grandis* were collected at six sites across the northern Gulf of Mexico (Fig.1). One site was located in
Louisiana (Grand Terre [GT]) two sites were in Mississippi (Bay St. Louis [BSL] and Belle Fontaine Point [BFP]) and three Alabama sites (Bayou La Batre [BLB], Mobile Bay [MB] and Fort Morgan [FMA]). A second sample was taken at all sites captured the peak of oiling that was observed only at the GT site [1] and a final sampling was collected two months after the exposure sampling. Fish from the GT site showed divergent genome expression through time, in comparison to the other non-oiled field sites, and this divergent expression coincided with the timing and location of oil contamination. Genome expression profiles from liver tissues were diagnostic of exposure to the toxic components of oil, and reflected the types of responses that are expected to precede long-term population-level effects [1, 17].

**Figure 1:** Location of the field study sampling sites from the experiments reported in Whitehead et. al, [1]. Sites include, Grand Terre (GT), Bay St. Louis (BSL), Belle Fontaine Point (BFP), Bayou La Batre (BLB), Mobile Bay (MB) and Fort Morgan (FMA).

The controlled laboratory exposure study reported here seeks to further characterize the biological response of *F. grandis* to weathered oil and contribute to further the interpretation of the biological responses observed in the field. South Louisiana crude oil, used as a surrogate for the Macando-252 well crude oil, was experimentally weathered to create a water-accommodated fraction (WAF). Fish were exposed to a range of sub-lethal dilutions of WAF, and were sampled three times following exposure. Gill and liver tissues were preserved for genome expression profiling, and blood samples were preserved for DNA strand break assays. Our data show that exposures to the WAF resulted in direct damage to DNA molecules. A low-concentration genome expression response was distinguishable from a high-concentration
response. Genome expression in the lab, and in particular the high-concentration response, was predictive of divergent genome expression associated with the timing and location of oil contamination in the field.
Materials and Methods

Fish Collection

Gulf Killifish (Fundulus grandis) were collected using minnow traps in Chauvin, Louisiana (29.360016 N, 90.625952 W), which was not impacted by DWH oil. Killifish collected from Chauvin, Louisiana, should be genetically similar to killifish from other Louisiana coastal marshes given patterns of genetic divergence across the northern Gulf of Mexico [18]. Adult males (5-8 cm in length) were collected and brought back to Louisiana State University’s Biological Sciences Aquatics Facility (Baton Rouge, LA). Fish were copper treated for parasite removal (0.75 ml Cuppermine per 39.7 L) for two weeks and were in a recirculating system at 24°C for three weeks prior to experimentation. Water salinity was kept at 10 ppt (reverse osmosis water mixed with Instant Ocean® Sea Salt), similar to the salinity at the field sites during the field study [1]. This clean brackish water source (10ppt artificial brackish water) was used for all experiments. Water concentrations for ammonia, nitrate, and nitrate were determined bi-weekly using API test kits. Nitrogenous wastes were undetectable during the acclimation period and dissolved oxygen levels were between 5-8 mg/L. Temperatures were kept between 22-24°C and lighting was kept on a 12 hours light, 12 hours dark cycle.

Range-Finding Experiment

Prior to experimental manipulations, a pilot study was performed to experimentally weather surrogate crude oil to create a water-accommodated fraction (WAF) used to determine the highest sub-lethal concentration for the definitive experimental exposures. Surrogate crude oil was weathered by a ratio of 1:10 of oil to clean brackish water [19, 20]. Three gallons (11.4 L), of south Louisiana crude oil supplied by Plane’s Marketing (Lafayette, Louisiana) (obtained in April, 2011 from an onshore oil platform in Southern Louisiana), was added to 30 gallons (113.5 L) of clean brackish water in a 400 gallon (1514 L) fiberglass tank and mixed twice daily for five minutes each time to increase emulsification. Between June 8th and July 8th, 2011, the 400 gallon tank was exposed to outside elements, including temperature and rain, and was always covered with a mosquito net (Table 1). To prevent sample dilution, a canopy tent was used to cover the tank during rain.

After 30 days of experimental weathering, the water fraction (hereafter referred to as the water accommodated fraction: WAF) was separated from the floating oil and a series of WAF dilutions was used at different concentrations in sublethal tests in F. grandis. The highest sub-lethal concentration was determined by exposing animals to four different dilutions of WAF to determine a concentration that fish could tolerate for 7 days without any mortality. The four dilutions tested were: 1) 100% WAF dilution (100% WAF + 0% clean brackish water), 2) 75% WAF dilution (75% WAF + 25% clean brackish water), 3) 50% WAF dilution (50% WAF + 50% clean brackish water), and 4) 25% WAF dilution (25% WAF + 75% clean brackish water). Fifty percent water changes and water quality measurements were performed daily. The only dilution that had complete survival during the 7 day exposure was from the 25% WAF dilution. This concentration was designated the highest sub-lethal concentration (Fig. 2) and used as the highest concentration for definitive exposure experiments (see below section).
**Table 1:** Temperatures and cloud coverage were noted during the duration of weathering pilot study crude oil (June 8-July 8, 2011). Samples were taken every two days, weather permitting.

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<th>Date</th>
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<th>Notes</th>
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</tr>
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</tr>
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</tr>
<tr>
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<td>No cloud coverage</td>
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<tr>
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Definitive Exposure Experiment

Following the pilot study, a second batch of surrogate South Louisiana crude oil was obtained from a chemical company, Nalco (Naperville, IL), which was collected from a deep-sea pipeline in the Northern Gulf of Mexico. This surrogate crude oil was used to mimic the weathered oil from the Macando well and to generate enough WAF for definitive fish exposure experiments. Similar to the pilot experiment, this oil was mixed with clean brackish water for 40 days. Using a 1:10 ratio of oil to water, 20 gallons (76 L) of south Louisiana crude oil was mixed with 200 gallons (757 L) of clean water in a 400-gallon (1514 L) fiberglass tank. The oil-water mixture was mixed three times daily for 10 minutes each to introduce chemicals from the crude oil into the water column to create the WAF. The weathering tank was kept outside and exposed to ambient sun and wind to mimic the weathering of spilled Deepwater Horizon oil in the field (Table 2). Once the original experimental water was removed from the tank, another 200 gallons (760 L) of clean brackish water was added to simulate a tidal exchange where contaminated waters were replaced with clean water. For two days, this fresh addition of clean brackish water was mixed with the remaining overlying weathered oil and a new WAF was drawn off from the tank (hereafter referred to as the “tidal” treatment).

Figure 2: Survivorship of *Fundulus grandis* after 7-day exposure range-finding experiment. Four different dilutions of WAF were tested to determine the highest sub-lethal concentration that fish: 100% WAF, 75% WAF, 50% WAF and 25%. The 25% dilution was the highest concentration that had no mortality for the seven-day duration of exposure.
Table 2: Weathering of crude oil during the definitive weathering experiment. Date, temperature, wind, humidity, UV Index and cloud coverage were noted. Samples were taken every two days weather permitting. Days where rain was observed is noted and the tank was covered with a tarp. *TS Lee is Tropical Storm Lee

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<th>UV Index</th>
<th>Cloud Cover</th>
<th>Rain?</th>
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<td>14%</td>
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<tr>
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<tr>
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<td>12.8 NE</td>
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Table 2 cont.

<table>
<thead>
<tr>
<th>Day #</th>
<th>Date</th>
<th>Temp (°C)</th>
<th>Wind (km/h)</th>
<th>Humidity</th>
<th>UV Index</th>
<th>Cloud Cover</th>
<th>Rain?</th>
</tr>
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<tbody>
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<td>9/15/11</td>
<td>30.6</td>
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<tr>
<td>34</td>
<td>9/16/11</td>
<td>28.3</td>
<td>11.2 NNE</td>
<td>39%</td>
<td>4</td>
<td>46%</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>9/17/11</td>
<td>29.4</td>
<td>11.2 ENE</td>
<td>48%</td>
<td>6</td>
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<tr>
<td>36</td>
<td>9/18/11</td>
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<td>11.2 ESE</td>
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<td>5</td>
<td>60%</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>9/19/11</td>
<td>32.2</td>
<td>12.8 SW</td>
<td>57%</td>
<td>5</td>
<td>58%</td>
<td>Rained</td>
</tr>
<tr>
<td>38</td>
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<td>5</td>
<td>62%</td>
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</tr>
<tr>
<td>39</td>
<td>9/21/11</td>
<td>28.9</td>
<td>6.4 NE</td>
<td>58%</td>
<td>0</td>
<td>46%</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>9/22/11</td>
<td>32.8</td>
<td>6.4 NNE</td>
<td>51%</td>
<td>4</td>
<td>79%</td>
<td>Remove WAF</td>
</tr>
<tr>
<td>47</td>
<td>9/28/11</td>
<td>33.9</td>
<td>8.0 W</td>
<td>43%</td>
<td>5</td>
<td>26%</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>9/29/11</td>
<td>33.9</td>
<td>8.0 W</td>
<td>43%</td>
<td>5</td>
<td>26%</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>9/30/11</td>
<td>28.9</td>
<td>16.0 NE</td>
<td>38%</td>
<td>5</td>
<td>2%</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>10/1/11</td>
<td>26.1</td>
<td>12.8 NE</td>
<td>28%</td>
<td>7</td>
<td>4%</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>10/2/11</td>
<td>23.9</td>
<td>12.8 ENE</td>
<td>36%</td>
<td>5</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>10/3/11</td>
<td>27.8</td>
<td>9.6 NE</td>
<td>27%</td>
<td>5</td>
<td>11%</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>10/4/11</td>
<td>28.3</td>
<td>9.6 ENE</td>
<td>22%</td>
<td>5</td>
<td>11%</td>
<td>Removed Tidal</td>
</tr>
</tbody>
</table>

The definitive exposure experiment consisted of three WAF dilutions: a high concentration (25% WAF), a low concentration, (2.5%), a tidal treatment (no dilution), and a control treatment consisting of clean brackish water. Water changes (50%) and water ammonia, nitrate, and nitrate concentrations were measured daily. Ammonia concentration was always below 0.50 ppm in all treatments, and nitrate and nitrate concentrations were always below detection limits of the assay kit (Table 3). Fish were sampled after 1 day, 3 days, and 7 days of exposure, and at each time, liver, gill and blood were sampled from six adult male fish per treatment. Liver is important in toxicology studies because it is the main tissue for xenobiotic metabolism in fish. Gills were also sampled because they have a large surface area in direct contact with the external aquatic environment. Liver and gill samples were preserved in RNAlater (Ambion, Inc.) and kept at 4 °C overnight before being transferred to a -20 °C freezer. Blood samples, for DNA strand break analysis, were drawn by capillary tubes from the caudal vein and ejected into a cryovial containing DMSO and RPMI 1640. Samples were then frozen in ethanol baths at -80 °C overnight before storing at -80 °C without an ethanol bath. Fish handling and dissections were in accordance with an Institutional Animal Care and Use Protocol approved by Louisiana State University (protocol # 10-066).
Analytical Chemistry

Definitive exposure experiment water samples from each WAF preparation (4 L) were collected in borosilicate glass amber bottles with Teflon-lined caps to characterize the oil-associated chemicals in the WAF. Analytical chemistry was performed at the Louisiana State University Department of Environmental Sciences. Organics were solvent-extracted from 4 liters of water sample per treatment using DCM, and concentrated down to 1 ml final volume using a nitrogen blow-down system. All sample extracts were analyzed using an Agilent 7890A Gas Chromatography (GC) system configured with a 5% diphenyl/95% dimethyl polysiloxane high resolution capillary column (30 meter, 0.25 mm ID, 0.25 micron film), an Agilent 7638B series Auto Injector, and directly interfaced to an Agilent 5975 inert XL MS detector system. The GC was operated in the temperature program mode with an initial column temperature of 55°C for 3 minutes then increased to 280°C at a rate of 5°C/minute and held for 3 minutes. The oven was then heated from 280°C to 300°C at a rate of 1.5°C/min and held at 300°C for two minutes. Total run time was 66.33 minutes per sample. The interface to the MS was maintained at 280°C. Ultra high purity (UHP) helium was the carry gas for the GC/MS system. Spectral data were processed by Chemstation™. Analyte concentrations were calculated based on the internal standard method. An internal standard mixture composed of naphthalene-d8, acenaphthene-d10, chrysene-dl2, and perylene-dl2 was spiked into the sample extracts just prior to analysis. The concentration of specific target oil analytes was determined by a 5-point calibration curve and an internal standard method. Standards containing parent (non-alkylated) hydrocarbons were used in the calibration curve.

DNA Damage

DNA damages after exposure were evaluated by the single cell gel electrophoresis assay (Trevigen’s CometAssay) [21]. Cells from collected whole blood were suspended at 2.5×10^5 per ml (counted by Beckman Coulter Z2) in PBS and combined with molten LMAgarose at a ratio of 1:10 (v/v). 50µL of this mixture was then spread onto a CometSlide (Trevigen). Slides were solidified at 4°C in the dark for 30 minutes and then lysed by immersion in pre-chilled lysis solution containing additional 10% DMSO at 4°C. After 1 hour, the slides were immersed in digestion solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10) with 1 mg/ml proteinase-K (Roche) for 2 hours at 37°C. Slides were washed by immersing into 50 mL pre-chilled 1X neutral electrophoresis buffer for 30 minutes at 4°C. Next, slides were placed in the CometAssay ES tank containing 1000 mL pre-chilled 1X neutral electrophoresis buffer, and electrophoresis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>25% WAF</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>2.5% WAF</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Tidal WAF</td>
<td>0.25</td>
<td>0.5</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Gene ontology enrichment analysis was performed using DAVID Bioinformatics. Significant and within ("analyzed using mixed model analysis of variance then quantile normalized normalized background intensity) and excluded from the n were too bright (maximum intensity) or too faint (below 2 standard deviations above captured Bioscience ScanArray Express scanner (PerkinElmer, Inc)). Each F. grandis sample was loaded on two CometSlide wells and a total of 100 randomly selected cells (50 cells from each of two replicate wells) scored from each individual. To evaluate the amount of DNA damage, the tail moments (tail moment = tail DNA% * length of tail) generated by CometScore from replicate F. grandis were analyzed [22]. Statistically significant differences between the control and the exposure groups were analyzed by one-way ANOVA (df=5, Graphpad Prism). When statistically significant differences were found, Tukey’s HSD post-hoc test was applied to determine which treatments were significantly different from control. Results were considered significant at P value less than 0.05.

**Genome Expression**

Genome expression profiling offers a top-down approach to understanding the mechanisms that animals utilize to respond to environmental stressors. Genome expression responses were measured using custom oligonucleotide microarrays (Agilent eArray Design ID 027999). This same microarray design and platform was used in killifish PCB exposure experiments [23] and field DWH oil exposure experiments [1], such that data from these studies are directly comparable. Liver and gill samples were used from five replicate individuals for each concentration-day treatment. Adult male fish were used to measure genome expression responses to varying dilutions of WAF. The genome response was determined in both livers and gills that were sampled at the control, low concentration (2.5% WAF), high concentration (25% WAF) and tidal treatments. Total RNA was extracted from gill and liver tissues using TRIzol (Invitrogen, Inc.) and further purified using Qiagen RNeasy kits. Antisense RNA (aRNA) was made using the amino allyl aRNA amplification kit (Ambion, Inc.) and purified aRNA was fluorescently labeled using Alexa Flour dyes (Alexa Flour 555, Alexa Flour 647, Invitrogen, Inc). Dye coupled samples were then hybridized to the microarrays for 18 hours at 60°C. These microarrays contain 6,800 unique EST sequences that are printed in duplicate on a 15,000-probe 8-plex microarray slide. Samples were paired together in a balanced loop design, including a dye swap, and then microarray slides were scanned by a Packard Bioscience ScanArray Express scanner (PerkinElmer, Inc) and spot intensities were captured using Imagene software (Biodiscovery, Inc). Spots were screened for those that were too bright (maximum intensity) or too faint (below 2 standard deviations above background intensity) and excluded from the normalization. Data were lowess-normalized, then mixed-model normalized with fixed (dye) and random (array) effects, then quantile normalized in JMP Genomics (SAS, Inc.). The normalized data were then analyzed using mixed model analysis of variance (in JMP Genomics) with “concentration” and “day” specified as main effects including an interaction term (concentration-by-day). In this model, “dye” was considered a fixed effect and “array” and within-treatment biological replicates (N=5) were treated as random effects. Significant treatment effects were determined by setting the p value threshold at < 0.01. Gene ontology enrichment analysis was performed using DAVID Bioinformatics.
Resources (http://david.abcc.ncifcrf.gov/), and network analysis performed using Ingenuity Pathway Analysis software (Ingenuity Systems, Inc.).
Results and Discussion

Range-Finding Experiment
The purpose of the range finding experiment was to determine the highest sub-lethal concentration following a month of weathering of surrogate South Louisiana crude oil. The highest sub-lethal concentration was determined from exposing *F. grandis* to varying dilutions of WAF for seven days (Fig. 2). Based on these data, the 25% WAF was the highest concentration of oil-contaminated water that caused no mortality after 7 days exposure.

Definitive Exposure Experiment
Adult male *F. grandis* (avg. 6.23g ± 3.48) were exposed to three WAF concentrations and a control. WAF concentrations included the highest sub-lethal concentration (25% WAF) as determined from the range-finding experiment and two additional treatments: 2.5% WAF and 100% tidal flush WAF. Three different time points were used (1d, 3d, 7d) to determine the time-course of exposure effects. No mortalities were observed over the seven-day experiment. Biological responses were measured and compared across time (sampling time point) and exposure treatment (WAF percentage), including DNA strand break analysis, and at the molecular level using genome expression profiling.

Analytical Chemistry
Water chemistry was analyzed to determine the alkane and aromatic composition in the WAF dilutions used for the definitive exposure experiment. Individual alkane concentrations that were above detection level were below 1 mg/L (Table 4) and the highest concentrations of individual compounds were C-3 and C-4 phenantrenes. Tidal and high concentrations had similar alkane and aromatic profiles and had higher concentrations to the control, whereas the low-concentration (2.5%) had few components above detection limit. Only a few aromatics were above detection limit (Table 5). The low concentration (2.5% WAF) exhibited the fewest alkane and aromatic components above detection limit, and for those above detection limit, the 2.5% WAF concentration had the lowest concentrations compared to the 25% and tidal WAFs compared to the control. The 25% WAF contained approximately a 10-fold higher concentration of total alkanes and approximately a 12-fold higher concentration of total aromatics, compared to the 2.5% WAF concentration. We predicted that the tidal preparation would be the lowest concentration but the tidal preparation had a similar profile and concentration of total alkanes to the 25% WAF. For aromatics, the tidal preparation had concentrations of aromatics that were approximately 3-fold higher than the 25% WAF. One possible reason for the difference in the aromatic concentration in the tidal preparation is from the formation of a new chemical equilibrium. It appears that once the original WAF was replaced with new clean brackish water, the toxic chemicals remained in the remaining oil and partitioned into the water column.
Table 4: Concentrations of a suite of alkanes from the control and WAF treatments from the definitive exposure experiment, as detected by GC-MS.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>TIDAL WAF</th>
<th>25% WAF</th>
<th>2.5% WAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Matrix</td>
<td>Water</td>
<td>Water</td>
<td>Water</td>
<td>Water</td>
</tr>
<tr>
<td>Sample Size (ml)</td>
<td>1,000 ml</td>
<td>1,000 ml</td>
<td>1,000 ml</td>
<td>1,000 ml</td>
</tr>
<tr>
<td>Sample Unit-Basis Units</td>
<td>mg/L</td>
<td>mg/L</td>
<td>mg/L</td>
<td>mg/L</td>
</tr>
<tr>
<td>nC-10 Decane</td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
</tr>
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<td>nC-15 Pentadecane</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>nC-16 Hexadecane</td>
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<td>0</td>
<td>0</td>
<td>0.021</td>
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<tr>
<td>nC-17 Heptadecane</td>
<td>0.695</td>
<td>0.184</td>
<td>0.809</td>
<td>0.116</td>
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<tr>
<td>Pristane</td>
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<td>0.628</td>
<td>0.545</td>
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<tr>
<td>nC-18 Octadecane</td>
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<td>Phytane</td>
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<td>0.394</td>
<td>0.317</td>
<td>0.041</td>
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<td>0.112</td>
<td>0.145</td>
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<td>0.057</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
</tr>
<tr>
<td>nC-22 Docosane</td>
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<td>0</td>
<td>0.018</td>
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<td>0.064</td>
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<td>0</td>
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<td>0</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
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<tr>
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<td><strong>2.81</strong></td>
<td><strong>3.41</strong></td>
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<td>Surrogate Recovery (%)</td>
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<tr>
<td>5 Alpha Androstane</td>
<td>81</td>
<td>80</td>
<td>85</td>
<td>83</td>
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</table>
**Table 5**: Concentrations of a suite of aromatics from the control and WAF treatments from the definitive exposure experiment, as detected by GC-MS.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>TIDAL WAF</th>
<th>25% WAF</th>
<th>2.5% WAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Matrix</td>
<td>Water</td>
<td>Water</td>
<td>Water</td>
<td>Water</td>
</tr>
<tr>
<td>Sample Size (ml)</td>
<td>1,000</td>
<td>1,000</td>
<td>1,000</td>
<td>1,000</td>
</tr>
<tr>
<td>Sample Unit-Basis Units</td>
<td>µg/L</td>
<td>µg/L</td>
<td>µg/L</td>
<td>µg/L</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>0</td>
<td>0</td>
<td>17.858</td>
<td>2.338</td>
</tr>
<tr>
<td>C1-Naphthalenes</td>
<td>0</td>
<td>28.165</td>
<td>15.708</td>
<td>1.594</td>
</tr>
<tr>
<td>C2-Naphthalenes</td>
<td>0</td>
<td>117.304</td>
<td>42.815</td>
<td>3.55</td>
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<tr>
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<td>0</td>
<td>134.339</td>
<td>71.564</td>
<td>6.91</td>
</tr>
<tr>
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<td>0</td>
<td>88.465</td>
<td>10.54</td>
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<td>Fluorene</td>
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<td>15.344</td>
<td>6.224</td>
<td>0.372</td>
</tr>
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<td>C2-Fluorenes</td>
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<td>0</td>
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</tr>
<tr>
<td>C3-Fluorenes</td>
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<td>150.355</td>
<td>16.4</td>
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<tr>
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<tr>
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<td>209.639</td>
<td>60.384</td>
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**DNA Damage**

To determine if WAF exposures cause direct damage to the DNA, comet assays were performed on nucleated red blood cells in fish. Data show that DNA strand breakage is significantly greater at higher concentrations. DNA damage is estimated by comparing the tail moment of WAF-treated fish to those from the control treatment (Fig. 3). Significant differences in tail moments were observed in the high concentration compared to the control for all time points (p < 0.001). Only at the 3-day time point did the low concentration show a subtle increase in tail moment compared to the control (p < 0.05). DNA strand breakage is a direct response to the exposure of the dissolved chemicals in the WAF dilutions that persisted through time and varied by treatment compared to a control. The high-concentration WAF showed significantly elevated DNA damage even after 1 day of exposure. Fish that were exposed to the different dilutions of WAF, which contain PAHs (polycyclic aromatic hydrocarbons), experienced an increase in DNA strand breakage compared to the control within 24 hours and this response persisted throughout the 7-day exposure. Several field studies have determined a cause and effect relationship between PAH specific contamination in oil and strand breakage in fish [24, 25] and mussel tissues [26-28]. DNA damage has been determined in a
laboratory study linking DNA strand breaks to the AHR pathway and induction of oxidative DNA lesions [29].

![DNA strand breakage graph](image)

**Figure 3**: DNA strand breakage after a time course exposure of 1 day, 3 days, and 7 days was assessed by comet assay and is expressed as tail moment (tail moment = tail DNA% * length of tail). Data were presented as means ± SD. *p<0.05 and ***p<0.001 denote significant differences. Throughout the time course the high concentration had the most DNA strand breakage compared to the control. The low concentration had slightly elevated DNA strand breakage in comparison to the control at the 3-day time point only.

**Genome Expression**

Profiling genome-wide transcriptional responses offers a top-down approach to understanding the mechanisms that animals utilize to respond to environmental stressors. A genome expression profile was characterized for gill and liver tissues to determine the genes regulated upon controlled exposure to a range of WAF concentrations in the laboratory. There were more concentration-responsive genes in the gill than in the liver (Fig. 4). Three main genomic expression patterns that were common for both tissues were observed. One cluster of co-expressed genes was up-regulated at all concentrations (gill = 17 genes, Fig. 4A, cluster 1a; liver = 17 genes, Fig. 4B, cluster 1a) or at least the high and tidal concentrations (gill = 10 genes, Fig. 4A, cluster 1b; liver = 14 genes, Fig. 4B, cluster 1b) compared to the control. The genome expression response is indistinguishable between the high-concentration and tidal treatments, even though some aromatic components were detected at higher concentrations in the tidal treatment than in the 25% WAF treatment (Table 4, 5). It is plausible that the molecular response is saturated at these relative high concentrations of aromatics and alkanes. The largest cluster of co-expressed genes (# of genes) are those that are up- and down-regulated in the low concentration only in both gill and liver (gill = 853 genes, Fig. 4A, cluster 2; liver = 95 genes, Fig. 4B, cluster 2).
Gene ontology enrichment analysis of sets of co-regulated genes can offer insight into the molecular mechanisms underpinning biological responses. The gene sets that were transcriptionally responsive in the high concentration treatments (25% WAF and tidal WAF; Fig. 4A cluster 1a + 1b) and in all three exposure treatments (Fig. 4A cluster 1a) were significantly enriched for genes associated with the KEGG pathway “metabolism of xenobiotics” (hsa00980) (p=0.004). These genes include well-known transcriptional targets of the toxicant-activated aryl hydrocarbon receptor (AHR) signaling pathway (CYP1A1, CYP1B, CYB5, UGT, FOXQ1). This AHR pathway is canonically activated by organic pollutants such as those found in oil and model toxicants such as the dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and the polycyclic aromatic hydrocarbon benzo[a]pyrene (BaP), and this pathway mediates much if not all of the toxic effects of these chemicals in diverse vertebrate taxa including for example, mammals, birds and fish including killifish [30]. The activation of this pathway is clear evidence of biologically-relevant exposure to the toxic components of weathered oil that are available in the WAF, even at the lowest dilution (2.5% WAF). Divergent genome expression associated with the timing and location of oil contamination in the field implicated activation of this same ARH signaling pathway [1].

Many genes were regulated in the low concentration treatment only, especially in gill. The gene set that was up-regulated at the low concentration only (Fig. 4A cluster 2a) was enriched for GO terms including nucleotide binding (p=3.0E-6, number of molecules = 90), positive regulation of transcription (p=2.8E-4, n=22), centrosome (p=6.2E-3, n=10), and negative regulation of apoptosis (p=0.04, n=15), whereas down-regulated genes at the low concentration only (Fig. 4 cluster 2b) were enriched for GO terms RNA processing and spliceosome (p=1.3E-3 and n=20, and p=4.7E-3 and n=10, respectively), response to DNA damage stimulus (p=0.01, n=8), and KEGG pathway oxidative phosphorylation (p=2.1E-3, n=16).

**Figure 4:** Patterns of expression for significantly responsive genes across treatments and time points for gill (A) and liver (B). Expression levels for specific genes are labeled as row and time points are within concentration columns. Cell color indicates an up regulation (yellow) or down-regulation (blue) compared to the control. Genes are grouped by patterns of co-regulation (Pearson correlation). Four patterns of co-regulation were observed relative to the control for both tissues: all concentrations up-regulated (cluster 1a), the high and tidal treatment up-regulated (cluster 1b), the low concentration up regulated (cluster 2a) and the low concentration down-regulated (cluster 2b). Scale indicates an up- or down-fold regulation.
A. Gill

Control \rightarrow WAF – 2.5\% \rightarrow WAF – 25\% \rightarrow WAF – Tidal

1a. 1b. 2a. 2b.

B. Liver

Control \rightarrow WAF – 2.5\% \rightarrow WAF – 25\% \rightarrow WAF – Tidal

1a. 1b. 2a. 2b.
The genes that were responsive in gill tissues connect to form three major sub-networks of interacting genes (Fig. 5). Gene functions that are enriched within networks of genes are consistent with the biological functions implicated by gene ontology enrichment analysis. The first sub-network (Fig. 5, top left cluster) includes almost all of the genes that were up-regulated at all or at least the high concentrations (Fig. 4A cluster 1, Fig. 5 red molecules), and is highly connected to AHR/ARNT/TCDD/BaP (Fig. 5). AHR and ARNT are key mediators of the toxic response to model toxicants such as TCDD and BaP in diverse taxa including fish [31]. The second sub-network (Fig. 5, top right cluster) primarily includes genes that were responsive to the low concentration only (Fig. 4A cluster 2, Fig. 5 yellow and blue molecules) and is united by UBC. This second sub-network is associated with the functions processing of RNA (p=8.2E-14) and splicing of RNA (p=2.0E-8). The third sub-network, also primarily including genes that were low concentration responsive only, is associated with functions cell cycle progression (p=7.5E-6) and tissue morphology (epithelial cells, p=2.0E-5).

Figure 5: Gill gene interaction network connected by aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT) hubs and model toxicants benzo-a-pyrene (BaP) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Genes up-regulated at high concentrations (Fig. 4 cluster 1) are colored red. Genes that are up- and down-regulated in the low concentration only are colored yellow and blue, respectively. Lines represent interactions between genes, and blue lines highlight genes that directly interact with AHR/ARNT/TCDD/BaP.
All three sub-networks are highly connected to AHR/ARNT/TCDD/BaP and to each other. This implies a complex coordinated molecular response to weathered oil, a response that is modular according to concentration, and a response that is functionally coupled to chemicals that are mechanistically related to those that comprise the toxic components of oil (e.g., TCDD and BaP). For example, sub-network 1 includes most of the genes up-regulated by all three concentrations, which includes many direct transcriptional targets of an activated AHR such as xenobiotic metabolism genes CYP1A1, CYP1B1, CYB5, and UGT, transcription factor NFE2L2, and transcription factor FOXQ1 which when up-regulated by TCDD-activated AHR is associated with developmental abnormalities in zebrafish [32]. Many genes in sub-network 2, which are primarily regulated in the low concentration only, are connected to AHR/ARNT/TCDD/BaP, including the hub UBC which binds both AHR and ARNT [33, 34]; indeed UBC is highly connected to genes in sub-network 1 and 3. In addition to UBC, this group of low-concentration-only genes is united by a group of translational regulators, including several known to interact with AHR/TCDD such as STAT1 [35], CTNNB1 [36, 37], NFE2L2 [38, 39], and HIF1A [40]. NFE2L2 may be particularly important, since it is highly connected in our network, it binds with and regulates expression of UBC [41], and is involved in many cell functions including apoptosis, cell death, and cellular response to injury and oxidative stress [42, 43]. Similarly, many genes in sub-network 3 are connected to AHR/ARNT/TCDD/BaP, including the hubs HNF4A which is regulated by ARNT [44], and TP53 which is regulated by AHR/ARNT, TCDD, and BaP [45, 46].

Many more genes were regulated at the low concentration only (Fig. 4). Clearly, pathways were activated at the low concentration that were not activated by higher concentrations. Given the identity of gene ontologies enriched in this low-concentration-only set (regulation of transcription, centrosome, cell cycle progression, RNA processing, DNA damage, apoptosis), we hypothesize that animals exposed to low concentrations were invoking cellular responses to compensate for activation of toxicity (AHR) pathways, and that these compensatory responses were overwhelmed or otherwise inhibited at higher concentrations. Indeed, this is consistent with little evidence of toxicity (DNA damage) in low-concentration fish compared to fish in the control and higher concentrations (Fig. 3). Compared to gill tissues, fewer genes were responsive in the liver. This may indicate different temporal dynamics of cellular response to environmental chemicals for organs that are in direct contact with the environment (e.g., gills) compared to organs that are internal (e.g., liver). One might predict that cells of gills, which are in direct contact with contaminated waters, might respond more dramatically over the short term to chemicals dissolved in the water, than cells of internal organs such as liver. Despite differences in the numbers of genes that were responsive between liver and gills, the biological functions that are implicated in response to oil-contaminated water exposures were similar between tissues, especially for high concentrations. For example, genes that were responsive at all three concentrations and the highest concentrations in liver (Fig. 4B cluster 1) were significantly enriched for genes associated with the KEGG pathway “metabolism of xenobiotics” (hsa00980) (p=0.004). As in gills, these genes include well-
known transcriptional targets of the toxicant-activated aryl hydrocarbon receptor (AHR) signaling pathway (e.g., CYP1A1, CYP1B, UGT, GST).

In liver, responsive genes connected to form two coupled sub-networks (Fig. 6). The first sub-network is primarily genes up-regulated at all concentrations and high+tidal concentrations, and largely represents genes activated by ligand-activated AHR, similar to the response in gill. The second network is united by UBC and TP53 hubs. These hubs were also implicated in the gill analysis, but in the liver the genes associated with the UBC hub are not associated with RNA processing functions as they were in the gill network. In fact, within the UBC-centered networks of both liver and gill, only 5 genes are shared between the two tissues (PLOD3, FOXC1, FARSB, ITSN1, and SRPK1).

**Figure 6:** Liver gene interaction network connected by aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT) hubs and model toxicants benzo-a-pyrene (BaP) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Genes up-regulated at high concentrations (Fig. 4 cluster 1) are colored red. Genes that are up- and down-regulated in the low concentration only are colored yellow and blue, respectively. Lines represent interactions between genes, and blue lines highlight genes that directly interact with AHR/ARNT/TCDD/BAP.

The molecular response was similar among tissues at high concentrations, but diverged at low concentrations. Genes that were responsive at high concentrations in liver and gill were largely overlapping in gene identity and gene function. In contrast, genes that were low-concentration responsive only were largely non-overlapping in identity or function between liver and gill. This implies that at high concentrations, the two tissues respond similarly at the molecular level, and this response is largely explained by activation of the AHR signaling pathway, while at low concentration the two tissues diverged in their molecular response. In gills, the low-concentration response appears to be associated with regulation of cell cycle, transcription, DNA damage, and apoptosis. In the liver, few functional categories were implicated, perhaps because the internal
concentration was insufficient to have induced compensatory responses over the duration of this experiment or the liver was more efficient at clearing damaging chemicals compared to gill. Reasons for tissue-specificity in molecular responses to contaminating oil merit further detailed study.

\[ \text{Figure 7: Trajectories for gill (left column) and liver (right column) transcriptional change through time and across sites in the field study [1] for subsets of genes identified in the laboratory study reported here. Rows are different subsets of genes selected from the laboratory study, including the 10% most significantly differentially expressed genes, the high-concentration responsive genes, and all the genes that were differentially expressed in the low concentration only. In the field study, liver responses were profiled across 6 sites at 3 time points, including Grand Terre Island (GT), Bay St. Louis (BSL), Bayou La Batre (BLB), Belle Fontaine Point (BFP), Mobile Bay (MB) and Fort Morgan Alabama (FMA) [1], before, during, and after peak oiling in 2010. The GT site was the only site directly oiled, and oil arrived between the first and second time-points. Gill responses were profiled at the oil-impacted GT site and two reference sites (BSL and BLB), at the same three time-points, plus an additional time-point at the GT site during summer 2011, one year after the third sampling time-point.}

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Genome-wide transcriptional responses from the laboratory study can help interpret genome expression responses observed coincident with the timing and location of oil contamination in the field study. In the field, the genome expression response for
liver was profiled across 6 field sites, and 3 time points, whereas for the gill we profiled across a subset (3) of field sites and included a fourth time-point for the GT site (August 2011, one year after time-point 3). We selected the sub-set of genes that were transcriptionally-responsive to the high-concentration and tidal treatment and explored the expression of those same genes across field sites and time-points in the field study. For these genes, a divergent trajectory of expression change through time was observed at the GT site at the second time-point, which coincides with the location and timing of oil (Fig. 7). Therefore, the genome expression response from controlled exposures to oil in the laboratory, especially the high-concentration response genes, predicted the genome response seen associated with contaminating oil in the field [1]. The largest sub-set of exposure-responsive genes is specific to the low concentration in both tissues (Fig. 4A cluster 2, Fig. 4B cluster 2) and the expression of these genes was explored across field sites and time-points in the field study. The low-concentration responsive genes were not divergently expressed with the major oiling events. Interestingly, these low-concentration responsive genes were divergent at GT time point 4 in gills (livers were not profiled at GT time 4) and were also divergent at FMA time 2 in livers (gills were not profiled at FMA) (Fig. 7). Satellite imagery data [1] and shoreline cleanup assessment technique (SCAT) data (http://gomex.erma.noaa.gov) indicated that oil came close to the FMA field site during time point 2, and by sampling time 4 at GT the contaminating oil had been weathering in situ for over 1 year. Therefore, patterns of expression of these low-concentration responsive genes in the field are consistent with possible exposure to low concentration responses experienced in the field. Despite much expected biological variation across space and time in the field, the laboratory response specifically to oil, especially the high-concentration response, was predictive of the response that was detected in the field. *F. grandis* that were sampled at the Grand Terre site were exposed to oil.
Summary

From what was observed in the field study, along with the water chemistry, comet assays, and the genome expression profiling from the laboratory study, we can conclude that the genomic response associated with oiling in the field was a direct consequence of exposure to contaminating oil in resident fish. From our experimental weathering of surrogate oil, water chemistry determined that components of crude oil had been dissolved into the clean brackish water. The high and tidal concentration responses were indistinguishable, although the tidal treatment contained higher concentrations of aromatic compounds. Comet assay data showed elevated DNA damage in the high concentration relative to the control at all time-points sampled, including just 1 day after exposure. In contrast, the lowest concentration (2.5% WAF) showed only slightly elevated strand breakage at the day 3 time point.

Genome expression profiling is a good test to use for studying environmental disasters, which offers a top-down approach to understanding the mechanisms that organisms are utilizing in response to their environment. In both the field and laboratory studies, responses from gill and liver tissues showed an increase in expression for genes known to be induced upon exposure to the toxic components of oil, including the induction of the AHR signaling pathway. The high and tidal concentrations of co-expressed genes were predictive of divergent expression at the GT site that coincides with the timing and location of oil exposure. The low-concentration responsive genes, in contrast, were not predictive of the major oiling event in the field. The low concentration response genes may represent a cellular compensatory response that may be overwhelmed or inhibited at higher concentrations. In the field, these low-concentration responsive genes may be predictive of low levels of exposure to contaminating oil.

Overall, the laboratory study showed differences in the genomic response to low and high concentrations of contaminating oil, and the gill genome expression profile was more dramatic compared to the liver, at least over a short time course. Importantly, a large response of what was observed in the field study was the same response determined in the laboratory, specifically at the high concentration. These results confirm that the response detected in the field was due to oil.
References


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38. Miao, W. M.; Hu, L. G.; Scrivens, P. J.; Batist, G., Transcriptional regulation of NF-E2 p45-related factor (NRF2) expression by the aryl hydrocarbon receptor-xenobiotic


Vita

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