Analysis of Sublethal Toxicity in Developing Zebrafish Embryos Exposed to a Range of Petroleum Substances

Bryan M. Hedgpeth, Aaron D. Redman, Rebecca A. Alyea, Daniel J. Letinski, Martin J. Connelly, Josh D. Butler, Heping Zhou, and Mark A. Lampia

Abstract: The Organisation for Economic Co-operation and Development (OECD) test guideline 236 (fish embryo acute toxicity test; 2013) relies on 4 endpoints to describe exposure-related effects (coagulation, lack of somite formation, tail-bud detachment from the yolk sac, and the presence of a heartbeat). *Danio rerio* (zebrafish) embryos were used to investigate these endpoints along with a number of additional sublethal effects (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 5-d exposures to 7 petroleum substances. The substances investigated included 2 crude oils, 3 gas oils, a diluted bitumen, and a petrochemical containing a mixture of branched alcohols. Biomimetic extraction–solid-phase microextraction (BE–SPME) was used to quantify freely dissolved concentrations of test substances as the exposure metric. The results indicated that the most prevalent effects observed were pericardial and yolk sac edema, tail curvature, and lack of embryo viability. A BE–SPME threshold was determined to characterize sublethal morphological alterations that preceded embryo mortality. Our results aid in the understanding of aquatic hazards of petroleum substances to developing zebrafish beyond traditional OECD test guideline 236 endpoints and show the applicability of BE–SPME as a simple analytical tool that can be used to predict sublethal embryo toxicity.

INTRODUCTION

Exposure to individual hydrocarbons, simple hydrocarbon mixtures, and complex hydrocarbon-based 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 et al. 1999; Incardona et al. 2004, 2013; Hicken et al. 2011; Philibert et al. 2016; Hodson 2017). Research characterizing toxic effects from exposure to individual hydrocarbons and hydrocarbon mixtures typically involves an acute endpoint (median lethal concentration [LC50]) or the observation of chronic effects (edema, cardiac malformation, craniofacial malformation, reduced swim speed, prey capture, or predator avoidance) that may impact organism fitness (Carls et al. 1999, 2008; Hicken et al. 2011; Incardona et al. 2013; Brown et al. 2016; Philibert et al. 2016; Hodson 2017). Organisation for Economic Co-operation and Development (OECD) test guideline 236 describes a fish embryo acute toxicity (FET) test that identifies 4 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 the tail bud from the yolk sac, and lack of a heartbeat; Organisation for Economic Co-operation and Development 2013; Braunbeck et al. 2015). When the focus is directed toward endpoints that only describe lethality, an opportunity to capture broader exposure-related sublethal effects is neglected. Often a suite of sublethal morphological effects precedes mortality,
which could provide a linkage between sublethal and lethal toxicity in developing zebrafish (Braunbeck et al. 2015). Furthermore, little research has attempted to identify exposure thresholds at which observation of sublethal effects becomes a practical predictor of hydrocarbon toxicity (Knight et al. 2009; Hoff et al. 2010; Kavlock and Dix 2010; Shukla et al. 2010; Kavlock et al. 2012; Rotroff et al. 2013).

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 et al. 2004; Fraysse et al. 2006; Brannen et al. 2010; Belanger, Rawlings, and Carr 2013). Identifying a level at which exposure to petroleum substances would consistently result in developmental abnormalities would provide an improved basis for risk assessment of petroleum substances in the environment, and would support laboratory-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 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 the most common methods of managing substances with limited aqueous solubility is to use a carrier solvent such as dimethyl sulfoxide. Using a carrier solvent can increase exposure concentrations above their solubility limit and can lead to artefactual effects due to physical oiling of the organism, as well as spurious interpretation of concentration–response data. With increasing regularity, data generated in one toxicity assay are being extrapolated to predict toxicity to other organisms. Read-across efforts such as these mandate correct dosing and analysis of petroleum substance toxicity. Recently, efforts to link zebrafish toxicity data to mammalian developmental toxicity have shown varying degrees of agreement (64–100%; Brannen et al. 2010; Hemsen et al. 2011; Hill et al. 2011; Padilla et al. 2011; Sipes et al. 2011; Selderslåghs et al. 2012). One of multiple drivers of variability among these studies could be dosing or analytical techniques (Sipes 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 on, data extrapolation will not be useful.

Zebrafish (Danio rerio) are a convenient model species widely used for toxicity testing and drug discovery (Hill 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 et al. 2005). Proper assessment of petroleum substance exposure can be difficult, because hydrocarbon constituents have widely different bioavailabilities based on their physicochemical properties such as water solubility and vapor pressure (Redman 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 to correlate morphological effects. Often metrics such as total petroleum hydrocarbon (TPH) or total polycyclic aromatic hydrocarbon (TPAH) are used to characterize petroleum constituents within exposure media. Both TPH and TPAH measure bulk hydrocarbons, which is not necessarily a measurement of the bioavailable fraction, and therefore such metrics can lead to an inaccurate assessment of the 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. This method 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 et al. 2000; Redman et al. 2014, 2018; Redman and Parkerton 2015). Because BE–SPME is a measure of the bioavailable fraction in an aqueous medium (Letinski 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 et al. 2002; Redman et al. 2014). The benefits of BE–SPME are attributed to the polydimethylsiloxane (PDMS)-coated fiber, which acts as a surrogate for organismal lipid (Verbruggen et al. 2000).

Individual hydrocarbons have been shown to have an adverse impact on developing fish embryos (Carls et al. 1999, 2008; Barron et al. 2004; Incardona et al. 2004; Carls and Meador 2009; Hicken et al. 2011; Pauka et al. 2011; Perrichon et al. 2016; Hodson 2017). However, attributing specific effects to individual constituents found within crude oil can be difficult when one is testing complex or undefined mixtures. When one is observing sublethal effects, compositional differences among fresh, weathered, and other fractions of crude oil might be expected to result in varying sublethal effects; however, differences in sublethal effects are seldom observed (Ernst et al. 1977; Carls et al. 1999; Incardona et al. 2004; Carls and Meador 2008; González-Doncel et al. 2008; Carls and Meador 2009; Hicken et al. 2011; Pauka et al. 2011; Belanger et al. 2013; Incardona et al. 2013; Jung et al. 2013; Perrichon 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 cardiotoxicity mediated
by the aryl hydrocarbon receptor (Barron et al. 2004; Incardona et al. 2004, 2006; Billiard et al. 2006; González-Doncel et al. 2008; Carls and Meador 2009; Van Tiem and Di Giulio 2011; Brown et al. 2016; Hodson 2017; Incardona 2017).

In an effort to better understand exposure to complex hydrocarbon substances, the present study describes the exposure of zebrafish embryos to increasing concentrations of 7 petroleum substances. We used sublethal acute endpoints, which cannot currently be linked to chronic or population-level effects; however, we felt that if we were able to make a connection/correlation to the 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 aimed 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 consistent endpoints that would help to characterize petroleum substance exposure. For this objective, isotridecanol (a branched alcohol) was used to compare sublethal effects from a nonhydrocarbon petrochemical with hydrocarbon-based petroleum substances. The second objective was to identify a bioavailable exposure threshold using BE-SPME that would aid in the prediction of sublethal effects. Concentration–response exposures for 7 petroleum substances were quantified using BE-SPME as a surrogate for critical body burden. With 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.

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 is a medium-weight crude oil from the Alaskan North Slope region (USA) varying in hydrocarbons from C5 to C80. Twenty percent weathered Troll oil is a light sweet crude from the Norwegian oil fields primarily made up of C15 to C80. The CRGO and VHGO fields primarily consist of saturated hydrocarbons ranging from C9 to C30, and SRGO is comprised of hydrocarbons primarily in the range of C9 to C25. Dilbit is an extensively bio-degraded bitumen diluted with natural gas condensate and consists primarily of asphaltenes in the C50 to C80 range, with the condensate ranging from C2 to C8. Isotridecanol consists of branched alcohols in the range of C10 to C14 (CONCAWE Classification and Labelling Task Force 2015).

Culture and generation of fertilized zebrafish embryos

Adult male and female D. rerio were acquired from Aquatic Research Organisms 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 (Pyrex®, Corning) were used as embryo collection vessels. Collection vessels were placed into breeding aquaria 1 h 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 vessels were removed from the aquaria, and eggs were collected and sorted for fertilization under an Olympus SZX12 stereo microscope. Sufficient fertilized embryos were collected and distributed into 50-ml beakers within 90 min of fertilization. Temporary holding vessels (50-mL beakers) were filled with treatment solution until individual embryos could be transferred into their respective replicate vials (20-mL scintillation vials). Temporary holding vessels were used to ensure that embryo exposure began within the OECD test guideline 236 recommended 90-min test initiation window (Organisation for Economic Co-operation and Development 2013).

Extended FET testing and identification of endpoints

The principle intention of OECD test guideline 236 is to identify lethality utilizing 4 observations (coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail bud from the yolk sac, and lack of a heartbeat) in the determination of an LC50 (Organisation for Economic Co-operation and Development 2013). In addition to the OECD test guideline 236 endpoints, we characterized developmental abnormalities to better understand the role that sublethal effects can have in the evaluation of toxicity from exposure to complex petrochemicals (Brannen et al. 2010). Each exposure was completed in general agreement with OECD test guideline 236 (Organisation for Economic Co-operation and Development 2013); 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. 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 et al. 2010; Panzica-Kelly 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 4 traditional OECD test guideline 236 endpoints (coagulation, tail bud detachment, somite development, and heartbeat; Organisation for Economic Co-operation and Development 2013) along with sublethal developmental endpoints (pericardial and yolk sac edema,
notochord, tail, fins, craniofacial, brain, swim bladder, viability, and heart rate) occurred using the 96-h observation data. The lack of viability was identified by embryos that were still alive at 96 h post fertilization (hpf) but did not hatch. After completion of the 96-h observation, 5 of the total 20 replicates in each treatment were euthanized 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. After completion of the 120-h observations, all remaining embryos/larvae were euthanized using a buffered tricaine methanesulphonate solution per OECD test guideline 236 (Organisation for Economic Co-operation and Development 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 post-test analysis. All control embryos and test substance–exposed embryos that displayed pericardial edema were imaged at 10× using an Olympus DP72 camera attached to an Olympus CKX41 inverted microscope. Images were analyzed using Olympus CellSens Dimension software Ver 1.3, 2010.

Postimage analysis (CellSens Dimensions) of pericardial edema allowed for area (µm²) measurements of the pericardial cavity. Pericardial edema occurrences were documented using 2 methods. The first method was recording either the presence or absence of edema; the second method was intended to quantify the pericardial cavity area (in µm²) to characterize both normal and abnormal pericardial cavity size in relation to exposure. All incidents of pericardial edema were compared with a library of control pericardial cavity areas compiled from the 7 test substances we discuss. 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 s to provide average beats/min. All observations were recorded at 96 hpf except for jaw development, which was assessed at 120 hpf.

**Statistical analysis**

Nonlinear regression analysis was used to calculate effect concentration, 25% (EC25) and LC50 values using JMP 13 (SAS, 2016) and graphed against plotted data using Graphpad 6 (Ver 6.07. 2015). The EC25 endpoint calculation used a nonlinear regression model of best fit, which was either Gompertz 3 P or Logistic 3 P from all models available (Probit, logistic, Gompertz, Hill, and Weibull). The LC50 calculation used a Gompertz 3 P nonlinear regression model. The EC25 was selected as the most appropriate effect threshold because there was clear and reliable differentiation from background sublethal effects when the EC10 did not provide sufficient separation and an EC25 was more sensitive than the EC50 (Brannen et al. 2010; Redman et al. 2018).

**Preparation and administration of test substance**

Soft water was prepared following “Standard methods for the examination of water and wastewater Table 8010:I!” (Eaton et al. 2005), and aliquoted into 4-L aspirator bottles with a tubing outlet (Pyrex, #1220-4 L; Coming). Each bottle was customized to fit a solid Teflon® screw cap and filled to the top, which allowed each bottle to be sealed to minimize loss via volatilization. Test solutions were prepared using a standard water accommodated fraction (WAF) method by adding a test substance to dilution water using glass and stainless steel gastight 1700 series syringes (Hamilton® Singer 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 isorotidecanol. 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 treatment level consisted of 20 individual scintillation vials with each vial containing one embryo. Each vial was sealed with no headspace to minimize volatilization using screw caps lined with polytetrafluoroethylene (Qorpak, cap-00544).

**BE–SPME**

When the WAFs were prepared for toxicity testing, the test substance was added to dilution water and stirred with a ≤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. Because the dilution water contained only the dissolved hydrocarbons, the subsequent toxicity testing avoided the confounding effects of physical oiling. The BE–SPME method was used to quantify the bioavailable fraction during exposure (Redman et al. 2014; Leslie et al. 2002).

The BE–SPME measurements can be used to evaluate petroleum substance exposure in part due to the target lipid model (TLM; Di Toro et al. 2000; Leslie et al. 2002; Redman 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 BE–SPME fiber is coated with PDMS, which acts as a surrogate for organismal lipid (Verbruggen et al. 2000; Leslie et al. 2002; McGrath and Di Toro 2009; Letinski et al. 2014; Redman et al. 2014). The rate of partitioning from water to PDMS is similar to that of water to lipid (Ding 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 is quantified in comparison with a standard of 2,3-dimethylnaphthalene (Redman et al. 2018). The BE–SPME method provided a convenient cross-comparison among 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**

The 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 of PDMS) 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). Petroleum substance sample GC–FID chromatographs were normalized against an external 2,3-dimethylnaphthalene standard derived from liquid solvent injection of hydrocarbon standards. The standard of 2,3-dimethylnaphthalene average molar response factor was used to convert the measured petroleum substance GC–FID response to micromoles of organic constituents on the PDMS fiber (µmol 2,3 dimethylnaphthalene/mL PDMS; Redman et al. 2018).

**RESULTS**

**Acute toxicity: LC50**

As a method of comparison between acute sublethal endpoints and traditional acute FET lethality-based endpoints, an LC50 was calculated for mortality by plotting the mortality for all test substances against BE (µM). The calculated LC50 was 36.6 (27.3–45.9) mM (Figure 1). In principle, BE-based measurements normalize for the different bioavailabilities of the test substances. Therefore, a dataset-wide LC50 was calculated, 36 mM, which is similar to previous findings by Redman et al. (2018), who noted that zebrafish embryos were of median sensitivity across 10 different species.

**Sublethal effects**

Although none seemed more informative than the others, 4 sublethal effects were observed across all test substances (Table 1). These were pericardial and yolk sac edema, tail curvature, and lack of viability (nonviable), described as embryos that were still alive at 96 hpf but did not hatch. Post-test analysis focused on these 4 endpoints.

As a consistent basis of comparison, EC25 values for Endicott crude oil were used as the x-axis for cross comparisons among test substances (Figure 2). 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 that 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 comparisons, the sublethal endpoints generally tracked 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 increased the total area under the curve by stretching the total detection area compared with individual measurement of bitumen or diluent (e.g., pentanes). 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). This finding could be driven by the constituents that comprise isotridecanol (C13 alcohol isomers with some C11–C14 isomers), which are more polar than hydrocarbons; therefore, when compared with hydrocarbon-based materials, isotridecanol appeared to have a lower BE (µmol as 2,3-dimethylnaphthalene/mL PDMS) response.

The 4 sublethal endpoints that occurred most frequently are not unique to these test substances (Hill et al. 2004; Jezierska et al. 2009; Knöbel et al. 2012). Multiple mechanisms seem to drive pericardial and yolk sac edema. Some are well understood, such as oxidative stress, and loss of expression of WW domain containing oxidoreductase (wwox) and leucine-rich repeat containing protein (LRRC10; Hill et al. 2004; Kim et al. 2007; Chen et al. 2008; Madison et al. 2015; Tsuruwaka et al. 2015). The initiating event of other mechanisms is less clear, such as failure to block water from entering the pericardial

**FIGURE 1:** Percentage of mortality for all test substances. Percentage of mortality consists of the 4 OECD test guideline 236 endpoints that indicate lethality (coagulation, lack of somite formation, tail bud detachment from the yolk sac, and the presence of a heartbeat). The median lethal concentration (LC50) was calculated based on the total percentage of mortality using Gompertz nonlinear regression. The LC50 was calculated to be 36.6 (mM), with 95% confidence intervals of 27.3 to 45.9. BE–SPME = biomimetic solid-phase microextraction; CRGO = cracked gas oil; SRGO = straight run gas oil; VHGO = vacuum hydrocracked gas oil.
cavity, developmental inhibition, or kidney failure (Villalobos et al. 1996; Incardona et al. 2004). Although many events may lead to either yolk sac or pericardial edema, the initiating event involved in either type of edema could be mediated through the aryl hydrocarbon receptor (Incardona et al. 2006). Tail curvature is less understood but is attributed to inhibition of both skeletal and musculature development (Fraysse et al. 2006). Finally, lack of hatch in nonviable embryos could be caused by other morphological abnormalities that inhibit the embryo’s ability to break through the chorion, or by a lack of zebrafish hatching enzyme 1 (ZHE1). This enzyme is responsible for partially digesting and swelling the chorion, to weaken it and enable the embryo to hatch (Sano et al. 2008).

The EC25 values were calculated for individual endpoints (pericardial edema, yolk sac edema, tail curvature, and lack of viability) for each test substance to allow for cross-test substance comparison (Table 1). This analysis indicated that the sensitivity of all the observed endpoints were comparable (i.e., that there was no single most sensitive sublethal endpoint; Table 1), and that the standard deviations of the majority of the

| Endpoint          | Endicott crude oil | Weathered Troll crude oil | CRGO    | VHGO    | SRGO    | Dilbit | Isotridecanol |
|-------------------|--------------------|---------------------------|---------|---------|---------|--------|---------------|
| Pericardial edema | 15.4 (14.9–15.9)   | 18.0 (16.0–20.0)          | 16.6    | 11.8 (10.0–13.5) | 13.0 (12.4–13.2) | 29.4 (28.1–30.8) | 11.2 (9.4–13.0) |
| Yolk sac edema    | 17.9               | 13.2 (12.3–14.1)          | 16.3    | 21.5 (20.4–22.7) | 17.9 (17.9–17.9) | 18.1 (15.8–20.4) | 11.2 (11.2–11.2) |
| Tail curvature    | 7.8                | 12.4 (12.4–12.4)          | 16.0 (12.9–15.0) | 15.5 (12.5–18.5) | 13.0 (11.3–14.9) | 23.8 (8.0–38.7) | NA             |
| Nonviability      | 14.9 (14.6–15.2)   | 18.0 (15.9–20.0)          | 20.8    | 23.3 (16.2–30.4) | 13.3 (9.5–17.1)  | 32.9 (32.8–33.0) | 6.9 (6.8–7.0)   |
| Total % effect    | 14.3 (12.3–16.3)   | 15.1 (13.5–16.8)          | 24.1 (6.7–41.7) | 21.1 (18.4–23.7) | 14.0 (12.4–15.6) | 19.8 (15.0–24.7) | 10.4 (7.6–13.1) |

*EC25 values calculated for individual endpoints (pericardial edema, yolk sac edema, tail curvature, and lack of viability) for each test substance to allow for cross-test substance comparison. All values are expressed in mM; range in parentheses. CRGO = cracked gas oil; VHGO = vacuum gas oil; SRGO = straight run gas oil; Dilbit = diluted bitumen; NA = no evaluation of this endpoint occurred.

FIGURE 2: Cross-plots for individual sublethal effect comparison among test substances. Each axis is the biomimetic extraction (BE; µmol as 2,3 dimethylnaphthalene/mL polydimethylsiloxane) scale. Endicott crude has been plotted as the standard metric for comparison and is plotted on all x-axes. The y-axis for each plot is of the remaining 6 test substances. Each data point within the figures is the calculated effect concentration, 25% (EC25) for the represented endpoint (blue = tail curvature; orange = nonviable; red = pericardial edema; green = yolk sac edema; black = combined sublethal effect EC25 calculation). The diagonal line is the 1:1 comparison to identify the relationship of sublethal endpoint occurrence when test substances are compared. VHGO = vacuum gas oil; CRGO = cracked gas oil; weathered Troll = weathered Troll oil; Dilbit = diluted bitumen; SRGO = straight run gas oil.
substances consistently, and these 4 sublethal effects were considered biologically plausible. These results are not surprising, because all substances tested were expected to impart toxicity via narcosis.

Our second objective was to identify a bioavailable exposure threshold using BE–SPME that would aid in the prediction of sublethal effects. Eleven sublethal endpoints were planned prior to test initiation, but following test completion, 4 of the 11 endpoints were identified to occur consistently in each test. On further analysis, all endpoints were seen to occur at a similar BE level across the 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 the relative sensitivity of sublethal and lethal endpoints for fish embryos exposed to hydrocarbons. The BE threshold identified in relation to sublethal endpoints can improve risk assessment by relating BE response to sublethal and lethal endpoints, providing a method to screen petroleum substances for potential hazard using BE–SPME (Hodson 2017).

Our third objective 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 4B), whereas no quantifiable response could be discerned when the effects were plotted against nominal loading (mg/L; Figure 4A). 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 was clear and reliable differentiation from background sublethal effects when the EC10 did not provide sufficient separation and an EC25 was more sensitive than the EC50 (Brannen et al. 2010; Redman et al. 2018). Because we observed similar sublethal responses across all substances tested, we suggest using a combined sublethal approach in the derivation of thresholds or effects data, because no one endpoint was more sensitive than another (identified by the large overlap in the standard deviations of the 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 the 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 the capabilities of FET tests compared with 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 that directly compare the FET with the acute and chronic fish toxicity tests, especially data from unknown or variable composition, complex reaction products, and biological materials of limited solubility. As highlighted earlier, Redman et al.
have the potential to act specifically when compared with individual or simple de-constituents (3-ring PAHs) within petroleum substances are used as the test substances com-promised mixtures. If particular constituents (3+ ring PAHs) were to drive toxicity, we would expect that petroleum substances with a greater number of 3+ ring PAHs would be inherently more toxic. This was not observed in the present study (Figure 6). All sublethal and lethal toxicity was observed within the same BE range when sublethal effects were combined as a percentage of effect rather than identifying them individually (Figures 1 and 3, respectively). Therefore, when one is assessing the toxicity of petroleum substances as a whole, the traditional “nonpolar narcosis” model is a better fit for risk assessment. Geier et al. (2018) noted a similar pattern when they assessed a variety of 123 individual PAHs, and they recognized that the relationship between toxicity and chemical structure was complex. Geier et al. (2018) emphasize the point that risk assessment should not be based on individual constituents found within a petroleum substance, but instead the whole substance should be evaluated when toxicity is assessed in an aqueous environment (Geier et al. 2018).

The associated toxic units of each fraction were calculated using PETROTOX Ver 3.06 (Redman et al. 2012). In terms of the present study, a toxic unit is described as the concentration in water divided by the observed endpoint (toxic unit = \( \Sigma C / LL50 \), where LL50 is the loading rate of a test substance resulting in 50% mortality). Each section of the chart is equal to the corresponding fraction’s contribution to the observed toxicity (effect loading, 50% [EL50]). As seen in Figure 5, the...
contributing fractions to toxicity are not limited to 3+‐ring PAHs. If toxicity assessments were to be based on the conclusion that 3+‐ring PAHs are the drivers of toxicity, then there is a potential to inaccurately estimate toxicity, because other fractions within the petroleum substance play an integral role in overall substance toxicity. The variable composition of petroleum substances can be seen in Figure 7.

CONCLUSIONS

No individual sublethal effect was a most sensitive indicator of toxicity across all petroleum substances tested. Therefore, when the toxicity of petroleum substances is being assessed, we recommend accounting for all sublethal effects rather than making risk assessment decisions based on individual sublethal responses. The varied sublethal effects indicated nonspecific mode of action; therefore, nonpolar 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 petroleum substances are being assessed for toxicity, associating sublethal and lethal effects with particular constituents is discouraged because 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 4 endpoints reinforces the nonspecific mode of action, nonpolar narcosis model. Finally, BE‐SPME was shown to be a convenient method to measure the bioavailable exposure of petroleum substances and equate BE response to observed lethal toxicity.
and sublethal effects across different classes of petroleum substances.

Acknowledgment—The present study was funded by ExxonMobil Biomedical Sciences, mutualized strategic program.

Data Accessibility—Data and calculation tools pertaining to this manuscript are available from the corresponding author (bryan.hedgpeth@exxonmobil.com).

REFERENCES

Baron MG, Carls MG, Heintz R, Rice SD. 2004. Evaluation of fish early life-stage toxicity models of chronic embryonic exposures to complex polycyclic aromatic hydrocarbon mixtures. Toxicol Sci 78:60–67.

Belanger SE, Rawlings JM, Carr GJ. 2013. Use of fish embryo toxicity tests for the prediction of acute fish toxicity to chemicals. Environ Toxicol Chem 32:1768–1783.

Billiard SM, Timme-Laragy AR, Wassenberg DM, Cockman C, Di Giulio RT. 2006. The role of the aryl hydrocarbon receptor pathway in mediating synergistic developmental toxicity of polycylic aromatic hydrocarbons to zebrafish. Toxicol Sci 92:526–536.

Brannek KC, Panzica-Kelly JM, Danberry TL, Augustine-Rauch KA. 2010. Development of a zebrafish embryo teratogenicity assay and quantitative prediction model. Birth Defects Res B Dev Reprod Toxicol 89:66–77.

Braunbeck T, Kais B, Lannier E, Otte J, Schneider K, Stengel D, Streecher R. 2015. The fish embryo test (FET): Origin, applications, and future. Environ Sci Pollut Res 22:16247–16261.

Brown DR, Bailey JM, Oliveri AN, Levin ED, Di Giulio RT. 2016. Developmental exposure to a complex PAH mixture causes persistent behavioral effects in naïve Fundulus heteroclitus (killifish) but not in a population of PAH-adapted killifish. Neurotoxicol Teratol 53:55–63.

Brusseau ML, Farnan SB, Artiola JF. 2004. Chemical contaminants. In Artiola JF, Pepper IL, & Brusseau ML, eds, Environmental Monitoring and Characterization. Academic, Cambridge, MA, USA.

Carls MG, Meador JP. 2009. A perspective on the toxicity of petrogenic PAHs to developing fish embryos related to environmental chemistry. Hum Ecol Risk Assess 15:1084–1099.

Carls MG, Rice SD, Hose JE. 1999. Sensitivity of Fish embryos are damaged by dissolved PAHs, not oil particles. Aquat Toxicol 88:121–127.

Chen J, Catey SA, Peterson RE, Heideman W. 2008. Comparative genomics identifies genes mediating cardiotoxicity in the embryonic zebrafish heart. Physiol Genom 33:148–158.

CONCAWE Classification and Labelling Task Force. 2015. Hazard classification and labelling of petroleum substances in the European economic area—2015. Report No. 9/15. Brussels, Belgium.

Toro Di, Dominic M, McGrath JoyA, Hansen DavidJ. 2000. Technical basis for narcotic chemicals and polycyclic aromatic hydrocarbon criteria. I. Water and tissue. Environ Toxicol Chem 19:1951–1970.

Ding Yiping, Landrum PeterF, You Jing, Harwood AmandaD, Lydi MichaelJ. 2012. Use of solid phase microextraction to estimate toxicity: Relating fiber concentrations to toxicity—part I. Environ Toxicol Chem 31:2159–2167.

Eaton AD, Clesceri LS, Franzon MAH, Greenberg AE, Rice EW. 2005. Standard Methods for the Examination of Water & Wastewater. American Public Health Association, Washington, DC, USA.

Emst VV, Neff JM, Anderson JW. 1977. The effects of the water-soluble fractions of no. 2 fuel oil on the early development of the estuarine fish, Fundulus grandis baird and girard. Environ Pollut 14:25–35.

Frayssé B, Mons R, Garcia J. 2006. Development of a zebrafish 4-day embryo-larval bioassay to assess toxicity of chemicals. Ecotoxicol Environ Saf 63:253–267.

French-McCay DP. 2002. Development and application of an oil toxicity and exposure model, OilToxEx. Environ Toxicol Chem 21:2080–2094.

Geer MC, Chlebowski AC, Truong L, Massey Smonich SL, Anderson KA, Tanguy RL. 2018. Comparative developmental toxicity of a comprehensive suite of polycyclic aromatic hydrocarbons. Arch Toxicol 92:571–586.

González-Doncel M, González L, Fernández-Torija C, Navas JM, Tarazona JV. 2008. Toxic effects of an oil spill on fish early life stages may not be exclusively associated to PAHs: Studies with Prestige oil and medaka (Oryzias latipes). Aquat Toxicol 87:289–298.

Henry JA. 1998. Composition and toxicity of petroleum products and their additives. Hum Exp Toxicol 17:111–123.

Hermens SA, van den Brandhof EJ, van der Ven LT, Piersma AH. 2011. Relative embryotoxicity of two classes of chemicals in a modified zebrafish embryo toxicity test and comparison with their in vivo potencies. Toxicol In Vitro 25:745–753.

Hicken CE, Linbo TL, Baldwin DH, Willis ML, Myers MS, Holland L, Larsen M, Stekoll MS, Rice SD, Collier TK, Scholz NL, Incardona JP. 2011. Sublethal exposure to crude oil during embryonic development alters cardiac morphology and reduces aerobic capacity in adult fish. Proc Natl Acad Sci U S A 108:7086–7090.

Hill AJ, Bello SM, Prasch AL, Peterson RE, Heideman W. 2004. Water permeability and TCDD-induced edema in zebrafish early-life stages. Toxicol Sci 78:78–87.

Hill AJ, Teroaka H, Heideman W, Peterson RE. 2005. Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol Sci 86:6–19.

Hill A, Jones M, Dodd A, Diekmann H. 2011. A review of developmental toxicity screening using zebrafish larvae. Toxicol Lett 205:5115.

Hudson PV. 2017. The toxicity to fish embryos of PAH in crude and refined oils. Arch Environ Contam Toxicol 73:12–18.

Hoff D, Lemmann W, Pease A, Raimondo S, Russom C, Steeger T. 2010. Predicting the toxicities of chemicals to aquatic animal species. US Environmental Protection Agency, Washington, DC.

Icardona JP. 2017. Molecular mechanisms of crude oil developmental toxicity in fish. Arch Environ Contam Toxicol 73:19–32.

Icardona JP, Collier TK, Scholz NL. 2004. Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. Toxicol Appl Pharmacol 196:191–205.

Icardona JP, Day HL, Collier TK, Scholz NL. 2006. Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P4501A metabolism. Toxicol Appl Pharmacol 217:308–321.

Icardona JP, Swarts TL, Edwards RC, Linbo TL, Aquilina-Beck A, Sloan CA, Gardner LD, Block LA, Scholz NL. 2013. Exxon Valdez to Deepwater Horizon: Comparable toxicity of both crude oils to fish early life stages. Aquat Toxicol 142–143:303–316.

Jezierska B, Bogowska K, Witoska M. 2009. The effects of heavy metals on embryonic development of fish (a review). Fish Physiol Biochem 35:625–640.

Jung JH, Hicken CE, Boyd D, Anulacion BF, Carls MG, Shim WJ, Icardona JP. 2013. Geologically distinct crude oils cause a common cardiotoxicity syndrome in developing zebrafish. Chemosphere 91:1146–1155.

Kavlock R, Dixon D. 2010. Computational toxicology as implemented by the U.S. EPA: Providing high throughput decision support tools for screening and assessing chemical exposure, hazard and risk. J Toxicol Environ Health B 13:197–217.

Kavlock R, Chandler K, Houck K, Hunter S, Judson R, Kleinstreuer N, Kavlock R, Dix D. 2012. Update on EPAs ToxCast program: Providing high throughput decision support tools for chemical risk management. Chem Res Toxicol 25:1287–1302.

Kim K-H, Antkiewicz DS, Yan I, Elicerio KW, Heideman W, Peterson RE, Lee Y. 2007. Lrrc10 is required for early heart development and function in zebrafish. Dev Biol 308:494–506.

Knight AW, Little S, Houck K, Dixon D, Judson R, Richard A, McCartney N, Akerman G, Yang C, Birrell L, Walsmey RM. 2009. Evaluation of high-throughput genotoxicity assays used in profiling the US EPA ToxCast™ chemicals. Regul Toxicol Pharmacol 55:188–199.

Knobbel M, FJM Busser, Rico-Rico A, Kramer NI, JLM Hermens, Hafner C, Tanneberger K, Schirmer K, Scholz S. 2012. Predicting adult fish acute lethality with the zebrafish embryo: Relevance of test duration, endpoints, compound properties, and exposure concentration analysis. Environ Sci Technol 46:9690–9700.
Leslie HA, Oosthoek AJP, Busser FJM, Kraak MHS, Hermens JLM. 2002. Biomimetic solid-phase microextraction to predict body residues and toxicity of chemicals that act by narcosis. Environ Toxicol Chem 21:229–234.

Letinski D, Parkerton T, Redman A, Manning R, Bragin G, Febbo E, Palandro E, Nedwed T. 2014. Use of passive samplers for improving oil toxicity and spill effects assessment. Mar Pollut Bull 86:274–282.

Madison BN, Hodson PV, Langlois VS. 2015. Diluted bitumen causes deformities and molecular responses indicative of oxidative stress in Japanese medaka embryos. Aquat Toxicol 165:222–230.

McGrath JA, Di Toro DM. 2009. Validation of the target lipid model for toxicity assessment of residual petroleum constituents: Monocyclic and polycyclic aromatic hydrocarbons. Environ Toxicol Chem 28:1130–1148.

Moore SF, Dwyer RL. 1974. Effects of oil on marine organisms: A critical review of the state of knowledge of the effects of oil spills on marine organisms. Mar Pollut Bull 5:226–232.

Organisation for Economic Co-operation and Development. 2013. Test No. 236: Fish embryo acute toxicity (FET) test. In OECD Guidelines for the Testing of Chemicals. Paris, France.

Padilla S, Hunter DL, Padnos B, Frady S, MacPhail RC. 2011. Assessing locomotor activity in larval zebrafish: Influence of extrinsic and intrinsic variables. Neurotoxicol Teratol 33:624–630.

Panza-Kelly JM, Zhang CX, Danberry Ti, Flood A, DeLan JW, Brannen KC, Augustine-Rauch KA. 2010. Morphological score assignment guidelines for the dechorionated zebrafish teratogenicity assay. Birth Defects Res B Dev Reprod Toxicol 89:382–395.

Pauk LM, Maceno M, Rossi SC, Silva, de Assis HC. 2011. Embryotoxicity and biotransformation responses in zebrafish exposed to water-soluble fraction of crude oil. Bull Environ Contam Toxicol 86:389–393.

Perrichon P, Le Menach K, Akcha F, Cachot J, Budzinski H, Bustamante P. 2016. Toxicity assessment of water-accommodated fractions from two different oils using a zebrafish (Danio rerio) embryo-larval bioassay with a multilevel approach. Sci Total Environ 568:952–966.

Philibert DA, Philibert CP, Lewis C, Tierney KB. 2016. Comparison of diluted bitumen (dilbit) and conventional crude oil toxicity to developing zebrafish. Environ Sci Technol 50:6091–6098.

Redman AD, Parkerton TF. 2015. Guidance for improving comparability and relevance of oil toxicity tests. Mar Pollut Bull 98:156–170.

Redman AD, Parkerton TF, McGrath JA, Di Toro DM. 2012. PETROTOX: An aquatic toxicity model for petroleum substances. Environ Toxicol Chem 31:2498–2506.

Redman AD, Parkerton TF, Letinski DJ, Manning RG, Adams JE, Hodson PV. 2014. Evaluating toxicity of heavy fuel oil fractions using complementary modeling and biomimetic extraction methods. Environ Toxicol Chem 33:2094–2104.

Redman AD, Butler JD, Letinski DJ, Di Toro DM, Paumen ML, Parkerton TF. 2018. Technical basis for using passive sampling as a biomimetic extraction procedure to assess bioavailability and predict toxicity of petroleum substances. Chemosphere 199:585–594.

Rotroff DM, Dix DJ, Houck KA, Knudsen TB, Martin MT, McLaurin KW, Reif DM, Crofton KM, Singh AV, Xia M, Huang R, Judson RS. 2013. Using in vitro high throughput screening assays to identify potential endocrine-disrupting chemicals. Environ Health Perspect 121:7–14.

Sano K, Inohaya K, Kagawuchi M, Yoshizaki N, Iuchi I, Yasumasu S. 2008. Purification and characterization of zebrafish hatching enzyme—An evolutionary aspect of the mechanism of egg envelope digestion. FEBS J 275:5934–5946.

Selderslaghs IWT, Blust R, Witters HE. 2012. Feasibility study of the zebrafish assay as an alternative method to screen for developmental toxicity and embryotoxicity using a training set of 27 compounds. Reprod Toxicol 33:142–154.

Shukla SJ, Huang R, Austin CP, Xia M. 2010. The future of toxicity testing: A focus on in vitro methods using a quantitative high-throughput screening platform. Drug Discov Today 15:997–1007.

Singer MM, Aurand D, Bragin GE, Clark JR, Coelho GM, Sowby ML, Tjeerdema RS. 2000. Standardization of the preparation and quantitation of water-accommodated fractions of petroleum for toxicity testing. Mar Pollut Bull 40:1007–1016.

Sipes NS, Padilla S, Knudsen TB. 2011. Zebrafish—As an integrative model for twenty-first century toxicity testing. Birth Defects Res C Embryo Today Rev 93:256–267.

Tsuruwaka Y, Konishi M, Shimada E. 2015. Loss of wwox expression in zebrafish embryos causes edema and alters Ca(2+) dynamics. Peer J 3:e727.

Van Tiem LA, Di Giulio RT. 2011. AHR2 knockdown prevents PAH-mediated cardiac toxicity and XRE- and ARE-associated gene induction in zebrafish (Danio rerio). Toxicol Appl Pharmacol 254:280–287.

Verbruggen EMJ, Vaes WHJ, Parkerton TF, Hermens JLM. 2000. Polyacrylate-coated SPME fibers as a tool to simulate body residues and target concentrations of complex organic mixtures for estimation of baseline toxicity. Environ Sci Technol 34:324–331.

Villalobos SA, Soimasauro R, the SW, Fan TWM, Higashi RM, Hinton DE. 1996. Mechanistic studies of pericardial edema (PE) in early life stages (ELS) of medaka (Oryzias latipes). Mar Environ Res 42:137.