Analysis of Sublethal Toxicity in Developing Zebrafish Embryos Exposed to a Range of Petroleum Substances using BE-SPME and Whole Transcriptome Microarray

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ANALYSIS OF SUBLETHAL TOXICITY IN DEVELOPING ZEBRAFISH EMBRYOS EXPOSED TO A RANGE OF PETROLEUM SUBSTANCES USING BE-SPME AND WHOLE TRANSCRIPTOME MICROARRAY

By

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Molecular Bioscience from the Department of Biological Sciences of Seton Hall University

May, 2019
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CHAIRPERSON,
DEPARTMENT OF BIOLOGICAL SCIENCES
ACKNOWLEDGEMENTS

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## TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii

TABLE OF CONTENTS iv

LIST OF TABLES vi

LIST OF FIGURES vii

LIST OF EQUATIONS ix

ABBREVIATIONS x

ABSTRACT OF THE DISSERTATION xi

INTRODUCTION 1

1.0 Petroleum Substances 1

1.1 Hazard and risk assessment 1

1.2 Background of fish toxicity testing 2

1.3 Supporting the 3Rs during environmental toxicology hazard and risk assessment 3

1.4 Background of fish embryo toxicity testing 4

1.5 Hydrocarbon metabolism 9

1.6 Hydrocarbon Mode of Toxic Action 10

1.7 Predicting hydrocarbon toxicity 12

1.8 Transcriptome profiling 13

1.9 Chemical analysis 14

2.0 Toxicity testing introduction 20

MATERIALS AND METHODS 26

Test Substances 26

Culture and generation of fertilized zebrafish embryos 26

Extended FET testing and identification of endpoints 27

Experimental evaluation of sublethal endpoints 28

Statistical analysis 29

Preparation and Administration of test substance 30
Biomimetic extraction – solid phase microextraction 31
BE-SPME exposure confirmation 32
Whole transcriptome microarray 34
Whole transcriptome microarray analysis 34
RESULTS 36
Acute Toxicity, LC50 36
Sublethal effects 38
Whole transcriptome microarray analysis 44
DISCUSSION 82
LIST OF TABLES

Table 1. Summary of individual sublethal endpoint EC25 calculations for each test substance.

Table 2. STRING analysis of up-regulated transcriptome profiles using KEGG pathway identifiers

Table 3. STRING analysis of down-regulated transcriptome profiles using KEGG pathway identifiers
LIST OF FIGURES

Figure 1. Fish embryo toxicity vs. acute fish toxicity test regression analysis from Belanger et al 2013.

Figure 2. Example of silicone coated GC fiber for use in BE-SPME analysis

Figure 3. Example of BE-SPME analysis chromatogram

Figure 4. Calculated LC50 for cumulative percent mortality across test substances

Figure 5. Cross plots comparing individual sublethal effects amongst test substances

Figure 6. Calculated EC25 for sublethal effects across test substances

Figure 7. Principal component analysis (PCA) from whole transcriptome analysis

Figure 8. Summary of differentially expressed genes following treatment comparison

Figure 9. Volcano plot comparing differentially expressed genes from Endicott high vs. Endicott control

Figure 10. Volcano plot comparing differentially expressed genes from CRGO high vs. CRGO control

Figure 11. Volcano plot comparing differentially expressed genes from Endicott low vs. Endicott control

Figure 12. Volcano plot comparing differentially expressed genes from CRGO low vs. CRGO control

Figure 13. Volcano plot comparing differentially expressed genes from Endicott high vs. Endicott low

Figure 14. Volcano plot comparing differentially expressed genes from CRGO high vs. CRGO low
LIST OF FIGUREs cont’d

Figure 15. Heatmap of Endicott low vs control differential expression

Figure 16. Heatmap of Endicott high vs control differential expression

Figure 17. Heatmap of Endicott high vs control differential expression

Figure 18. Heatmap of Endicott low vs Endicott high differential expression

Figure 19. Heatmap of CRGO low vs control differential expression

Figure 20. Heatmap of CRGO high vs control differential expression

Figure 21. Heatmap of CRGO high vs control differential expression

Figure 22. Heatmap of CRGO low vs CRGO high differential expression

Figure 23. Legend for STRING analysis of whole transcriptome microarray

Figure 24. STRING analysis profile of up-regulated genes for Endicott high vs. Endicott control comparison

Figure 25. STRING analysis profile of up-regulated genes for CRGO high vs. CRGO control comparison

Figure 26. STRING analysis profile of down-regulated genes for Endicott high vs. Endicott control comparison

Figure 27. STRING analysis profile of down-regulated genes for CRGO high vs. CRGO control comparison

Figure 28. STRING analysis profile of up-regulated genes for Endicott low vs. Endicott control comparison

Figure 29. STRING analysis profile of up-regulated genes for CRGO low vs. CRGO control comparison
LIST OF FIGUREs cont’d

Figure 30. STRING analysis profile of up-regulated genes for Endicott high vs. Endicott low comparison

Figure 31. STRING analysis profile of up-regulated genes for CRGO high vs. CRGO low comparison

Figure 32. STRING analysis profile of down-regulated genes for Endicott high vs. Endicott control comparison

Figure 33. STRING analysis profile of down-regulated genes for CRGO high vs. CRGO control comparison

Figure 34. All sublethal effects combined to visualize total percent effects in reference to nominal loading (mg/L) vs. measured BE-SPME (mM)

Figure 35. Acute sublethal effects data plotted with chronic fish toxicity data using BE-SPME as exposure metric

Figure 36. Fractional composition of petroleum substances used during testing

Figure 37. Toxic Unit (TU) contribution calculation of petroleum substance fractions using PETROTOX

LIST OF EQUATIONS

Equation 1. Calculation of BE-SPME from silicone coated fiber
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BE-SPME</td>
<td>Biomimetic Extraction - Solid Phase Microextraction</td>
</tr>
<tr>
<td>OECD</td>
<td>Organization for Economic Co-operation and Development</td>
</tr>
<tr>
<td>ECHA</td>
<td>European Chemicals Agency</td>
</tr>
<tr>
<td>CONCAWE</td>
<td>Conservation of Clean Air and Water in Europe</td>
</tr>
<tr>
<td>UVCB</td>
<td>Chemical substance of Unknown or Variable Composition, Complex Reaction Products, and Biological Materials</td>
</tr>
<tr>
<td>PNEC</td>
<td>Predicted No Effect Concentration</td>
</tr>
<tr>
<td>K_{ow}</td>
<td>n-octanol/water partitioning coefficient</td>
</tr>
<tr>
<td>3Rs</td>
<td>reduce, replace, refine</td>
</tr>
<tr>
<td>FET</td>
<td>Fish Embryotoxicity test</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>TPH</td>
<td>Total Petroleum Hydrocarbon</td>
</tr>
<tr>
<td>TPAH</td>
<td>Total Petroleum Aromatic Hydrocarbon</td>
</tr>
<tr>
<td>TLM</td>
<td>Target Lipid Model</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatography-Mass Spectrometry</td>
</tr>
<tr>
<td>GC-FID</td>
<td>Gas Chromatography-Flame Ionization Detector</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>AHR</td>
<td>Aryl Hydrocarbon Receptor</td>
</tr>
<tr>
<td>CRGO</td>
<td>Cracked Gas Oil</td>
</tr>
<tr>
<td>Dilbit</td>
<td>Diluted Bitumen</td>
</tr>
<tr>
<td>SRGO</td>
<td>Straight Run Gas Oil</td>
</tr>
<tr>
<td>VHGO</td>
<td>Vacuum Gas Oil</td>
</tr>
<tr>
<td>WAF</td>
<td>Water Accommodated Fraction</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>TAC</td>
<td>Transcriptome Analysis Console</td>
</tr>
<tr>
<td>.CEL</td>
<td>Raw data file from transcriptome microarray</td>
</tr>
<tr>
<td>STRING</td>
<td>Functional Protein association networks</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
</tr>
<tr>
<td>TU</td>
<td>Toxic Unit</td>
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ABSTRACT OF THE DISSERTATION

The OECD 236 Fish Embryo Acute Toxicity Test guideline relies on four endpoints to describe exposure related effects (coagulation, lack of somite formation, tail-bud detachment from yolk-sac, and presence of heartbeat). *Danio rerio* (zebrafish) embryos were used to investigate these endpoints along with a number of additional sublethal effects (i.e. cardiac dysfunction, pericardial edema, yolk sac edema, tail curvature, hatch success, pericardial edema area, craniofacial malformation, swim bladder development, fin development, and heart rate) following five day exposures to one of seven petroleum substances. The substances investigated included two crude oils, three gas oils, a diluted bitumen, and a petrochemical containing a mixture of branched alcohols. Biomimetic extraction via solid phase microextraction (BE-SPME) was used to quantify freely dissolved test substances as the exposure metric. The most prevalent effects observed were pericardial and yolk sac edema, tail curvature, and lack of embryo viability. Whole transcriptome microarray was used to profile gene expression following exposure to two petroleum substances. Meaningful downregulated differential expression was localized to concentrations that already displayed sublethal morphological effects; therefore, whole transcriptome profiling did not provide sufficient data to be able to predict sublethal morphological effects. A BE-SPME threshold was determined to characterize sublethal morphological alterations that preceded embryo mortality. Overall, this work aids in the understanding of aquatic hazards of petroleum substances to developing zebrafish beyond traditional OECD 236 test endpoints and shows applicability of BE-SPME as an analytical tool to predict sublethal embryotoxicity.
INTRODUCTION

1.0 Petroleum Substances

Crude oil is comprised of thousands of individual constituents, each constituent varying by presence and abundance from petroleum to petroleum (Henry 1998, Redman and Parkerton 2015). Crude oil is a complex combination of hydrocarbons consisting of aliphatic, alicyclic, and aromatic compounds and may also contain small amounts of nitrogen, oxygen, and sulfur compounds (CONCAWE 2015). Due to the complex composition of crude oil and petroleum substances it is not possible to characterize petroleum substances in terms of consistent chemical composition; therefore, petroleum substances are classified as substances of unknown or variable composition, complex reactions products, or biological materials (UVCB) and are grouped into categories based on manufacturing processes and basic physical properties (CONCAWE 2015).

1.1 Hazard and risk assessment

Hydrocarbons found within petroleum substances are common environmental contaminants originating from natural and anthropogenic sources (Volkman, Holdsworth et al. 1992, Tuvikene 1995, Abdel-Shafy and Mansour 2016). Due to their physicochemical properties, hydrocarbons can be challenging substances to perform accurate hazard and risk assessment; many hydrocarbons are sparingly soluble, volatile or both. Risk assessment as defined by the Organization for Economic Co-operation and Development (OECD) is a four step process: hazard identification, hazard characterization, exposure assessment, and risk characterization. Hazard identification
and hazard characterization are encompassed by hazard assessment, which is the identification and characterization of chemical substance properties to inform environmental risk on potential ecotoxicological effects and environmental fate. Exposure assessment is used to estimate the extent of chemical exposure to target species and the environment during chemical production, transportation, use, and disposal. Lastly, risk characterization is the qualitative and quantitative determination of the probability of occurrence of adverse effects of chemicals to the environment (OECD 2014). Standardized environmental toxicity testing is one key to informing risk assessment decisions. The OECD has developed standardized toxicity testing guidelines so that data may be generated through adherence to common practices. Following common practices ensures that any data generated is consistent by decreasing potential discrepancies in methodology.

1.2 Background of fish toxicity testing

Acute fish toxicity testing is a regulatory requirement for three primary reasons: fish toxicity data is used for hazard classification and labeling of chemicals; regulating bodies require data on chemical toxicity to fish to inform on hazards to higher tier vertebrate organisms. For these reasons, acute fish toxicity data is often used in combination with data from other aquatic organisms such as daphnia or algae to calculate predicted no effect concentrations (PNEC) for regulatory use (OECD 2012).

Chronic fish toxicity testing serves to provide data on long term chemical exposure and derive a PNEC that is protective of long term exposure to sensitive
organisms. Data collected can be survival, growth, development, reproduction, or mechanistic mode of action. Mechanistic endpoints are used by agencies that desire to regulate substances based on intrinsic hazard rather than on their environmental risks which are typically estimated by comparing effect level with predicted exposure in the environment (OECD 2012). Mechanistic endpoints add an additional level of detail to exposure hazard, but interpretation of data is cautioned because mechanistic activity does not always equal an adverse response.

Regulatory acute and chronic fish toxicity testing is designed to provide sufficient data for hazard and risk assessment of aquatic species and potential secondary exposure to humans indirectly exposed via aquatic organism consumption. The amount of fish toxicity testing and fish toxicity data produced for an individual chemical is driven by the end use of chemical substance. Chemicals that are predicted to heavily interact with the aquatic environment will require a more robust fish toxicity data set than chemicals that have limited aquatic interaction (OECD 2012).

1.3 Supporting the 3Rs during environmental toxicology hazard and risk assessment

Fish regulatory compliance testing has long been dependent on using whole organisms to provide sufficient data for hazard and risk assessment. A transition to alternative methods in toxicity test design have increased the opportunity to update current testing method to align with the 3Rs, which include reduction, refinement, and replacement when possible (Scholz, Sela et al. 2013, Hutchinson, Wheeler et al. 2016,
Lillicrap, Belanger et al. 2016). Reduction can be accomplished through minimizing the number of fish used in testing by supplementing or informing test design with toxicity modeling prior to performing toxicity testing. This allows for a reduction in concentrations needed within the test and ultimately reduces the number of fish used for each test. Refinement can be achieved by shifting focus from traditional lethality indicating endpoints to sublethal endpoints to inform decision making. Replacement can be achieved through the selection of alternative tests rather than relying on traditional acute fish toxicity testing, which requires the use of whole organisms (Hutchinson, Wheeler et al. 2016). However, caution should be taken when promoting the 3Rs in environmental toxicity testing to ensure the degree of environmental protection is not diluted (OECD 2012). Sufficient evidence should be provided in defense of alternative methods when suggesting them as replacement, reduction, or refinement of current methods. If sufficient evidence is provided, then the alternative method should be at a minimum equally weighted to the data of traditional fish acute toxicity testing. One alternative test suggested as a replacement for the acute fish toxicity test is the fish embryo toxicity test (FET) (Belanger, Rawlings et al. 2013, Rawlings, Belanger et al. 2019).

1.4 Background of fish embryo toxicity testing

Fish embryos have been suggested through multiple lines of evidence as a replacement for fish acute toxicity testing (OECD 2012, Belanger, Rawlings et al. 2013, Hutchinson, Wheeler et al. 2016, Rawlings, Belanger et al. 2019). Fish embryos can be
used as an ecotoxicity model because they represent a complex biological system during a sensitive developmental stage. However, fish embryotoxicity testing is predicated on regulatory definitions of protected versus unprotected fish life stages (OECD 2012). The typical life-stage used to differentiate a fish from a fish embryo is based on the eleutheroembryo stage, which occurs after the embryo has emerged from the chorion but is still dependent on the yolk-sac for nutrients. After the embryo has absorbed the yolk-sac and moved into the free feeding stage, it will be classified as a fish. The determination of when the embryo becomes a fish varies by regulating body, but usually before and during the eleutheroembryo stage is considered to be developing and therefore classified as a “non-animal” and will not be considered a protected organism (Halder, Léonard et al. 2010, OECD 2012).

The FET test was designed to determine acute toxicity following chemical exposure to embryonic stages of fish. The validation of the FET was based on studies performed using *Danio rerio* (zebrafish). The principles of the FET test are to expose zebrafish embryos that are less than 90 min post fertilization to a toxicant and use four lethality indicating endpoints (coagulation of fertilized egg, lack of tail-bud detachment from the yolk sac, lack of somite formation, and lack of heartbeat) to derive an LC50 following 96 h of exposure. Observation for any of the four lethality indicating endpoints occurs every 24 h and all observations are enumerated per concentration at 96 h (OECD 2013).

Evidence supporting the FET test as a replacement for the fish acute toxicity test has been building over the past decade. In 2010 Embry et al. summarized a workshop on
the “Application of the Fish Embryo Test as an Animal Alternative Method in Hazard and Risk assessment and Scientific Research” in which representatives from academia, non-government organizations, regulating bodies, and industry agreed that if enough evidence were to be gathered the FET test could act as a replacement for the acute fish toxicity assay (Embry, Belanger et al. 2010). However, the group agreed that there was insufficient data to replace the acute fish toxicity assay with the FET at the time. The outcome of the meeting did identify sensitive areas of the FET assay that if further developed would bolster the FET as a replacement. Areas identified that needed improvement were to increase the FET beyond embryonic stage and to include the eleutheroembryo stage to include additional endpoints beyond the four already identified to facilitate additional toxicity interpretation beyond acute testing, and to increase the amount of data to support read-across between the FET test and the acute fish toxicity test (Embry, Belanger et al. 2010, Belanger, Rawlings et al. 2013). Following the workshop, the OECD guideline for FET was finalized and included the extension of testing from 48 h to 96 h to incorporate the eleutheroembryo stage. Since the 2010 summary a large amount of work has been done to link the FET and acute fish tests by gathering exposure data from both assays and comparing endpoint concentrations for similarities and potential predictive applications (Belanger, Rawlings et al. 2013).

In 2013 Belanger et al. published a robust data review that compared FET and acute fish toxicity data. This work combined data from previous literature reviews, OECD validation efforts, peer reviewed literature, summary results made publically available, data presentations from conferences, and targeted emails to researchers
working in the field. Overall, the compiled data set included 985 FET studies covering 229 compounds and 1531 acute fish studies covering 151 compounds. In total there were 151 compounds for which parallel data existed for both FET and acute fish tests. Belanger et al. 2013 presented multiple iterations of the dataset, all of which support a correlation between FET and acute fish data. Within this dataset two figure present a significant correlation. Figure 1 plots 144 compounds with FET LC50 data on the x-axis and 96 h acute fish toxicity LC50s on the y-axis. A significant correlation was observed, but the data scatter across an order of magnitude depending on the compound (figure 1A and 1B) (Belanger, Rawlings et al. 2013). Regressions from figure 1A and 1B correlate well between fish acute toxicity tests and fish embryo toxicity tests (Belanger, Rawlings et al. 2013). The correlation relationship declines when the data is restricted to zebrafish only data; however, Belanger et al. noted that this is expected due to the limited number of corresponding datasets and that the relationship would be expected to improve as more data become available (Belanger, Rawlings et al. 2013).
Figure 1. Figure from Belanger et al. 2013 showing the relationship between acute fish toxicity and fish embryo toxicity (FET) assays. Open circles are individual test data and solid orange circles are geometric means of LC50 for each chemical. (A) FET versus 96 h acute fish toxicity assays. (B) 96 h FET versus 96 h acute fish toxicity (Belanger, Rawlings et al. 2013). Licensed content from John Wiley and Sons, Environmental Toxicology and Chemistry journal license number 4532011450821.
The Belanger et al. 2013 analysis provides sufficient evidence that the FET is predictive of acute fish toxicity across a range of chemical classes. However, there is a need to increase species diversity and include compounds such as chemical substances of unknown or variable composition, complex reaction products, or biological materials (UVCB) (Incardona, Collier et al. 2004, Fraysse, Mons et al. 2006, Brannen, Panzica-Kelly et al. 2010, Belanger, Rawlings et al. 2013). Petroleum substances fall within the UVCB category and are difficult to test and analyze in aqueous environments due to limited solubility and varying compositions. Due to testing difficulties, petroleum substance are often misrepresented in toxicity interpretation or underrepresented due to lack of reliable data (Knight, Little et al. 2009, Hoff, Lehmann et al. 2010, Kavlock and Dix 2010, Shukla, Huang et al. 2010, Kavlock, Chandler et al. 2012, Rotroff, Dix et al. 2013).

1.5 Hydrocarbon metabolism

Hydrocarbons are ubiquitous environmental contaminants and preferentially adsorb to organic and inorganic particulate matter (Tuvikene 1995). Following adsorption, hydrocarbons become more stable and are at lower risk of oxidation and nitration reactions (Tuvikene 1995). Fish and other aquatic organisms readily adsorb hydrocarbons following aquatic or sediment contamination, and the rate of uptake is dependent upon the lipid content of the organism (Tan and Melius 1986, Tuvikene 1995, Di Toro, McGrath et al. 2000). This means that an increase in the lipid fraction of the organism will correspond to an increase in hydrocarbon uptake and require a greater
accumulation of hydrocarbons in the target tissue to elicit a toxic response (Tuvikene 1995, Di Toro, McGrath et al. 2000).

Following organism uptake, hydrocarbons are subject to biotransformation, which a critical step influencing toxicity, distribution, and excretion (Tuvikene 1995). Through biotransformation, hydrocarbons are converted to more water soluble compounds, which assists in excretion (Tuvikene 1995). The enzymatic process of biotransformation involves Cytochrome P450 enzymes (CYP), epoxide hydrolase, and conjugating enzymes in categories labeled phase I and phase II reactions (Jiminez and Stegeman 1990, Pritchard 1993, Tuvikene 1995). Phase I enzymes utilize oxidative, reductive, or hydrolytic processes through the introduction of polar groups into the hydrocarbon. Phase II reactions leverage conjugation of hydrocarbons or the metabolites created in Phase I reactions (Goksøy and Förlin 1992, Tuvikene 1995).

1.6 Hydrocarbon Mode of Toxic Action

Acute hydrocarbon toxicity is typically associated with narcosis (Di Toro, McGrath et al. 2000, Parkerton, Stone et al. 2000, Barron, Carls et al. 2004). Narcosis is a reversible anesthetic effect caused by hydrocarbons and other hydrophobic compounds partitioning into cell membranes and disrupting normal function (Barron, Carls et al. 2004). Narcotics or chemicals acting via narcosis are generally thought to be less toxic than chemicals that act via a specified mode of action because narcotics are reliant upon accumulation within the organism to elicit a response (Barron, Carls et al. 2004). Generally, the narcosis model is predictive of fish toxicity in both juvenile and adult life.
stages. However, Barron et al. 2004 compiled literature data and suggested that narcosis is not predictive of fish embryotoxicity (Barron, Carls et al. 2004). In the Barron et al. 2004 review, the narcosis model narcosis was predictive of mortality but was not a good predictor of sublethal effects in fish embryos (Barron, Carls et al. 2004).

Barron et al. 2004 suggested that fish embryo toxicity may not follow a narcotic mode of action but instead that fish embryos or early life stages of fishes might be more sensitive to specific hydrocarbon classes such as alkyl phenanthrene or aryl hydrocarbon receptor (AhR) agonists which can result in blue sac disease (Barron, Carls et al. 2004). Though the datasets compiled in Barron et al. 2004 provide reasonable evidence that narcosis is not the best predictor of fish embryo sublethal effects, the overall analytical methods used to determine embryo exposure may not have been ideal (total petroleum hydrocarbon (TPH) and total petroleum aromatic hydrocarbon (TPAH). Current analytical methods such as biomimetic extraction-solid phase microextraction (BE-SPME) provide a more robust representation of the bioavailable exposure leading to toxicity (Redman, Parkerton et al. 2014). TPH and TPAH focus on a core set of approximately 40 parent PAHs and their alkyl homologs, and thus only comprise a portion of the bioavailable exposure. As such, the contribution of the approximately 40 parent PAHs to toxicity is misrepresented because it does not account for all compounds contributing to toxicity (Redman, Butler et al. 2018).
1.7 Predicting hydrocarbon toxicity

In cases where limited or no data exists on the toxicity of a particular chemical, models can be used to inform risk assessments (Hoff, Lehmann et al. 2010). The application of models for use in environmental risk assessment has become commonplace due to the abundance of chemicals in the marketplace and the limited availability of toxicity data (Hoff, Lehmann et al. 2010). However, modeling petroleum substance toxicity can be difficult due to the substances complex and variable composition (King, Lyne et al. 1996). Each constituent contained within a petroleum substance may contribute to toxicity based on its physicochemical properties (Di Toro, McGrath et al. 2000). The physicochemical properties define the constituent’s ability to solubilize in the media and become bioavailable to the organism (Di Toro, McGrath et al. 2000, Parkerton, Stone et al. 2000, Redman, Butler et al. 2018). Various environmental fate and effects properties such as degradation or sorption to sediments play an integral role in bioavailability and toxicity to aquatic organisms (King, Lyne et al. 1996).

Hydrocarbons, being non-polar and hydrophobic, readily partition into the lipid fraction of an organism, where hydrocarbons can accumulate until a threshold concentration is reached and toxicity occurs (Di Toro, McGrath et al. 2000). The accumulation of hydrocarbons within an organism is predictable based on physicochemical properties. The accumulation threshold that is necessary to elicit toxic response is organism-specific, and based on the lipid fraction within each organism (Di Toro, McGrath et al. 2000). Therefore, if the lipid fraction of the organism and the constituents within the hydrocarbon mixture are known, toxicity becomes predictable.
Though individual constituents within petroleum substances can result in varying levels of toxicity, closely-related constituents can impart similar toxicity (King, Lyne et al. 1996, Redman, Parkerton et al. 2012). In the case of petroleum substances, closely-related constituents are grouped into blocks. Each hydrocarbon block estimates the environmental impact of a group or block of hydrocarbons by associating known toxicity values for individual hydrocarbons within that block (King, Lyne et al. 1996).

PETROTOX is a spreadsheet model used to predict aquatic toxicity of petroleum substances using the hydrocarbon block method (Redman, Parkerton et al. 2012). Using a three-phase fate model, the distribution of hydrocarbons are predicted within water-air- and oil-phase liquid within the experimental vessel (Redman, Parkerton et al. 2012). The toxicity of hydrocarbons is then computed based on the predicted aqueous concentration, associated toxicity, and the target lipid model (Redman, Parkerton et al. 2012). Overall, hydrocarbon toxicity is reasonably well-behaved and predictable if physicochemical and bioavailability parameters are considered.

1.8 Transcriptome profiling

Transcriptomics can be used as a part of exposure analysis to provide additional insight into how an organism responds to different chemical exposures (Brockmeier, Hodges et al. 2017, Sauer, Deferme et al. 2017, Schüttler, Reiche et al. 2017). Transcriptome profiling looks for consistent changes in RNA expression following chemical exposure. Consistent alteration in the organism’s transcriptome can be used to inform and classify chemical exposure impact. However, the use of transcriptomics or
any other ‘omics technology (genomics, proteomics, metabolomics) to observe shifts in omic profiles must not always be interpreted as an adverse effect (Sauer, Deferme et al. 2017). Alterations in molecular response can indicate organism variability or compensatory, reversible changes that may not result in adverse or observational effects (Sauer, Deferme et al. 2017). Additionally, biological variability has the potential to mislead data interpretation as gene expression can vary amongst organisms (Sauer, Deferme et al. 2017). Understanding of when gene expression is biologically meaningful is imperative for application beyond research (Walhout 2011).

1.9 Chemical analysis

Total petroleum hydrocarbon (TPH) analytical methods are used to measure the amount of petroleum hydrocarbons in environmental media. Because environmental media can dictate the availability of petroleum hydrocarbons through sorption and degradation properties, the amount of measureable petroleum hydrocarbons is dependent on the composition of environmental media (Gustafson, Tell et al. 1997, CDC 1999). TPH measurement relies on solvent extraction of environmental media to isolate hydrocarbons. The solvent is then analyzed using gas chromatography (GC), and hydrocarbons are quantified based on the resolved and unresolved components (Reddy and Quinn 1999). To measure the aromatic fraction (total petroleum aromatic hydrocarbons (TPAH)), which is frequently associated with toxicity, gas chromatography coupled with mass spectrometry (GC-MS) is used (Reddy and Quinn 1999). Both TPH and TPAH are frequently used to represent petroleum hydrocarbons in environmental

An alternative measurement technique that quantifies the bioavailable fraction is biomimetic extraction solid phase microextraction (BE-SPME). BE-SPME uses a polydimethylsiloxane (PDMS)-coated fiber to quantify bioavailable non-polar organic compound, such as hydrocarbons, in environmental media (Parkerton, Stone et al. 2000, Leslie, Oosthoek et al. 2002, Letinski, Parkerton et al. 2014, Redman, Parkerton et al. 2014, Redman and Parkerton 2015, Redman, Butler et al. 2018). Sorption of hydrocarbons into the PDMS is dependent upon the bioavailability in environmental media. If the hydrocarbon has partitioned into the media, it is deemed as being bioavailable to the organism. The rate of partitioning and accumulation into the organisms is the same as the rate of partitioning into the PDMS. For this reason the accumulation of hydrocarbons into the PDMS-coated fiber is representative of hydrocarbon accumulation into the organism (Parkerton, Stone et al. 2000, Leslie, Oosthoek et al. 2002, Letinski, Parkerton et al. 2014, Redman, Parkerton et al. 2014, Redman and Parkerton 2015, Redman, Butler et al. 2018). Since hydrocarbons act via narcosis, the correlation of toxicity to a BE-SPME measurement will allow for understanding the hydrocarbon concentration within the organism that elicits a toxic response. To measure the bioavailable portion of the hydrocarbon exposure, the PDMS-coated fiber is placed within the environmental media and allowed to equilibrate (figure 2). The fiber is then removed and extracted using gas chromatography coupled with
flame ionization detection (GC-FID) (figure 3). The resulting chromatograph produced from GC-FID is analyzed by area under the curve in relation to a 2,3-dimethylnaphthalene standard and multiplied by the volume of silicone on the fiber (equation 1) (Parkerton, Stone et al. 2000, Leslie, Oosthoek et al. 2002, Letinski, Parkerton et al. 2014, Redman, Parkerton et al. 2014, Redman and Parkerton 2015, Redman, Butler et al. 2018).

BE-SPME measures the abundance of constituents through quantification of total hydrocarbons that partition into the SPME fiber as well as individual fiber-water partition coefficients (Di Toro, McGrath et al. 2000, Redman, Parkerton et al. 2014, Redman and Parkerton 2015, Redman, Butler et al. 2018). As BE-SPME is a measure of bioavailable fraction within aqueous media, assessing petroleum substance effects on aquatic organisms in relation to a BE-SPME measurement has shown to correlate across substances with comparable composition (Leslie, Oosthoek et al. 2002, Letinski, Parkerton et al. 2014, Redman, Parkerton et al. 2014).
Figure 2. Example of silicone coated fiber equilibrating to bioavailable constituents in environmental media. (This figure is used under with permission from Creative Commons Attribution 3.0 License which permits unrestricted use, distribution, and reproduction in any medium provided the original work is properly cited (Rosero-Moreano 2018) License agreement: https://creativecommons.org/licenses/by/3.0/legalcode).
Figure 3. Example of a chromatogram produced following extraction of silicone coated fiber. Blue line indicates sample chromatogram where area under the curve is compared to area under the curve of a standard of 2,3-dimethylnaphthalene (red line).
\[ C_{\text{fiber}} = \frac{\text{Peak area}_{\text{sample}}}{\text{Peak area}_{\text{standard}}} \times \text{fiber volume} \]

Equation 1. Calculation of BE-SPME following extraction of PDMS-coated fiber.
2.0 Toxicity testing introduction

Exposure to individual hydrocarbons, simple hydrocarbon mixtures, and complex hydrocarbon-based UVCBs (chemical substances of unknown or variable composition, complex reaction products, and biological materials) such as crude oil, can be toxic to fish species at varying life stages (Carls, Rice et al. 1999, Incardona, Collier et al. 2004, Hicken, Linbo et al. 2011, Incardona, Swarts et al. 2013, Philibert, Philibert et al. 2016, Hodson 2017). Research characterizing toxic effects from exposure to individual hydrocarbons and hydrocarbon mixtures typically involve an acute endpoint (LC50) or the observation of chronic effects (edema, cardiac malformation, craniofacial malformation, reduced swim speed, prey capture or predator avoidance) which may impact organism fitness (Carls, Rice et al. 1999, Carls, Holland et al. 2008, Hicken, Linbo et al. 2011, Incardona, Swarts et al. 2013, Brown, Bailey et al. 2016, Philibert, Philibert et al. 2016, Hodson 2017). The Organization for Economic Co-operation and Development (OECD) test guideline 236 describes a fish embryo acute toxicity test (FET) that identifies four key endpoints to evaluate acute or lethal toxicity of chemicals in embryonic zebrafish (coagulation of fertilized embryo, lack of somite formation, lack of detachment of tail-bud from yolk sac, and lack of heartbeat) (OECD 2013, Braunbeck, Kais et al. 2015). Directing focus on endpoints which only describe lethality neglects an opportunity to capture broader exposure-related sublethal effects. Often a suite of sublethal morphological effects precede mortality, which could provide linkage between sublethal and lethal toxicity in developing zebrafish (Braunbeck, Kais et al. 2015). Furthermore, little research has attempted to identify exposure thresholds at which

An extensive amount of research supporting the identification of developmental abnormalities is available. However, the majority of this research focuses on single chemicals or simple defined mixtures (Incardona, Collier et al. 2004, Fraysse, Mons et al. 2006, Brannen, Panzica-Kelly et al. 2010, Belanger, Rawlings et al. 2013). Identifying an exposure level at which petroleum substances consistently result in developmental abnormalities would provide an improved basis for risk assessment of petroleum substances in the environment, and support lab to field extrapolations. Therefore, it is important to characterize threshold responses to provide reasonable guidance for risk management measures.

Not only is the identification of endpoints in relation to exposure concentration necessary for threshold development, but understanding the exposure is vital to the correct derivation of thresholds. Petroleum substances are complex and variable: each constituent that makes up a petroleum substance has different physiochemical properties (e.g. solubility, volatility) that will dictate how it will interact with the aqueous media (Brusseau, Famisan et al. 2004). Incorrect dosing and analytical characterization can obscure interpretation by associating unachievable or unrealistic chemical concentrations with observed toxicity due to the natural limitations of compound aqueous solubility (Redman and Parkerton 2015).
One of most common methods of managing substances with limited aqueous solubility is to use a carrier solvent such as dimethylsulfoxide (DMSO). Using a carrier solvent can often increase exposure concentrations above maximum aqueous solubility, and can lead to artefactual effects due to physical oiling of the organism, and subsequent spurious interpretation of concentration-response data. With increasing regularity, data generated in one toxicity assay is being extrapolated to predict toxicity to other organisms. Read-across efforts such as these require high quality data going in, and thus the importance of correct dosing and analysis of petroleum substance toxicity is evident. Recently, efforts to link zebrafish toxicity data to mammalian developmental toxicity have shown varying degrees of agreement (64-100%) (Brannen, Panzica-Kelly et al. 2010, Hermsen, van den Brandhof et al. 2011, Hill, Jones et al. 2011, Padilla, Hunter et al. 2011, Sipes, Padilla et al. 2011, Selderslaghs, Blust et al. 2012). One of multiple drivers of variability amongst these studies could be dosing or analytical techniques (Sipes, Padilla et al. 2011). As an end result, even if the same test substances were used through the different research projects, if a common method of dosing and detection is not agreed upon, data extrapolation will not be useful.

Zebrafish (*Danio rerio*) are a convenient model species widely used for toxicity testing and drug discovery (Hill, Teraoka et al. 2005). Their well-known life cycle, and the ease with which they can be maintained in the laboratory, promote their use in aquatic toxicity testing, and an abundance of biological exposure data exists with which to compare results (Hill, Teraoka et al. 2005). Proper assessment of petroleum substance exposure can be difficult as hydrocarbon constituents have widely different
bioavailabilities based on their physicochemical properties such as water solubility and vapor pressure (Redman, Butler et al. 2018), and it becomes difficult to identify whether a single confounding factor leads or contributes to toxicity (Moore and Dwyer 1974, Henry 1998, French-McCay 2002, Redman and Parkerton 2015). This creates a need to normalize exposures in order to correlate morphological effects. Often metrics such as TPH (total petroleum hydrocarbon) or TPAH (total polycyclic aromatic hydrocarbon) are used to characterize petroleum constituents within exposure media. TPH and TPAH measure bulk hydrocarbons, which is not necessarily a measurement of the bioavailable fraction, and therefore can lead to an inaccurate assessment of constituents contributing to toxicity (Redman and Parkerton 2015). Biomimetic extraction using solid phase microextraction (BE-SPME) offers a convenient method to measure dissolved hydrocarbons based on their respective bioavailable fraction in aqueous media. BE-SPME measures the abundance of constituents through quantification of total hydrocarbons that partition into the SPME fiber as well as individual fiber-water partition coefficients (Di Toro, McGrath et al. 2000, Redman, Parkerton et al. 2014, Redman and Parkerton 2015, Redman, Butler et al. 2018). As BE-SPME is a measure of the bioavailable fraction in an aqueous medium (Letinski, Parkerton et al. 2014), assessing petroleum substance effects on aquatic organisms in relation to a BE-SPME measurement (accumulation of hydrocarbons in polymer) correlates across substances with comparable composition (Leslie, Oosthoek et al. 2002, Redman, Parkerton et al. 2014). BE-SPME benefits are attributed to the PDMS coated fiber which acts as a surrogate for organismal lipid (Parkerton, Stone et al. 2000).
Individual hydrocarbons have been shown to have an adverse impact on developing fish embryos (Carls, Rice et al. 1999, Barron, Carls et al. 2004, Incardona, Collier et al. 2004, Carls, Holland et al. 2008, Carls and Meador 2009, Hicken, Linbo et al. 2011, Pauka, Maceno et al. 2011, Perrichon, Le Menach et al. 2016, Hodson 2017). However, attributing specific effects to individual constituents found within crude oil can be difficult when testing complex or undefined mixtures. When observing sublethal effects, compositional differences between fresh, weathered, and other fractions of crude oil, might be expected to result in varying sublethal effects; however, little difference in sublethal effects are often observed (Ernst, Neff et al. 1977, Carls, Rice et al. 1999, Incardona, Collier et al. 2004, Carls, Holland et al. 2008, González-Doncel, González et al. 2008, Carls and Meador 2009, Hicken, Linbo et al. 2011, Pauka, Maceno et al. 2011, Belanger, Rawlings et al. 2013, Incardona, Swarts et al. 2013, Jung, Hicken et al. 2013, Perrichon, Le Menach et al. 2016, Hodson 2017). This lack of sublethal effect variability between fresh and weathered crude oil may be attributed to the bioavailable fraction of compounds equally contributing to toxicity despite the overall variable composition of crude and weathered oil (Carls and Meador 2009). Sublethal embryotoxicity from oil exposure typically, but not exclusively, results in pericardial and yolk-sac edemas, craniofacial and cardiac malformations, spinal curvature, circulatory failure, and fin erosion (Hodson 2017). Particular fractions of crude oil have been associated with various effects observed in embryotoxicity studies with a more direct focus being on aryl hydrocarbon receptor (AhR)-mediated cardiotoxicity (Barron, Carls et al. 2004, Incardona, Collier et al. 2004, Billiard, Timme-Laragy et al. 2006, Incardona, Day et al.

In an effort to better understand complex hydrocarbon substances exposure, this work describes the exposure of zebrafish embryos to increasing concentrations of seven petroleum substances. The sublethal acute endpoints described in the study cannot currently be linked to chronic/population level effects, however if we are able to make a connection/correlation to chronic effects seen in literature data, this type of test could be used to inform testing decisions, while decreasing the number of animals used in testing. We aim to investigate sublethal endpoints often identified in toxicity tests but rarely used to inform future testing decisions or to establish relevant exposure thresholds for complex substances primarily due to the shortage of relevant data. The first objective was to identify endpoints that are characteristic of hydrocarbon exposure. For this objective, isotridecanol, a branched alcohol, was used to compare sublethal effects from a non-hydrocarbon petrochemical to hydrocarbon-based petroleum substances. The second objective was to identify a BE-SPME exposure threshold that aids in the prediction of sublethal effects. Concentration-response exposures for seven petroleum substances, were quantified using BE-SPME as a surrogate for critical body burden (CBB). Using BE-SPME, we can correlate the compound concentration directly to toxicity (narcosis), regardless of substance composition (Hodson 2017). The third objective was to identify one or more endpoints that could be used as a leading indicator of toxicity. Finally, the fourth objective was to describe whole transcriptome profile following exposure to petroleum substances.
MATERIALS AND METHODS

Test Substances

The following complex substances were used: Endicott crude oil, Weathered Troll oil, Cracked Gas Oil (CRGO), diluted bitumen (dilbit), Straight Run Gas Oil (SRGO), Vacuum Hydrocracked Gas Oil (VHGO), and isotridecanol. Endicott crude, a medium weight crude oil from the Alaskan North Slope region varying in hydrocarbons from C$_6$ – C$_{80}$; 20% weathered Troll oil, a light sweet crude from the Norwegian oil fields primarily made up of C$_{10}$ – C$_{80}$; CRGO and VHGO primarily consisted of saturated hydrocarbons ranging from C$_9$ – C$_{30}$; SRGO was comprised of hydrocarbons primarily in the range of C$_9$ – C$_{25}$; dilbit, an extensively biodegraded bitumen diluted with natural gas condensate and consisted primarily of asphaltenes in the C$_{50}$ – C$_{80}$ range with the condensate ranging from C$_2$ – C$_8$; isotridecanol consisted of branched alcohols in the range of C$_{10}$ – C$_{14}$ (CONCAWE 2015).

Culture and generation of fertilized zebrafish embryos

Adult male and female D. rerio were acquired from Aquatic Research Organisms (Hampton, NH) and housed in separate 40 L aquaria on a 12:12 h light:dark cycle with biological and mechanical filtration. Fish were maintained at 26 ± 1°C and fed TetraMin Tropical Flakes (Tetra) with supplemental Brine shrimp nauplii, Platinum grade 0 (Argent Aquaculture) twice daily. One day prior to embryo collection, female and male D. rerio at a 2:1 female to male ratio were placed into a 40 L aquarium. Marble-filled glass evaporation dishes (Corning, PYREX®) were used as embryo collection vessels.
Collection vessels were placed into breeding aquaria one hour prior to the onset of light. Fish were allowed to spawn for 45 min at the onset of light. Following the 45 min spawn time, the embryo collection vessel was removed from the aquaria, and eggs were collected and sorted for fertilization under an Olympus SZX12 stereoscope. Sufficient fertilized embryos were collected and distributed into 50 mL beakers within 90 min of fertilization.

*Extended FET testing and identification of endpoints*

The principle intention of the OECD 236 guideline is to identify lethality utilizing four observations (coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat) in the determination of an LC50 (OECD 2013). In addition to the OECD 236 endpoints, developmental abnormalities were characterized to better understand the role sublethal effects can have in the evaluation of toxicity from exposure to complex petrochemicals (Brannen, Panzica-Kelly et al. 2010). Each exposure was completed in general agreement with OECD 236; however, instead of targeting lethality, concentrations that were expected to result in sublethal effects were selected. Exposure trials were used to identify morphological abnormalities that occurred repeatedly across test substances. Each exposure was conducted in a walk-in environmental chamber where lighting and temperature could be remain constant. Sublethal endpoints evaluated included pericardial and yolk sac edema, lack of viability (heartbeat present but did not hatch from embryo), notochord development, tail, fin, brain, jaw, and swim bladder development, craniofacial
features, and heart rate (Brannen, Panzica-Kelly et al. 2010, Panzica-Kelly, Zhang et al. 2010). All observations were made in 24 h increments following test initiation using an Olympus CKX41 inverted microscope.

**Experimental evaluation of sublethal endpoints**

Experimental evaluation of the four traditional OECD 236 endpoints (coagulation, tail-bud detachment, somite development, and heartbeat) along with sublethal developmental endpoints (pericardial and yolk-sac edema, notochord, tail, fins, craniofacial, brain, swim bladder, viability, and heart rate) occurred at 96 h. The lack of viability was identified by embryos that were still alive at 96 hpf (hours post fertilization) but did not hatch. Upon completion of the 96 h observation, 5 of the total 20 replicates in each treatment were sacrificed for aqueous BE-SPME analysis. The remaining 15 replicates in each treatment were carried through an additional 24 h of exposure. Following the final 120 h of exposure, jaw/gill arches were assessed for development. Upon completion of the 120 h observations, all remaining embryos/larvae were euthanized using a buffered tricaine methanesulphonate solution (MS-222) per OECD 236 (OECD 2013).

Observations were conducted in 24 ± 1 h increments for up to 120 hpf. At each 24 h period any abnormal morphological alteration was documented for posttest analysis. All control embryos and test substance exposed embryos that displayed pericardial edema were imaged at 10X using an Olympus DP72 camera attached to an Olympus CKX41
inverted microscope. Images were analyzed using Olympus CellSens Dimension software version 1.3, 2010.

Post image analysis (CellSens Dimensions) of pericardial edema allowed for area (µm²) measurements of the pericardial cavity. Pericardial edema occurrences were documented using two methods. The first method was recording either presence or absence of edema; the second method was intended to quantify the pericardial cavity area (µm²) in order to characterize both normal and abnormal pericardial cavity size in relation to exposure. All incidents of pericardial edema were compared to a library of control pericardial cavity areas compiled from the seven test substances discussed herein. Heart rate was analyzed at 96 h by recording the time required to reach 20 beats. Three individual times were recorded for each replicate and averaged to give average time to 20 heartbeats. This time was then extrapolated to 60 seconds to provide average beats per minute. All observations were recorded at 96 hpf except jaw development, which was assessed at 120 hpf.

**Statistical analysis**

Non-linear regression analysis was used to calculate EC25 and LC50 using JMP 13 (SAS, 2016) and plotted using Graphpad 6 (version 6.07, 2015). EC25 endpoint calculation used a non-linear regression model of best fit, which was either Gompertz 3P or Logistic 3P. The LC50 calculation used a Gompertz 3P non-linear regression model. The EC25 was selected as the most appropriate effect threshold because there is clear
differentiation from background sublethal effects where the EC10 did not provide sufficient separation and an EC25 was more sensitive than the EC50.

**Preparation and Administration of test substance**

Soft water was prepared following “Standard methods for the examination of water and wastewater Table 8010:I” (Eaton, Clesceri et al. 2005), and aliquoted into 4 L aspirator bottles with a tubing outlet (Corning, PYREX®, #1220-4L). Each bottle was customized to fit a solid Teflon® screw cap and filled to the top, which allowed each bottle to be sealed in order to minimize loss via volatilization. Test solutions were prepared using a standard water accommodated fraction (WAF) method by adding test substance to dilution water using glass and stainless steel gastight 1700 series Hamilton® syringes (Singer, Aurand et al. 2000). Test substance loading rates were 3.2, 11, 36, 120, 400 and 1000 mg/L for SRGO, 2.5, 8.0, 27, 90, 300 and 1000 mg/L for dilbit, 22.5, 51, 116, 264 and 600 mg/L for Endicott crude, 4.1, 14, 45, 150 and 500 mg/L for weathered Troll, 0.83, 2.5, 7.6, 23, and 162 mg/L for CRGO, 1.3, 3.2, 8.0, 20, and 50 mg/L for VHGO, and 0.5, 0.75, 1.0 and 3.25 mg/L for isotridecanol. Following the addition of test substance, solutions were stirred at ≤ 20% vortex of static liquid depth using a magnetic stir plate and a Teflon®-coated stir bar for 24 ± 1 h. Following the allotted mixing time, stirring was stopped and the WAF was allowed to settle for 1 h ± 15 min. The WAF solutions were then drawn from the outlet at the bottom of each aspirator bottle and distributed to each corresponding replicate container (n=20). Replicate containers were 20 mL scintillation vials (VWR #66022-128). Each vial was sealed with no headspace to minimize volatilization using PTFE-lined screw caps (Qorpak®, cap-00544).
Biomimetic extraction – solid phase microextraction

When preparing WAFs for toxicity testing, the test substance was added to dilution water and stirred with a $\leq 20\%$ vortex for 24 h. Provided that the test substance was sufficiently hydrophobic, the settling period provided an opportunity for any undissolved test material to return to the surface. This method of mixing relied on partitioning of the water-soluble portion of the test substance into the dilution water over the 24 h mixing period. Since dilution water contained only the dissolved hydrocarbons, the subsequent toxicity testing avoided the confounding effects of physical oiling. BE-SPME was used to quantify the bioavailable fraction during exposure (Leslie, Oosthoek et al. 2002, Redman, Parkerton et al. 2014).

BE-SPME measurements can be used to evaluate petroleum substance exposure in part due to the target lipid model (TLM) (Di Toro, McGrath et al. 2000, Leslie, Oosthoek et al. 2002, Redman, Parkerton et al. 2014). The TLM asserts that toxicity can be estimated based on the octanol-water partitioning coefficient ($K_{ow}$) of the test compound and the lipid content of the organism. This inference is translatable to BE-SPME. The SPME fiber is coated with polydimethylsiloxane (PDMS), which acts as a surrogate for organismal lipid (Verbruggen, Vaes et al. 2000, Leslie, Oosthoek et al. 2002, McGrath and Di Toro 2009, Letinski, Parkerton et al. 2014, Redman, Parkerton et al. 2014). The rate of partitioning from water to PDMS is similar to that of water to lipid (Ding, Landrum et al. 2012). This means that when the PDMS-coated fiber is placed into the exposure water, the bioavailable fraction will partition from the water onto the PDMS
fiber. The fiber is then thermally desorbed and analyzed via gas chromatography with flame ionization detection (GC-FID) and the area under the response curve quantified in comparison to a standard of 2,3-dimethylnaphthalene (Redman, Butler et al. 2018). The BE-SPME method provided a convenient cross comparison amongst petroleum substances for relating bioavailable fractions of each test substance to toxicity by quantifying the bioavailable fraction of each test substance to the observed lethal or sublethal effects.

**BE-SPME exposure confirmation**

BE-SPME samples were taken in triplicate at test initiation and termination. Each sample was automatically extracted with a 30 µm PDMS (0.132 µL) SPME fiber (Supelco) for 100 min at 30°C with rapid agitation (250 RPM) and analyzed by GC-FID (Perkin Elmer AutoSystem GC-FID equipped with dual Gerstel MPS2 Rail). Sample FID responses were normalized against 2,3-dimethylnaphthalene derived from liquid solvent injection of the hydrocarbon standards. BE-SPME results were normalized to the volume of PDMS and reported as µmol 2,3-dimethylnaphthalene/mL PDMS.

**Zebrafish embryo selection for microarray**

Observations were conducted to identify all visible sublethal effects. All sublethal observations were documented and graphed for visualization of effects and selection of embryos for whole transcriptome analysis. Following every 24 h observation, a sacrificial set of embryos were pooled together into three replicates, each replicate containing five
embryos. Pooled embryos were milled into RNAlater™ stabilization solution using sterile plastic pestels (DWK Life Sciences Kimble™ Kontes™ Pellet Pestle™) and stored at -20°C until RNA extraction.

The 96 hpf samples were used to select the concentrations based on observed sublethal effects. However, the embryos used for microarray analysis came from 48 hpf samples. The 48 hpf samples were selected for two reasons: one, the 48 hpf time point was late enough in development that the majority of biological function has been established (Mathavan 2005, Vesterlund, Jiao et al. 2011), and at 48 h there was less mortality so RNA integrity was not in question as all embryos selected for microarray were alive. Embryos from two petroleum substances (Endicott crude and CRGO) at control, toxic, and non-toxic concentrations were selected for analysis. Toxic and non-toxic concentrations were selected following identification of sublethal effects in the toxic concentration. The non-toxic concentration selected was identified as a concentration that produced no observed morphological abnormalities. A comparison of whole transcriptome response to these three conditions was aimed at observing transcriptome shifts of control organisms, which would be indicative of a transcriptome during normal development, non-toxic concentration, which documents normal biological response to petroleum substance exposure, and a toxic concentration, which documents transcriptome shifts from organisms that displayed developmental morphological abnormalities.
Whole transcriptome microarray

Whole transcriptome microarray was completed in partnership with Albert Einstein College of Medicine, Bronx, New York. In brief, whole embryos were homogenized in RNALater™ (Invitrogen) following exposure to petroleum substances. Total RNA was extracted using an RNeasy Micro Kit (Qiagen), which is designed for extraction and purification of total RNA from small cell and tissue samples. Total RNA was shipped to Albert Einstein College of Medicine where whole transcriptome microarray was performed using Affymetrix GeneChip Zebrafish gene 1.0 Array (Thermo Fisher Scientific).

Whole transcriptome microarray analysis

Both labeling and hybridization controls were included in microarray analysis for quality control. Poly-A RNA spike-ins were added to each sample in the beginning of processing. Labeling controls serve as independent controls for assay performance. Hybridization controls were added to the samples prior to array hybridizations and used to check that hybridization to the array was successful. After completion of QC analysis, each replicate within each test substance treatment level was treated as a group and compared amongst test substances and treatment levels.

Primary data analysis was with the transcriptome analysis console (TAC) 4.0 (ThermoFisher). Upon receipt of microarray data from Albert Einstein College of Medicine, .CEL files were imported into TAC 4.0. TAC 4.0 is software that acts as a graphical interface for visualization and analysis of microarray data. Each treatment
replicate was associated with an individual .CEL file, allowing direct comparison between either treatments or replicates depending on which .CEL files were selected. Secondary analysis was performed using STRING version 10.5 (Franceschini, Szklarczyk et al. 2013, Szklarczyk, Franceschini et al. 2015, Szklarczyk, Morris et al. 2017). STRING is a database comprised of known and predicted protein-protein interactions. Both direct and indirect protein interactions are included in the database. All recorded interactions come from computational prediction, knowledge transfer between organisms, and interactions aggregated from other databases (genomic context predictions, high-throughput lab experiments, conserved co-expression, automated textmining, and previous knowledge in databases) (Franceschini, Szklarczyk et al. 2013, Szklarczyk, Franceschini et al. 2015, Szklarczyk, Morris et al. 2017).

For STRING analysis, each gene symbol identified as being differentially expressed through TAC analysis was entered into the STRING website (https://string-db.org/cgi/input.pl?sessionId=LL53xoM9kuEj&input_page_show_search=on) and D. rerio was selected as the organism. Initial STRING results displayed limited protein interaction due to the somewhat limited gene input from differential expression analysis. To better understand potential interactions, the “+ more” function was used. The “+ more” function allows additional interactions to be added to the query to better capture complete pathway interactions. Each STRING analysis was then processed using the “clusters” k-means clustering function. K-means clustering in terms of protein-protein interaction allows for grouping of related proteins by function as long as knowledge of
the protein function is already known (Wagstaff, Cardie et al. 2001). Clustering analysis increased visual interpretation of STRING analysis.

RESULTS

Acute Toxicity, LC50

As a method of comparison between acute sublethal endpoints and traditional acute FET lethality based endpoints, LC50s were calculated for mortality by plotting mortality for all test substances (SRGO, CRGO, VHGO, Endicott crude oil, weathered crude oil, Dilbit, and Isotridecanol) against BE (mM). The calculated LC50 was 36.6 (27.3-45.9) mM (Figure 4). In principle, BE-based measurements normalize for the different bioavailabilities of the test substances. Therefore, a dataset-wide LC50 was calculated, 36.6 mM, which is similar to previous findings by Redman and others (2018) who noted that zebrafish embryos were of median sensitivity across ten different species.
Figure 4. Cumulative percent mortality for all test substances (SRGO, CRGO, VHGO, Endicott crude oil, weathered crude oil, Dilbit, and Isotridecanol). Percent mortality consists of the four OECD 236 endpoints which indicate lethality (coagulation, lack of somite formation, tail-bud detachment from yolk-sac, and presence of heartbeat). LC50 was calculated based on total percent mortality using Gompertz non-linear regression. The LC50 was calculated to be 36.6 (mM) with 95% confidence intervals of 27.3 – 45.9.
Sublethal effects

Several sublethal morphological abnormalities were documented across all test substances. Though none seemed more informative than the others, four sublethal effects occurred across all test substances (Table 1). These were pericardial and yolk sac edema, tail curvature, and lack of viability (nonviable) defined as embryos that were still alive at 96 hpf (hours post fertilization) but did not hatch. Post-test analysis focused on these four endpoints. The four sublethal endpoints occurred over a BE range spanning from 6.9 for non-viable in isotridecanol to 32.9 for non-viable in dilbit.
Table 1. Summary of individual sublethal endpoint EC25 calculations for each test substance

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Endicott crude oil</th>
<th>Weathered Troll crude oil</th>
<th>CRGO</th>
<th>VHGO</th>
<th>SRGO</th>
<th>Dilbit</th>
<th>Isotridecanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pericardial Edema</td>
<td></td>
<td></td>
<td>15.4</td>
<td>18.0</td>
<td>16.6</td>
<td>11.8</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>(14.9-15.9)</td>
<td>(16.0-20.0)</td>
<td></td>
<td></td>
<td></td>
<td>(10.0-13.5)</td>
<td>(12.4-13.2)</td>
</tr>
<tr>
<td>Yolk sac Edema</td>
<td></td>
<td></td>
<td>17.9</td>
<td>13.2</td>
<td>16.3</td>
<td>21.5</td>
<td>17.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(20.4-22.7)</td>
<td>(17.9-17.9)</td>
</tr>
<tr>
<td>Tail Curvature</td>
<td></td>
<td></td>
<td>7.8</td>
<td>12.4</td>
<td>14.0</td>
<td>15.5</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(12.4-12.4)</td>
<td>(12.9-15.0)</td>
</tr>
<tr>
<td>Non Viable</td>
<td></td>
<td></td>
<td>14.9</td>
<td>18.0</td>
<td>20.8</td>
<td>23.3</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(16.2-30.4)</td>
<td>(9.5-17.1)</td>
</tr>
<tr>
<td>Total % effect</td>
<td></td>
<td></td>
<td>14.3</td>
<td>15.1</td>
<td>24.1</td>
<td>21.1</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td>(12.3-16.3)</td>
<td>(13.5-16.8)</td>
<td></td>
<td></td>
<td></td>
<td>(6.7-41.7)</td>
<td>(18.4-23.7)</td>
</tr>
</tbody>
</table>

CRGO = Cracked gas oil; VHGO = Vacuum gas oil; SRGO = Straight run gas oil; Dilbit = Diluted bitumen; NA = No evaluation of this endpoint occurred.
As a consistent basis of comparison, EC25s for Endicott crude oil were used as the x-axis for cross comparison amongst test substances (Figure 5). A 1:1 line was overlaid in each figure to visualize sublethal endpoint sensitivity to each test substance. If sublethal endpoints were to cluster below the 1:1 line, this would indicate sublethal endpoints were more sensitive to the y-axis test substance. Conversely, if sublethal endpoints were to cluster above the 1:1 line, they would be more sensitive to the x-axis test substance.

In the majority of test substance comparisons, the sublethal endpoints generally track along the 1:1 line, indicating similar sensitivity between endpoints and substances. Sublethal endpoints occurring at higher BE-SPME numbers for dilbit could be related to the strong bimodal distribution of constituents that make up this product (light diluent and bitumen). The light diluent mixed with the bitumen increases the total area under the curve by stretching the total detection area compared to if bitumen or diluent (e.g. pentanes) were measured individually. Isotridecanol elicited sublethal responses on the BE-SPME scale before any of the other test substances (Endicott crude, diluted bitumen, SRGO, CRGO, VHGO, weathered troll). The lower BE detection for isotridecanol could be driven by the constituents that comprise isotridecanol (C\textsubscript{13} alcohol isomers with some C\textsubscript{11}-C\textsubscript{14} isomers) being more polar than hydrocarbons; therefore, when compared to hydrocarbon-based materials, isotridecanol appears to have a lower BE (µmol as 2,3 dimethylnaphthalene/mL PDMS) response.
Figure 5. Cross plots for individual sublethal effect comparison amongst test substances (VHGO = Vacuum gas oil, CRGO = cracked gas oil, weathered Troll = weathered Troll oil, dilbit = diluted bitumen, SRGO = straight run gas oil). Each axis is the BE (µmol as 2,3 dimethylnaphthalene/mL PDMS) scale. Endicott crude has been plotted as the standard metric for comparison and is plotted on all x-axis. The y-axis for each plot is of the remaining six test substances. Each data point within the figure are the calculated EC25 for the represented endpoint (blue = tail curvature, orange = non-viable, red = pericardial edema, green = yolk sac edema, black = combined sublethal effect EC25 calculation). The diagonal line is the 1:1 comparison to identify relationship of sublethal endpoint occurrence when comparing test substances.
Individual EC25s were calculated for each test substance to allow for cross test substance comparison of individual endpoints (Table 1). This analysis indicates that the sensitivity of all the observed endpoints are comparable—no single most sensitive sublethal endpoint was observed (Table 1), and the standard deviations of the majority of the endpoints within each test substance overlapped. The four sublethal endpoints were therefore combined and analyzed together, and a BE-based EC25 was calculated and considered as an exposure threshold that would help predict sublethal and lethal effects (Figure 6). The calculated EC25 was 13.3 (11.9-14.6) mM (Figure 3) with a characteristically steep BE-response slope.
Figure 6. Cumulative percent effect for all test substances. Percent effect consists of the four primary identified sublethal endpoint (pericardial and yolk sac edema, tail curvature, and non-viable embryo). EC25 was calculated based on total percent effect using Gompertz or logistic non-linear regression. The EC25 was calculated to be 13.2 (black horizontal line intersect with green linear regression) with 95% confidence intervals of 11.9-14.6.
Whole transcriptome microarray analysis

Whole transcriptome microarray was used to profile the developing zebrafish transcriptome following control, low, and high level exposure to two petroleum substances (Endicott crude oil and CRGO). Low and high level exposure was determined based on the phenotypic response observed during the sublethal morphology assessment experiments. Low level exposure was identified by organisms that were exposed to a petroleum substance but did not exhibit any abnormal sublethal response, whereas high level exposure was characterized by embryos that did exhibit abnormal sublethal effects. The BE response for Endicott crude at treatment levels selected for transcriptome analysis were 0, 8, and 21 mM for control, low and high respectively and for CRGO the BE response was 0, 6.8, and 33 mM for control, low, and high respectively.

Differential expression of transcriptome data was completed using default settings in the TAC software (Array type: ZebGene-1_0-st, Analysis type: Expression (Gene), Analysis version: 1, Summarization method: Gene level – RMA, Genome version: zv9, Annotation: ZebGene-1_0-st-v1.na36.zv9.transcript.csv). Differential expression was characterized using less than -2 or greater than 2 fold change when compared to another group and statistical analysis of expression data was completed with an empirical bayes (ebayes) method using a 0.05 P-value.

Transcriptome profiling for Endicott crude and CRGO was done by comparing each treatment level within each test substance (e.g. Endicott control was compared to Endicott low and Endicott high, Endicott low was compared to Endicott high; CRGO control was compared to CRGO low and CRGO high, CRGO low was compared to
CRGO high). An initial principal component analysis indicated grouping of each treatment level based on BE response and petroleum substance (figure 7). The summary of differential gene expression can be found in figure 8 and visualized in figure 9-14.
Figure 7. Principal component analysis (PCA) of treatment and replicate variability. Separation amongst treatments (different colors) shows varying expression profiles when exposed to increasing concentrations of petroleum substances.
Figure 8. Differentially expressed genes following comparison amongst treatments within petroleum substances. Y-axis is the treatment levels compared to provide differential expression profiles. X-axis is number of genes differentially expressed. Differential expression is ≤ -2 or ≥ 2 fold change. Grey bars are the sum of up and down-regulated genes, red bars are up-regulated genes, and green are down-regulated genes.
Following initial identification of differential expression, volcano plots were used to visualize changes in the transcription profile amongst treatment comparisons. As expected, the greatest differential expression was observed when comparing each substance treatment level high to the respective control (Endicott high vs. Endicott control and CRGO high vs. CRGO control) figure 9 and 10. The expression profile is dominated by up-regulation of genes primarily responsible for xenobiotic metabolism.
Figure 9. Volcano plot of whole transcriptome profile following Endicott high vs. Endicott control treatment comparison. Genes showing no significant expression difference from the control treatment (grey), ≥ 2 fold up-regulated genes (red) and ≤ -2 fold down-regulated genes (green). X-axis is fold change; Y-axis if p-value. (-log₁₀).
Figure 10. Volcano plot of whole transcriptome profile following CRGO high vs. CRGO control treatment comparison. Genes showing no significant expression difference from the control treatment (grey), ≥ 2 fold up-regulated genes (red) and ≤ -2 fold down-regulated genes (green). X-axis is fold change; Y-axis if p-value. (-log10).
The number of differentially expressed genes decreased for test substances when comparing the low treatment level to their respective control treatment (figure 11 and 12). Again, as observed in the high treatment levels, the profile is dominated by up-regulated genes primarily responsible for xenobiotic metabolism. This result is expected as the embryos exposed at this treatment level did not exhibit any sublethal morphological alterations compared to the control, so the primary expression response is focused on xenobiotic response genes.
Figure 11. Volcano plot of whole transcriptome profile following Endicott low vs. control treatment comparison. Genes showing no significant expression difference from the control treatment (grey), ≥ 2 fold up-regulated genes (red) and ≤ -2 fold down-regulated genes (green). X-axis is fold change; Y-axis if p-value. (-log_{10}).
Figure 12. Volcano plot of whole transcriptome profile following CRGO low vs. CRGO control treatment comparison. Genes showing no significant expression difference from the control treatment (grey), ≥ 2 fold up-regulated genes (red) and ≤ -2 fold down-regulated genes (green). X-axis is fold change; Y-axis if p-value. (-log₁₀).
Though comparison of differential expression between treatment levels and control is valuable for providing insight into how organisms are responding to low and high levels of petroleum substance exposure, a more interesting comparison is how the transcriptome profile changes when comparing the high treatment levels to the low treatment levels (figure 13 and 14). The low treatment levels did not exhibit any sublethal morphological effects and therefore, from an observational standpoint, can be said to be successfully managing exposure to petroleum substances. When high treatment levels are compared to low treatment levels, the expression profile differs from the initial comparison to control treatment levels in that the expression is more evenly distributed between down and up-regulated genes. The previous dominance by xenobiotic response elements is no longer differentially expressed.
Figure 13. Volcano plot of whole transcriptome profile following Endicott high vs. Endicott low treatment comparison. Genes showing no significant expression difference from the control treatment (grey), ≥ 2 fold up-regulated genes (red) and ≤ -2 fold down-regulated genes (green). X-axis is fold change; Y-axis if p-value. (-log_{10}).
Figure 14. Volcano plot of whole transcriptome profile following CRGO high vs. CRGO low treatment comparison. Genes showing no significant expression difference from the control treatment (grey), ≥ 2 fold up-regulated genes (red) and ≤ -2 fold down-regulated genes (green). X-axis is fold change; Y-axis if p-value. (-log₁₀).
Heatmaps identifying expression profiles were generated using differential gene expression (Log2) following treatment comparison substance (e.g. Endicott control was compared to Endicott low and Endicott high, Endicott low was compared to Endicott high; CRGO control was compared to CRGO low and CRGO high, CRGO low was compared to CRGO high) (Figure 15-22).
Figure 15. Heatmap of differentially expressed genes (≥ 2 fold up-regulated genes and ≤ -2 fold down-regulated) following Endicott exposure. Endicott Low treatment was compared to the Endicott Control treatment. Up-regulated pathways are red with an increase in activity indicated by deepening in red color and down-regulated pathways are blue with an inhibition in activity indicated by deepening in blue color.
Figure 16. Heatmap 1 of 2 of differentially expressed genes (≥ 2 fold up-regulated genes and ≤ -2 fold down-regulated) following Endicott exposure. Endicott High treatment was compared to the Endicott Control treatment. Up-regulated pathways are red with an increase in activity indicated by deepening in red color and down-regulated pathways are blue with an inhibition in activity indicated by deepening in blue color.
Figure 17. Heatmap 2 of 2 of differentially expressed genes (≥ 2 fold up-regulated genes and ≤-2 fold down-regulated) following Endicott exposure. Endicott High treatment was compared to the Endicott Control treatment. Up-regulated pathways are red with an increase in activity indicated by deepening in red color and down-regulated pathways are blue with an inhibition in activity indicated by deepening in blue color.
Figure 18. Heatmap of differentially expressed genes (≥ 2 fold up-regulated genes and ≤ -2 fold down-regulated) following Endicott exposure. Endicott Low treatment was compared to the Endicott High treatment. Up-regulated pathways are red with an increase in activity indicated by deepening in red color and down-regulated pathways are blue with an inhibition in activity indicated by deepening in blue color.
Figure 19. Heatmap of differentially expressed genes (≥ 2 fold up-regulated genes and ≤ -2 fold down-regulated) following CRGO exposure. CRGO Low treatment was compared to the CRGO Control treatment. Up-regulated pathways are red with an increase in activity indicated by deepening in red color and down-regulated pathways are blue with an inhibition in activity indicated by deepening in blue color.
Figure 20. Heatmap 1 of 2 of differentially expressed genes (≥ 2 fold up-regulated genes and ≤ -2 fold down-regulated) following CRGO exposure. CRGO High treatment was compared to the CRGO Control treatment. Up-regulated pathways are red with an increase in activity indicated by deepening in red color and down-regulated pathways are blue with an inhibition in activity indicated by deepening in blue color.
Figure 21. Heatmap 2 of 2 of differentially expressed genes (≥ 2 fold up-regulated genes and ≤ -2 fold down-regulated) following CRGO exposure. CRGO High treatment was compared to the CRGO Control treatment. Up-regulated pathways are red with an increase in activity indicated by deepening in red color and down-regulated pathways are blue with an inhibition in activity indicated by deepening in blue color.
Figure 22. Heatmap of differentially expressed genes (≥ 2 fold up-regulated genes and ≤ -2 fold down-regulated) following CRGO exposure. CRGO Low treatment was compared to the CRGO High treatment. Up-regulated pathways are red with an increase in activity indicated by deepening in red color and down-regulated pathways are blue with an inhibition in activity indicated by deepening in blue color.
To better understand expression patterns, STRING profiling was used to display known, predicted, and co-expression gene-gene interactions. The same comparisons were used for STRING analysis as were for the volcano plots except up-regulation and down-regulation were separated to differentiate up-or-down expression patterns. Figure 23 represents the types of interactions possible using STRING analysis. Based on Kyoto encyclopedia of genes and genomes (KEGG), pathways involved in xenobiotic metabolism and steroid hormone biosynthesis are similarly up regulated between the high treatment levels and control (figure 24 and 25). The STRING analysis indicated additional pathways that are up-regulated in the Endicott high treatment but not present for CRGO high treatment (glutathione metabolism, arachidonic acid metabolism, and primary bile acid biosynthesis, other metabolism pathways) and pathways that were up-regulated for CRGO high, but not Endicott were related to MAPK signaling and regulation of actin cytoskeleton (figure 24 and 25).
Figure 23. STRING analysis legend colored nodes are queried genes including first shell of interactions with queried genes. Colored lined connecting nodes indicate known interactions (light blue and pink), predicted interactions (green, red, and blue), and interactions defined from other sources such as text mining (yellow) and co-expression (black). (This figure is used with permission from Creative Commons Attribution 4.0 License. License agreement: https://creativecommons.org/licenses/by/4.0/legalcode)
Figure 24. Control vs. Endicott high up-regulated STRING analysis expression profile. Colored nodes are queried genes including first shell of interactions with queried genes. Colored lines connecting nodes indicate known interactions (light blue and pink), predicted interactions (green, red, and blue), and interactions defined from other sources such as text mining (yellow) and co-expression (black). (This figure is used with permission from Creative Commons Attribution 4.0 License. License agreement: https://creativecommons.org/licenses/by/4.0/legalcode)
Figure 25. Control vs. CRGO high up-regulated STRING analysis expression profile. Colored nodes are queried genes including first shell of interactions with queried genes. Colored lines connecting nodes indicate known interactions (light blue and pink), predicted interactions (green, red, and blue), and interactions defined from other sources such as text mining (yellow) and co-expression (black). (This figure is used with permission from Creative Commons Attribution 4.0 License. License agreement: https://creativecommons.org/licenses/by/4.0/legalcode).
The transcriptome profile is more similar between Endicott high and CRGO high when comparing down-regulated pathways (Figures 26 and 27). The same differentially expressed KEGG pathways are purine metabolism, one carbon pool by folate, phototransduction, and metabolic pathways. Melanogenesis related pathways were down-regulated in the CRGO high treatment was not shared between the two substances (melanogenesis).
Figure 26. Control vs. Endicott high down-regulated STRING analysis expression profile. Colored nodes are queried genes including first shell of interactions with queried genes. Colored lines connecting nodes indicate known interactions (light blue and pink), predicted interactions (green, red, and blue), and interactions defined from other sources such as text mining (yellow) and co-expression (black). (This figure is used with permission from Creative Commons Attribution 4.0 License. License agreement: https://creativecommons.org/licenses/by/4.0/legalcode).
Figure 27. Control vs. CRGO high down-regulated STRING analysis expression profile. Colored nodes are queried genes including first shell of interactions with queried genes. Colored lines connecting nodes indicate known interactions (light blue and pink), predicted interactions (green, red, and blue), and interactions defined from other sources such as text mining (yellow) and co-expression (black). (This figure is used with permission from Creative Commons Attribution 4.0 License. License agreement: https://creativecommons.org/licenses/by/4.0/legalcode)
Differential expression of low treatment levels compared to the control were less apparent than for the high treatment levels. There were not any identified pathways available for STRING analysis in down-regulated low level exposures, so all analysis was focused on up-regulated gene pathways. Pathway expression profile similarities between CRGO and Endicott low levels were xenobiotic metabolism, steroid hormone biosynthesis, tryptophan metabolism, and arachidonic acid metabolism. The Endicott low treatment exhibited eight different expression pathways than CRGO (metabolic pathways, retinol metabolism, drug metabolism via CYP P450, primary bile acid biosynthesis, ascorbate and aldarate metabolism, pentose and glucuronate interconversions, porphyrin and chlorophyll metabolism, drug metabolism – other enzymes, and starch and sucrose metabolism). CRGO only exhibited one different expression pathway from Endicott (proteasome) (figure 28 and 29).
Figure 28. Control vs. Endicott low up-regulation STRING analysis expression profile. Colored nodes are queried genes including first shell of interactions with queried genes. Colored lines connecting nodes indicate known interactions (light blue and pink), predicted interactions (green, red, and blue), and interactions defined from other sources such as text mining (yellow) and co-expression (black). (This figure is used with permission from Creative Commons Attribution 4.0 License. License agreement: https://creativecommons.org/licenses/by/4.0/legalcode).
Figure 29. Control vs. CRGO low up-regulation STRING analysis expression profile. Colored nodes are queried genes including first shell of interactions with queried genes. Colored lines connecting nodes indicate known interactions (light blue and pink), predicted interactions (green, red, and blue), and interactions defined from other sources such as text mining (yellow) and co-expression (black). (This figure is used with permission from Creative Commons Attribution 4.0 License. License agreement: https://creativecommons.org/licenses/by/4.0/legalcode)
STRING analysis of genes that were differentially expressed between low and high treatment levels profile the differences between an embryo is capable of managing exposure to low levels of petroleum substances and when the organism displays sublethal morphological alterations from high petroleum substance exposure. No KEGG pathways were identified in the Endicott low vs. Endicott high profile comparison. Alternatively, KEGG pathways for adherenes junction, WNT signaling, VEGF signaling, focal adhesion, regulation of actin cytoskeleton, ubiquitin mediated proteolysis, and MAPK signaling were identified for the CRGO low vs. CRGO high comparison (figure 30 and 31).
Figure 30. Endicott low vs. Endicott high up-regulated STRING analysis expression profile. Colored nodes are queried genes including first shell of interactions with queried genes. Colored lines connecting nodes indicate known interactions (light blue and pink), predicted interactions (green, red, and blue), and interactions defined from other sources such as text mining (yellow) and co-expression (black). (This figure is used with permission from Creative Commons Attribution 4.0 License. License agreement: https://creativecommons.org/licenses/by/4.0/legalcode).
Figure 31. CRGO low vs. CRGO high up-regulation STRING analysis expression profile. Colored nodes are queried genes including first shell of interactions with queried genes. Colored lines connecting nodes indicate known interactions (light blue and pink), predicted interactions (green, red, and blue), and interactions defined from other sources such as text mining (yellow) and co-expression (black). (This figure is used with permission from Creative Commons Attribution 4.0 License. License agreement: https://creativecommons.org/licenses/by/4.0/legalcode).
The final profile comparison was between the low and high treatment levels for down-regulated pathways. Similarities amongst CRGO and Endicott KEGG pathways include phototransduction, melanogenesis, and tyrosine metabolism. The Endicott profile also indicated differential expression in purine metabolism, metabolic, and one carbon pool by folate pathways. The CRGO profile indicated differences in ubiquinone and other terpenoid-quinone biosynthesis, phenylalanine metabolism, progesterone-mediated oocyte maturation, gap junction, tight junction, and adrenergic signaling in cardiomyocytes pathways (figure 32 and 33).
Figure 32. Endicott low vs. Endicott high down-regulation STRING analysis expression profile. Colored nodes are queried genes including first shell of interactions with queried genes. Colored lines connecting nodes indicate known interactions (light blue and pink), predicted interactions (green, red, and blue), and interactions defined from other sources such as text mining (yellow) and co-expression (black). (This figure is used with permission from Creative Commons Attribution 4.0 License. License agreement: https://creativecommons.org/licenses/by/4.0/legalcode)
Figure 33. CRGO low vs. CRGO high down-regulation STRING analysis expression profile. Colored nodes are queried genes including first shell of interactions with queried genes. Colored lines connecting nodes indicate known interactions (light blue and pink), predicted interactions (green, red, and blue), and interactions defined from other sources such as text mining (yellow) and co-expression (black). (This figure is used with permission from Creative Commons Attribution 4.0 License. License agreement: https://creativecommons.org/licenses/by/4.0/legalcode).
DISCUSSION

Though test substances spanned a range of petrochemical products from crude oil (Endicott crude) to a branched alcohol (isotridecanol), reasonably consistent sublethal morphological abnormalities were observed amongst all substances tested. The first objective was to identify endpoints characteristic of hydrocarbon exposure. Isotridecanol was used as a non-hydrocarbon petrochemical to provide data on whether sublethal effect varied compared to hydrocarbon substances. Other endpoints such as craniofacial malformation, abnormal cardiac function, and fin deformity were observed throughout testing but did not occur at a BE response lower than pericardial and yolk sac edema, nonviable, or tail curvature or did not occur with any consistency. Sublethal effects observed in isotridecanol were similar to those of hydrocarbon exposure at relatively similar BE response levels. Though the BE response was somewhat lower than the rest of the petroleum substances, this was attributed to the slightly more polar nature of isotridecanol. We successfully identified four sublethal effects that occurred across six substances consistently, and these four sublethal effects were considered biologically plausible. These results are not surprising, as all substances tested were expected to impart toxicity via narcosis.

The second objective of this work was to identify a sublethal threshold using BE-SPME, which would aid in the prediction of sublethal effects. Eleven sublethal endpoints were planned prior to test initiation, but following test completion, four of the 11 endpoints were identified to occur consistently in each test. Upon further analysis, all endpoints occurred at a similar BE level across petroleum substances, so the sublethal
endpoints were combined and defined as a cumulative effect. Cumulative effect was plotted against BE (mM) to establish an exposure threshold to characterize relative sensitivity of sublethal and lethal endpoints for FET exposed to hydrocarbons. The identified BE threshold in relation to sublethal endpoints can improve risk assessment by relating BE response to sublethal and lethal endpoints and by providing a method to screen petroleum substances for potential hazard using BE-SPME (Hodson 2017).

The third objective of this work was to identify an endpoint or endpoints that could be used as a leading indicator of toxicity. An EC25 was calculated from the combined sublethal effects observed across all test substances. A sharp and consistent sublethal response curve was observed when sublethal effects were plotted against BE (mM) (figure 34B), while no quantifiable response could be discerned when the effects were plotted against nominal loading (mg/L) (34A). The sharp response indicates little difference between sublethal effects regardless of endpoint (EC10, EC25, EC50) chosen. The EC25 was selected as the most appropriate effect threshold because there is clear differentiation from background sublethal effects where the EC10 did not provide sufficient separation and an EC25 is more sensitive than the EC50.

The fourth objective of this work was to describe the whole transcriptome profile of developing zebrafish following exposure to petroleum substances. The transcriptome profile clearly shows differential expression in relation to petroleum substance exposure. As expected, the expression profile indicates a strong xenobiotic response profile in up-regulated genes; however, when high treatment levels are compared to low level exposure, the majority of xenobiotic response pathways are no longer differentially
expressed, and expression profiles shift to consistent differential expression in down-regulated pathways such as purine metabolism, phototransduction, melanogenesis, metabolic pathways, and one carbon pool by folate (Table 2 and 3).
Figure 34. All sublethal effects combined for each test substance to visualize total percent effect in reference to nominal loading (mg/L) of test substance and against measured BE-SPME (µmol as 2,3-dimethylnaphthalene/mL PDMS) response. (A) Total percent sublethal effect against nominal loading (mg/L) of test substances. (B) Total percent sublethal effect against BE-SPME measurements of test substance.
Since we observed similar sublethal responses across all substances tested, we suggest using a combined sublethal approach in the derivation of thresholds or effects data, as no one endpoint is more sensitive than another (identified by the large overlap in standard deviations of EC25 calculations). The EC25 observed from combined sublethal effects was 13.3 (11.9-14.6) mM, whereas the EC50 for lethality was 36.6 (27.3-45.9) mM. Each test was designed to limit mortality and assess sublethal endpoints expected to occur most frequently following exposure to petroleum substances. The goal was to balance exposure concentrations on the edge of mortality to elicit a clear sublethal response.

Developmental sublethal endpoints are of interest for their potential to increase read across capabilities from fish embryo toxicity tests to fish acute/chronic toxicity tests. The most recurrent argument against using lethal and sublethal fish embryo data is predicated on the small number of reliable data sets which directly compare the FET to the acute and chronic fish toxicity tests, especially data from UVCBs. As highlighted earlier, Redman and others (2018) showed that zebrafish embryos are of median sensitivity when plotted amongst other organisms.

When plotting our data (acute sublethal fish embryo) with chronic toxicity data from Redman and others (2018), we see a similar pattern of acute zebrafish embryo sublethal effects occurring at a similar BE response as chronic 28 d growth inhibition (EC20) in rainbow trout. A similar response when comparing sublethal acute data to chronic fish data indicates that sublethal effects observed in the zebrafish embryo tests could be predictive of chronic toxicity (figure 35) (Redman, Butler et al. 2018).
Figure 35. Chronic rainbow trout growth inhibition data (red squares) plotted with acute sublethal effect (blue circles) data.
Describing toxicity in relation to individual constituents or fractions found within petroleum substances can be difficult, and we advise against this practice. Individual constituents and fraction ratios can vary amongst different petroleum substances as well as internally between substances of the same class (Redman and Parkerton 2015) depending on how the WAF is generated. Therefore, associating specific toxicity (e.g. cardio, morphological, developmental) to particular constituents or fractions within petroleum substances is cautioned as it can be difficult to isolate the observed toxicity and associate it with the corresponding constituent or fraction.

Previous research has identified specific PAH structures (3+ ring PAHs) within petroleum substances as drivers of toxicity, specifically cardiotoxicity (Incardona, Collier et al. 2004, Incardona, Day et al. 2006, Incardona 2017). We do not disagree that individual PAHs have the potential to be specifically acting, but we do not see the same targeted effects as the primary source of toxicity when petroleum substances are used as the test substances compared to individual or simple defined mixtures. If particular constituents (3+ ring PAHs) were to drive toxicity, we would assume those petroleum substances with a greater number of 3+ ring PAHs would be inherently more toxic. This was not observed in this research (figure 36).
Figure 36. Fractional composition of petroleum substances used in testing.
All sublethal and lethal toxicity was observed within the same BE range when sublethal effects were combined as percent effect rather than identified individually (figure 3 and 1, respectively). Therefore, when assessing toxicity of petroleum substances as a whole, the traditional “nonpolar narcosis” model is a better fit for risk assessment. Geier and others (2018) noted a similar pattern when assessing a variety of 123 individual PAHs by recognizing that the relationship between toxicity and chemical structure was complex. Geier and others (2018) emphasize the point that risk assessment should not be based on individual constituents found within a petroleum substance, but instead that the whole substance should be evaluated when assessing toxicity in an aqueous environment.

The associated toxic units (TUs) of each fraction was calculated using PETROTOX version 3.06 (Redman, Parkerton et al. 2012). A TU in terms of this paper is described as the concentration in water divided by the observed endpoint (TU = \( \Sigma C_w/LL50 \)). Each section of the chart is equal to the corresponding contribution of each fraction to the observed toxicity (EL50). As observed in figure 5, the contributing fractions to toxicity are not limited to 3+ ring PAHs. If toxicity assessments were based upon the conclusion that 3+ ring PAHs are the driver of toxicity, there is potential to inaccurately estimate toxicity as other fractions within the petroleum substance play an integral role in overall substance toxicity. The variable composition of petroleum substances can be observed in figure 37.
Figure 37. Toxic Unit (TU) contribution calculation of petroleum substance fractions using PETROTOX. TU is the concentration in water divided by the observed endpoint (TU=\(C_w/LL50\)). Each section of the chart is equal to the corresponding fractions contribution to the observed toxicity (EL50).
The principal component analysis from figure 7 shows grouping of treatment levels (control, low, and high) within each test substance (CRGO and Endicott crude). Grouping of treatment levels per test substances indicates distinct gene expression profiles dependent upon exposure concentration. Distinct expression profiles were somewhat expected since samples selected for transcriptome analysis were based on observed sublethal effects. Generally, low level exposure in both CRGO and Endicott crude would be expected to result in baseline xenobiotic response. Baseline xenobiotic response could include phase I and II metabolism enzymes such as those belonging to the Cytochrome P450 family (Donnarumma, De Angelis et al. 1988, Xu, Li et al. 2005, Matsuo, Gallagher et al. 2008, Wassmur 2011). Primarily in low level exposure we observed a transcriptome profile that is primarily dominated by up-regulation of metabolism enzymes (Table 2). There were no sublethal effects observed in these embryos, so the transcriptomic response would be expected to resemble one that successfully mediates petroleum substance exposure.

Alternatively, when comparing high treatment level exposure to their respective exposure control, the transcriptome profile captures the expected metabolism enzymes that we observed in the low level exposure, but additionally, high exposures shift to include some biological development pathways that are down-regulated during exposure (Table 3). Down-regulated pathways in high treatment levels were purine synthesis, one carbon pool by folate, phototransduction, and metabolic pathways for both Endicott and CRGO. Additionally, melanogensis was down-regulated in the CRGO high treatment. Purine metabolism, phototransduction, and melanogensis are of particular interest.
because clear and related effects have been identified across multiple organisms. Errors in purine metabolism have been linked to a range of phenotypic, neurologic, and hematologic abnormalities (Kamatani, Jinnah et al. 2014, Hubert and Sutton 2017). Visual phototransduction is the translation of light into an electrical signal in the retina via a photochemical reaction (Chong, Smith et al. 2010). Inhibition of phototransduction could lead to visual impairment through degradation of eyesight or reduction in visual development. Melanogenesis is the production of melanin pigments and abnormal production of melanin can lead to acute dermatological problems lending the organism susceptible to skin damage (D'Mello, Finlay et al. 2016, Pillaiyar, Namasivayam et al. 2018). Initial transcriptome profile comparison of both low and high treatment exposure to the control provided insight into how embryonic zebrafish responded to petroleum substance exposure compared to a control environment. However, comparing petroleum substance exposure to a control may not be the most appropriate analysis to fully understand environmental impact since organisms are able to successfully manage low levels of petroleum substance exposure (Yang, Kemadjou et al. 2007, Wang, Biales et al. 2016, Schüttler, Reiche et al. 2017).

To better assess high exposure, the high treatment level transcriptome profile was compared to the low level transcriptome profile. As expected, this comparison obscured the majority of baseline metabolism response observed following control comparison to low and high level exposures. Up-regulated pathways not originally apparent in CTRL vs. CRGO comparisons that were now apparent from the CRGO low vs. high comparison included pathways related to cell-cell interaction (adherens junction), cell fate
determination, cell migration, cell polarity, neural patterning, and organogenesis during embryonic development (Wnt signaling) (Komiya and Habas 2008), and angiogenesis (VEGF signaling pathway) (Holmes and Zachary 2005, Sherbet 2011). Interestingly, the Endicott low vs. Endicott high comparison did not reveal any new differentially expressed pathways (Table 2).

Down-regulated pathways observed when comparing low and high treatments reinforced some of the same pathways originally observed in the high vs. control comparison. Pathways that were similarly impacted in the Endicott comparison were Purine metabolism, one carbon pool by folate, phototransduction, and metabolic pathways. Pathways that were not originally were melanogensis and tyrosine metabolism. The CRGO high vs. low comparison shared two differentially expressed pathways (phototransduction and melanogensis) but also identified seven new pathways not observed in the CRGO high vs. control comparison (tyrosine metabolism, ubiquinone and other terpenoid-quinone biosynthesis, phenylalanine metabolism, progesterone-mediated oocyte maturation, gap junction, tight junction, and adrenergic signaling in cardiomyocytes). Overall, it seems down-regulation may provide better insight into exposure impact due to the relative consistency in transcriptome alteration observed across treatment comparison (Table 3).

The consistent alteration in purine metabolism, phototransduction, and melanogensis observed in down-regulated pathways following exposure to high treatments has previously been observed following thyroid related inhibition (Bagci, Heijlen et al. 2015, Baumann, Ros et al. 2016). Thyroid activity has been linked to a
number of key physiological development processes in zebrafish (Power, Llewellyn et al. 2001, Bagci, Heijlen et al. 2015, Nelson, Schroeder et al. 2016, Stinckens, Vergauwen et al. 2016). As thyroid-related effects are well-documented it is reasonable to think sustained alteration in thyroid activity could lead to an impact on zebrafish embryo development. However, exposure concentration is one key aspect of assessing either lethal or sublethal toxicity and in this research, thyroid-related activity is not predictive nor does it precede the phenotypic effects observed during exposure to petroleum substances or petrochemicals.

The four sublethal endpoints which occurred most frequently are not unique to these test substances (Hill, Bello et al. 2004, Jezierska, Ługowska et al. 2009, Knöbel, Busser et al. 2012). Multiple mechanisms seem to drive pericardial and yolk sac edema. There are mechanisms that are well-understood are oxidative stress, loss of wwox (WW Domain Containing Oxidoreductase) expression, and Leucine-rich Repeat Containing protein (LRRC10) (Hill, Bello et al. 2004, Kim, Antkiewicz et al. 2007, Chen, Carney et al. 2008, Madison, Hodson et al. 2015, Tsuruwaka, Konishi et al. 2015). Then there are other mechanisms where the initiating event is less clear, such as failure to block water from entering the pericardial cavity, developmental inhibition, or kidney failure (Villalobos, Soimasuo et al. 1996, Incardona, Collier et al. 2004). Though many events may lead to either yolk sac or pericardial edema, the initiating event involved in either edema could be mediated through the aryl hydrocarbon receptor (Incardona, Day et al. 2006). Tail curvature is less understood but is attributed to inhibition of both skeletal and musculature development (Fraysse, Mons et al. 2006). Finally, lack of hatch in non-
viable embryos could be the inability to break though the chorion due to inhibiting morphological abnormalities, or the lack of zebrafish hatching enzyme 1 (ZHE1). The hatching enzyme ZHE1, is responsible for partially digesting and swelling of the chorion, to weaken and enable the embryo to hatch (Sano, Inohaya et al. 2008)

For this research we were unable to conclude that thyroid activity or any other specific pathway drives a portion of the sublethal effects noted during this research, as the reported transcriptomic data are insufficient to link transcriptome activity to phenotypic effects. The transcriptome profiling was meant to be an exploratory effort and identify potential areas of interest. We do have sufficient data to indicate thyroid activity would be an interesting area of research to explore and that additional research would be needed to link the phenotypic effects observed during zebrafish embryo testing to thyroid inhibition. Additionally, we must not rely on one individual pathway, regardless of how well defined the pathway is, to describe or predict sublethal effects or mortality due to petroleum substances acting via a non-specific mode of action (narcosis) (Knapen, Vergauwen et al. 2015).
Table 2. STRING analysis of up-regulated transcriptome profiles using KEGG pathway identifiers.

<table>
<thead>
<tr>
<th>STRING analysis KEGG pathway up-regulation</th>
<th>CTRL vs. Endicott low</th>
<th>CTRL vs. CRGO low</th>
<th>CTRL vs. Endicott high</th>
<th>CTRL vs. CRGO high</th>
<th>CRGO low vs. CRGO high</th>
<th>Endicott low vs. Endicott high</th>
</tr>
</thead>
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<tr>
<td>Steroid hormone biosynthesis</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>Metabolic pathways</td>
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<td></td>
<td></td>
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<td>X</td>
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<td>Retinol metabolism</td>
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<td>Drug metabolism –CYP450</td>
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<tr>
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<tr>
<td>Ascorbate and aldarate metabolism</td>
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<td>Pentose and glucuronate Interconversions</td>
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<td>Porphyrin and chlorophyll metabolism</td>
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<td>Drug metabolism –other enzymes</td>
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<td>Starch and sucrose metabolism</td>
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<tr>
<td>Proteasome</td>
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<tr>
<td>Adherens junction</td>
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<td></td>
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<td>X</td>
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<td>Wnt signaling pathway</td>
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<td>VEGF signaling pathway</td>
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<td>X</td>
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<tr>
<td>Focal adhesion</td>
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<td>X</td>
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<tr>
<td>Ubiquitin mediated pathway</td>
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<td>X</td>
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</table>
Table 3. STRING analysis of down-regulated transcriptome profiles using KEGG pathway identifiers.

<table>
<thead>
<tr>
<th>STRING analysis KEGG pathway down-regulation</th>
<th>CTRL vs. Endicott low</th>
<th>CTRL vs. CRGO low</th>
<th>CTRL vs. Endicott high</th>
<th>CTRL vs. CRGO high</th>
<th>CRGO low vs. CRGO high</th>
<th>Endicott low vs. Endicott high</th>
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</thead>
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<tr>
<td>Purine metabolism</td>
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<td>One carbon pool by folate</td>
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<td>X</td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Metabolic pathways</td>
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<td></td>
<td></td>
<td></td>
<td>X</td>
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<tr>
<td>Melanogenesis</td>
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<td>X</td>
<td>X</td>
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<td>Tyrosine metabolism</td>
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<td>X</td>
<td>X</td>
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<tr>
<td>Ubiquinone and other terpenoid-quinone biosynthesis</td>
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<td>X</td>
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<td>Phenylalanine metabolism</td>
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<td>X</td>
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<td>Progesterone-mediated oocyte maturation</td>
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<td>Gap junction</td>
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<td></td>
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<td>X</td>
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<tr>
<td>Tight junction</td>
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<td></td>
<td>X</td>
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<tr>
<td>Adrenergic signaling in cardiomyocytes</td>
<td></td>
<td></td>
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</tbody>
</table>
CONCLUSIONS

No individual sublethal effect was more sensitive than the others as an indicator of toxicity across the substances tested. Therefore, when assessing toxicity of petroleum substances we recommend accounting for all sublethal effects rather than making risk assessment decisions based on individual sublethal responses. The varied sublethal effects indicated non-specific mode of action; therefore, non-polar narcosis can explain both the lethal and sublethal toxicity observed during testing. There were no observed connections between the sublethal and lethal response and a particular fraction within these petroleum substances. Therefore, when assessing petroleum substances for toxicity, associating sublethal and lethal effects with particular constituents is discouraged as it can lead to inaccurate assessment of toxicity.

Four sublethal effects (pericardial and yolk sac edema, tail curvature, and lack of embryo viability) occurred consistently across all test substances. The varied occurrence of these four endpoints reinforces the non-specific mode of action, nonpolar narcosis model. Finally, BE-SPME was shown to be convenient method to measure the bioavailable exposure of petroleum substances and equate BE response to observed lethal and sublethal effects across different classes of petroleum substances.

Whole transcriptome profiling indicated up-regulated pathways primarily comprise of metabolism and xenobiotic response enzymes. The observed up-regulated gene response was expected as organismal response to petroleum substances and petrochemicals is well-documented. Down-regulated pathways that show consistent response across high concentrations indicated potential thyroid related activity. However,
all differential expression in the down-regulated pathways was observed at concentrations which already resulted in sublethal effects. Therefore, we cannot conclude down-regulated differential expression is predictive of sublethal effects.

Overall, this research provides additional data to support the current body of fish embryo toxicity data in a step forward to adopting the zebrafish embryo test for alternative uses such as a replacement for the fish acute toxicity test. In addition to an acute fish toxicity test replacement, the sublethal BE-SPME response data in comparison to chronic fish toxicity data indicates a potential application of alternative fish embryo endpoints to be indicative of chronic fish toxicity. Additional BE-SPME chronic fish toxicity data is needed to support this conclusion, but this first step provides a base of evidence that has identified an analytical method as well as petroleum substances and a petrochemical for chronic fish toxicity comparison.
Reference:


OECD (2012). "Fish Toxicity Testing Framework." Joint meeting of the chemicals committee and the working party on chemicals, persistent and biotechnology Series on Testing and Assessment.


109


