Characterizing Crude Oil Toxicity to Early-Life Stage Fish Based On a Complex Mixture: Are We Making Unsupported Assumptions?

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Abstract

Numerous studies of the water-soluble fraction (WSF) from crude oil have concluded that polycyclic aromatic hydrocarbons (PAHs) are the primary causative agents for early-life stage (ELS) fish toxicity. Noteworthy is the lack of studies demonstrating that the sum of PAHs are capable of causing toxic effects in ELS fish at the low levels claimed (0.1 - 5 µg/L) without being part of a complex crude oil mixture. Crude oil and the WSF are composed of thousands of other compounds that co-occur and likely contribute to crude oil toxicity. Based on the available data, it appears that the syndrome of effects (lower heart rate, edemas, and morphological abnormalities) for ELS fish exposed to the aqueous fraction of a crude oil mixture is commonly observed in studies exposing fish embryos to high concentrations of a variety of compounds and may be a non-specific response. We conclude that the available data support the hypothesis that this syndrome of effects is likely the result of baseline toxicity (not receptor based) due to membrane disruption and resulting alteration in ion (e.g. calcium and potassium) homeostasis. We acknowledge the possibility of some compounds in the WSF capable of causing a specific receptor based toxicity response to ELS fish; however, such compounds have not been identified nor their receptor characterized. Concluding that PAHs are the main toxic compounds for crude oil exposure is misleading and does not result in guideline values that can be useful for environmental protection. Water quality guidelines for any single chemical or suite of chemicals must be based on a complete understanding of exposure concentrations, mechanism of action, potency, and resulting response. This review focuses on the toxic effects reported for fish embryos and the purported toxic concentrations observed in the aqueous phase of an oil/water mixture, the known levels of toxicity for individual PAHs, a toxic unit approach for characterizing mixtures, and the potential molecular initiating event for ELS toxicity in fish. This review also has implications for a

large number of studies exposing ELS fish to a variety of compounds at high concentrations that result in a common baseline toxic response.

1. INTRODUCTION

Over the past decade there has been a rapid increase in the number of studies attempting to characterize the toxicity of crude oil. Specifically, the focus has been on the aqueous compounds present after an oil spill, which is known as the water-soluble fraction (WSF), water-accommodated fraction (WAF) and others (e.g., chemically-enhanced WAF, [CEWAF]) and high-energy WAF [HEWAF]). We know that ELS fish are responding to the dissolved fraction[1] and not oil droplets, hence we will use the term WSF for this article to denote the aqueous phase containing crude oil compounds.

Many of these ELS fish studies concluded that polycyclic aromatic hydrocarbons (PAHs) were the component responsible for the observed toxic response, especially for WAFs or WSFs that were "weathered" (i.e. loss of volatile compounds)[2–5]. This conclusion is based on the supposition that as the "lighter" relatively "less toxic" components volatilize, the remaining hydrophobic compounds causing ELS fish to exhibit abnormal development are mostly the more toxic PAHs.

A large number of investigators have conducted toxicity experiments with crude oil and ascribed the toxicity to a limited group of PAHs, specifically the tricyclic aromatics acting by an AhR-independent mechanism[3–10]. Frequently, toxicity is reported for ELS fish in terms of the sum of PAHs (∑PAHs) without regard to compound potency or relative abundance, a topic that has been approached previously by others[11,12]. A key point here is that no studies exist demonstrating that ∑PAHs are capable of causing toxic effects in ELS fish at the low levels claimed (0.1 – 5 µg/L)[2,3,13–16] without being part of a crude oil WSF or WAF. As noted by many authors, PAHs are just a small fraction of the aqueous concentration of organic compounds found in this

complex mixture; hence, the toxic potential of the non-PAH fraction is not considered. Consequently, we believe that ∑PAHs may not be the most appropriate dose metric for crude oil toxicity.

There are several methods for preparing a WSF or WAF that will have a substantial impact on the composition of the aqueous fraction used for exposure studies. One common method is to coat gravel with crude oil at various rates of loading, air dry or heat (weathering), then flow water over the gravel to achieve a water soluble fraction. The degree of weathering can be controlled to achieve a different mix of PAHs and other compounds. Several issues noted for this technique include variable aqueous concentrations over time, variable compound profiles, and the potential for microbial production of metabolites[17]. Other methods include a high and low energy water accommodated fraction (LEWAF and HEWAF) in addition to chemically enhanced WAF that includes oil dispersants at various concentrations to facilitate solubilization of crude oil. Each one of these methods (with or without weathering) can produce vastly different water soluble fractions of crude oil varying in component concentration and profile. All of these methods will certainly confound the interpretation of the results especially with an inappropriate or poorly defined dose metric.

The intent of this review is to examine the assumption that tricyclic PAHs are primarily responsible for all adverse effects due to a crude oil WSF exposure and to enumerate the reasons why this conclusion is not strongly supported by the available literature. Our working hypothesis is that most of the current studies characterizing ELS toxicity in fish exposed to a WSF from crude oil are describing a non-specific baseline toxic effect that is characterized by a common syndrome of effects elicited by a large variety of organic compounds at relatively high exposure concentrations. This is the simplest explanation for the observed responses and is the appropriate working hypothesis until more definitive data can be collected that includes

critical experiments and identifying a defined molecular initiating event (MIE). We also acknowledge the possibility that uncharacterized compounds in the WSF are acting by unidentified specific mechanisms, with greater potency than observed for the tricyclic PAHs.

Our goal is to foster a critical evaluation of this paradigm and highlight the inappropriate and misleading conclusions regarding the toxic components found in crude oil. Without a scientifically defensible framework for characterizing the potential toxic response resulting from exposure to petroleum compounds, achieving environmental protection based on faulty assumptions will be counterproductive. We also encourage a greater focus on those uncharacterized aqueous-phase compounds that are likely important contributors to the toxic response that act by specific or non-specific modes of action, especially the polar fraction.

2. CRUDE OIL, THE WATER-SOLUBLE FRACTION, AND MAJOR COMPONENTS

Crude oil contains thousands of compounds[18] that range in water solubility from highly soluble to essentially insoluble. This includes a labile fraction known as volatile organic compounds (VOCs), which comprises about 15% of the total hydrocarbon load in whole oil[19]. These VOCs evaporate quickly after an oil spill; however, a majority of the hydrocarbons and polar compounds remain and dissipate slowly depending on their physicochemical characteristics, such as the octanol-water partition coefficient (K_{ow}). Crude oil from various geological formations exhibit very different profiles of compounds[20,21]. For example, Faksness et al.[21] noted that two Norwegian oils (Goliat and Heidrun) varied 2 to 4-fold for many chemical classes including 2 – 3 ring PAHs, C3 benzenes, and total petroleum hydrocarbons. Crude oil contains a wide variety of organic compounds other than hydrocarbons (carbon and hydrogen only). There are a very large number of

polar compounds containing sulfur, nitrogen, and oxygen and these can constitute a major portion of the aqueous fraction[18,22]. Many of these are heterocyclic compounds resembling PAHs in structure and properties and only a few have been identified, many with alkyl groups (C1 – C4). Examples of these include carbazoles, xanthones, and thioxanthones, in addition to dibenzothiophenes all with C1-C4 alkyl groups[23]. It was also noted by Sauer and Uhler[24] that the percentage composition for many heterocyclic compounds in a WSF is enhanced via weathering.

Polycyclic aromatic hydrocarbons (PAHs) constitute a very large group possibly reaching 10,000 unique compounds[25]. Only about 100 PAHs have been identified and studied[26] and they vary widely in physical-chemical properties and toxic potency[27]. However, in most crude oils PAHs comprised less than 1% of the total petroleum hydrocarbons[19] and most of the compounds are unidentified and commonly known as the unresolved complex mixture (UCM)[28]. For example, the data in Sammarco et al.[19] show that weathered field samples contained aqueous concentrations of total petroleum hydrocarbons (TPHs) that were 2 orders of magnitude higher than reported for total PAHs, which is common for many such studies[22,29].

The UCM from whole crude oil may have up to 250,000 compounds, which has been described as the most complex mixture of organic molecules in the environment[30]. It is important to note that even after separation into aliphatic, aromatic, and polar fractions, each one of those groups has its own UCM with a large number of unidentified compounds[23,30,31]. It is also important to note that the UCM of a WSF can constitute a high percentage of the total petroleum hydrocarbon fraction, with some ranging from 90 – 98%[21]. As noted by Melbye et al.[22], the UCM is resistant to weathering and likely to persist in the environment. Several authors also noted that the UCM constituted approximately 70% of the WSF and it contains high levels of polar compounds, including cyclic and aromatic sulfoxide compounds[22,32].

This is supported by Lang et al.[33] who noted that polar compounds can dominate a weathered WSF (98% of total). Recent studies have attempted to characterize the unknown compounds occurring in weathered oil and the UCM[28,34,35]. High concentrations (100 – 1,000 µg/L) of alkylphenols, alkylbenzenes (C3 – C6), alkylated aromatic heterocycles (quinolines, carbazoles, thiophenes, benzothiophenes, and benzofurans) were observed in WAFs by Barron et al.[36] and were generally far more abundant than PAHs.

3. CURRENT UNDERSTANDING AND MISUNDERSTANDING OF CRUDE OIL TOXICITY IN ELS FISH

The syndrome associated with ELS toxicity in fish exposed to crude oil WSF includes fluid accumulation (edema) around the heart and yolk sac, body axis and craniofacial abnormalities, and heart beat abnormalities[3,13,15,37]. Many authors have reported these responses for a variety of larval fish species exposed to crude oil and the vast majority express toxicity in terms of ∑PAHs, which is based on a limited list of 40 – 50 PAHs[16,38,39]. The prevailing conclusion among most researchers is that the tricyclic PAHs (specifically alkylated phenanthrenes) are the most toxic components in crude oil and responsible for this syndrome of effects in larval fish[13,15,37]. Interestingly, naphthalenes are included in the ∑PAHs dose metric[8,40,41]; however, they are not expected to contribute to the ELS fish toxicity syndrome[6].

The recent hypothesis regarding the molecular initiating event (MIE) for PAH toxicity highlights abnormal calcium cycling and alterations to the cellular flux of potassium[9,37,42]. Specific targets are thought to be rectifying potassium channels and sarcoplasmic reticulum calcium channels[16]. The main focus is on tricyclic PAHs and the MIE is assumed to be aryl hydrocarbon receptor (AhR)-independent[15,37]. This is a separate mechanism from that described for other PAHs (such as retene [an alkylphenanthrene]

194 and many high-molecular weight PAHs) known to act by AhR-dependent
195 toxicity causing toxicity to ELS fish at very low water and tissue
196 concentrations[43], and is known as blue-sac disease. These recent studies
197 highlighting the disruption of ion homeostasis in cardiac myocytes exposed
198 to crude oil mixtures[9,42]; however do not provide direct evidence of the
199 responsible compounds or group of compounds that cause these effects and
200 the exact MIE (i.e. specific or non-specific mode of action).

201 **3.1. Single Compound Toxicity Versus Mixture Toxicity**

202 There is only circumstantial data to support the conclusion that the ∑PAHs,
203 specifically the tricyclic PAHs, in the range of 0.1 – 5 µg/L are causing
204 toxicity in ELS fish, which is observed only when they are exposed to a
205 complex mixture (WSF or WAF). Studies examining developmental toxicity in
206 fish exposed to individual PAHs indicate toxic concentrations orders of
207 magnitude above this level[5,44,45]. Geier et al.[45] tested 123 PAHs for more
208 than 20 developmental endpoints in zebrafish (*Danio rerio*) and all
209 compounds (except 1,3 dinitropyrene) elicited responses above 0.5 µM, or
210 approximately 100 µg/L (most were 5 - 50 µM).

211 A number of studies attempting to characterize the mechanism of toxicity for
212 PAHs exposed ELS fish to mg/L (ppm) aqueous concentrations often with
213 DMSO allowing the compounds to exceed aqueous solubility[6,7,46]. These
214 highly cited single-compound studies at high exposure concentrations
215 describing the syndrome of ELS fish toxicity in great detail for PAHs
216 (specifically tricyclic PAHs) are the basis for dozens of subsequent studies
217 supporting the conclusion of PAH toxicity as the primary causative agents for
218 a complex mixture of hydrocarbons found in the WSF. Most of these high-
219 dose studies have been performed with warm-water species and one author
220 has claimed that zebrafish (*D. rerio*) are not sensitive to PAHs[37]. Even
221 though these high-dose studies were conducted with zebrafish, mackerel,

and tuna (*D. rerio*, *Scomber japonicas*, *Thunnus albacares*, *Thunnus orientalis*), there is no reason to assume these warm-water species are less sensitive than other species[47–49]. For example, Petersen and Kristensen[50] found similar bioconcentration factors for zebrafish, cod, herring, and turbot (*D. rerio*, *Gadus morhua*, *Clupea harengus*, and *Scophthalmus maximus*) larvae exposed to phenanthrene. Additionally, a warm-water species such as mahi-mahi exhibited similar effect concentrations in terms of ∑PAH within a WAF compared to cold-water species such as herring (*Clupea pallasi*)[3].

Several researchers have examined the toxicity of phenanthrene and alkylphenanthrenes and found ELS fish responses at water concentrations generally in the range of 40 – 100 µg/L and higher[13,44,51,52]. The target PAHs (tricyclics such as phenanthrene and alkylphenanthrenes) are present in a WSF at concentrations in the range of 0.1 - 1 µg/L, and claimed to be the sole cause of the ELS toxicity syndrome[2,13,15,37,42,53]. Obviously, there is a large discrepancy between what single-compound studies demonstrate and these same compounds in a complex crude-oil derived WSF.

Because most studies reporting ∑PAHs as the dose metric contain the putative highly toxic tricyclic PAHs at sub ppb concentrations, the discrepancy between high-dose single compound responses and orders of magnitude lower concentrations in a mixture must be rationalized as either a synergistic effect among components or the presence of compounds with far greater potency. There are no data to support either of these potential hypotheses for tricyclic PAHs acting by an AhR-independent mechanism. Also, using ∑PAHs as a marker for the actual toxic component of an oil WSF (e.g., alkylated phenanthrene) would not necessarily translate among different WSF mixtures in time and space as a result of differential weathering and other physicochemical properties. Crude oils are known to contain vastly different profiles of hydrocarbons and PAHs[20,21] and a toxic value based on ∑PAHs for one WSF would likely not translate to another.

Also noteworthy is the analysis for a variety of PAHs and their ability to cause toxicity for ELS fish. Some PAHs such as chrysene produce no effects and others characteristically lead to different responses as a function of AhR dependence when exposed to high concentrations[37,54]. Each compound exhibits variability in water solubility, uptake kinetics, tissue diffusion kinetics, and rates of metabolic transformation that determine bioaccumulation and toxicity. Without measured tissue concentrations, categorization of any toxic response is far more difficult due to compound and species differences[55,56]. For example, there is large interspecific variability for biotransformation of PAHs as well as large variability (e.g. 100 fold) among PAHs for a given species[57].

Fractionation studies that isolate the fraction of crude oil that is most toxic and quantify the alkanes and PAHs are appealing, but often fall short. For example Bornstein et al.[29] determined the most toxic fractions (F3-1-2, F3-1-3, and F3-1-4) contained high levels of PAHs (parent and alkylated forms), but only accounted for a small percentage of the total known analytes in each fraction that likely exhibited similar physicochemical properties and probably contributed to the toxic response. Interestingly, in that study the most toxic fraction (F3-1-3) contained ∑PAH concentrations that were 8x lower and 28x higher than two other fractions exhibiting similar toxicity. Bornstein et al.[29] acknowledged that compounds other than PAHs could have contributed to the toxicity for ELS fish exposed to a weathered crude oil WAF. Interestingly, a few studies have shown reduced variability in toxicity metrics when plotting these against the sum of tricyclic PAHs instead of ∑PAHs[7,40]; however this does not provide strong support for tricyclic PAHs as the responsible compounds. Such grouping provides a set of compounds with a narrower range of Kow values, which may reduce variability. Also, as seen for the Esbaugh et al.[40] data, the correlation between the sum of tricyclic and tetracyclic PAHs is high (r=0.85) among WAF fractions, which

suggests that variability would also be reduced with tetracyclic PAHs over that seen for ∑PAHs as the dose metric. The latter group have been implicated in developmental abnormalities in ELS fish by a different MIE (AhR-dependent) as that for tricyclic PAHs[54]; however they occur at concentrations 12x – 26x lower than observed for the tricyclic PAHs and are too low to cause effects.

3.2. Myriad Compounds in the Water-Soluble Fraction Not Considered

There is currently no support to infer the primary causative agent for ELS fish toxicity of a crude oil WSF is limited to only the quantifiable compounds (PAHs), given that myriad highly bioaccumulative, non-PAH compounds are present in the WSF of crude oils. Studies such as Melbye et al.[22] demonstrated that the polar fraction can dominate the toxicity of a WSF and is important in characterizing crude oil toxicity.

Recent studies have proposed that the UCM contains a number of toxic compounds including branched alkyl benzenes, indanes, and tetralines[35,58,59]. Many other compounds in the UCM that exhibit $\log_{10}$Kow values in the range of 4 – 6 may also be toxic and co-occur with the commonly quantified PAHs including such groups as aliphatic naphthalenes, aliphatic monocyclic acids, monocyclic thiophenic carboxylic acids, and monoaromatic hydrocarbons[35,58,59]. Some of these compounds can be toxic to fish hepatocytes at low concentrations[59] and at sublethal concentrations for mussels (*Mytilus edulis*)[35,58]. The 22 compounds listed in Petersen et al.[59] are very hydrophobic with most exhibiting $\log_{10}K_{ow}$ values in the 3 – 6 range indicating a high potential for bioaccumulation. Booth et al.[35] determined an EC20 of 7 µg/L for mussels (reduced feeding) exposed to a mixture of branched alkyl benzenes, a major component of the UCM. This occurred at a tissue concentration of approximately 2 mg/Kg wet weight (converted from

dry wt.), indicating that these compounds are very toxic and may be important for ELS fish toxicity. If these compounds are representative of the hundreds of compounds found in the UCM, high tissue concentrations would be expected for relatively low water concentrations (µg/L) from a WSF based on simple partitioning models.

3.3. Genomic Changes Do Not Confirm the MIE

A number of studies have characterized myriad genomic changes elicited by a crude oil WSF as they relate to the ELS fish toxicity syndrome[60,61]; however, these responses were observed after exposure to a mixture of thousands of compounds. One proteomic study examined the dose-response relationship between phenanthrene exposure (5 – 345 µg/L) and detected proteins in zebrafish embryos[62]. Of the 716 proteins examined, only two exhibited an expression profile (benchmark concentration for protein abundance alteration; $BMCp_{20}$) corresponding to an exposure concentration of 1.4 and 5 µg/L (calculated), respectively, which were the lowest values for the study and far above those claimed by other authors for tricyclic PAHs.

The WSF mixture studies report a large number of up-regulated and down-regulated genes that may be responsible for the observed phenotypic responses. These likely vary by species or major taxonomic group, and many may be a result of multiple MIEs. Many of these genomic changes are likely caused by altered calcium cycling and K^+ flux, which may result from both specific and non-specific toxicity. While these data are interesting and potentially useful as biomarkers, many of these are downstream events elicited by the unknown MIE, which are likely a consequence of altered ionic homeostasis. Of course, within the matrix of transcriptional changes are potentially specific changes resulting from receptor-based alterations from known or unknown WSF compounds. It would be informative to examine the genomic changes for other compounds causing the ELS fish toxicity

syndrome **(Table 1)** and compare these results to those for a crude oil WSF. Perhaps markers unique to crude oil toxicity can be identified and utilized.

4. BASELINE TOXICITY

4.1. An Alternative MIE for Crude Oil WSF Toxicity in ELS Fish

Baseline toxicological effects, also known as narcosis or non-specific toxicity[82,83], is best described for aquatic species and occurs at very predictable whole-body concentrations. The purported action for all organic compounds acting by a non-specific action is membrane disruption and impaired ion homeostasis[82,84]. As noted by many authors, baseline toxicity occurs when internal concentrations of organic compounds achieve a defined level in membranes and disrupt normal function such as ion transport[84–86]. The action of baseline toxicants is known to cause alterations in ion homeostasis (calcium and potassium) as a consequence of high concentrations of compounds in the membrane leading to reduced fluidity and damage[87–89]. Escher et al.[88] concluded that disturbance of membrane bound proteins is a likely result of baseline toxicity and add that ligand-gated ion channels are the most important targets in baseline toxicity for mammals. They also note that Na+/K+ ATPase activity is generally unaffected, as shown by van Wezel et al.[90], which may be an important observation for physiology studies. Unfortunately, baseline toxicity has not been studied with the same sophistication as that for crude oil toxicity in ELS fish. Consequently, there are many unknowns regarding sublethal outcomes, physiologic change, and compromised biochemical pathways for baseline toxicity.

The baseline lethal response has been demonstrated for hundreds of organic compounds[64,91,92] and occurs for whole-body organisms at approximately 2 – 8 mmol/Kg (wet weight) or 40 – 160 mmol/Kg lipid. Sublethal responses are rarely reported (but see Table 1) and occur in the 0.2 – 0.8 mmol/Kg

range[64,91,93,94]. Depending on the molecular weight, sublethal responses should occur at approximately 40 – 160 mg/Kg whole-body wet weight for a compound of 200 daltons, which is approximately 10 times lower than observed mortality. Consider the $\log_{10}K_{ow}$ for many PAH compounds and the water concentration required to result in high tissue burdens causing such baseline toxicity. For example, a PAH with a $\log_{10}K_{ow}$ of 5 (most alkylated phenanthrenes) would result in a tissue concentration approaching 40 - 200 mg/Kg for an aqueous concentration of 10 - 50 µg/L, which is the range for baseline toxicity (mortality and sublethal effects), especially for low lipid content fish embryos. Coincidently, this is the aqueous concentration range reported by Turcotte et al.[44] for a series of alkylated phenanthrenes causing toxic effects in ELS medaka (*Oryzias latipes*) (Table 1). This relationship would hold for any organic compound exhibiting a $\log_{10}K_{ow}$ in this range and many occur in the UCM. Of course, metabolism of these compounds must be considered when predicting tissue concentrations; however even if 90% of the compound is metabolized, predicted sublethal tissue concentrations would still be within the range observed for baseline toxicity. It is important to note that the unmeasured metabolites for such single compound studies may also contribute to the toxic response and may be as toxic or more toxic compared to the parent compound[95].

Within an organism, the tissue concentration for the baseline toxicity response is remarkably consistent. The main reason baseline toxicity occurs within a narrow range of internal concentrations is that membranes will lose fluidity when these lipophilic compounds achieve a critical concentration[88] and this is a common feature among all organisms. Once this critical membrane concentration is reached, many normal membrane functions are altered, such as ionophore regulation of internal and external cell concentrations of important ions, such as potassium, chloride, and calcium. These imbalances rapidly lead to cell injury and ultimately organism death at

these well-defined internal concentrations. At sublethal concentrations, cell function is inhibited resulting in a variety of responses, including edemas, cardiac dysfunction, and skeletal abnormalities, as seen for a variety of organic compounds (Table 1).

The toxicity of most compounds acting by a baseline mode of action is correlated with K_{ow} indicating a lack of specificity and more likely a physicochemical partitioning from aqueous exposure to the internal concentration. As noted by Turcotte et al.[44] and Hodson[13] a high correlation exists between ELS fish toxicity and logK_{ow} for a variety of individual PAHs indicating the likelihood of baseline toxicity. These correlations exhibit slopes that are very close or identical to the universal narcosis slope of -0.945 for non-polar compounds described by Di Toro et al.[91]. Petersen et al.[59] also observed a high correlation between the fish hepatocyte toxicity and logK_{ow} for a variety of compounds associated with the UCM suggesting a non-specific mode of action; however, it is possible that some of these compounds may cause specific toxic effects via undetermined receptors.

Two recent studies highlighted the role of calcium cycling in baseline toxicity. One study, Antczak et al.[84] provided compelling data that inhibition of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) plays an important role for the MIE in baseline toxicity. Another study is a meta-analysis of the transcriptome for zebrafish embryo studies as a function of chemical exposure (60 different compounds), which noted a striking similarity among studies for downregulation of genes related to calcium homeostasis[96]. Other studies relating alterations in calcium homeostasis to membrane disruption include Farber[87] and Rojanasakul et al.[97]. It is our hypothesis that this syndrome of effects (deformities, heart rate alteration, spinal curvature, growth effects, pericaridal edema and abnormalities, and yolk sac edema) described for larval fish exposed to crude oil and other compounds appears to be the result of baseline toxicity. This is the simplest model for the

observed responses from a crude oil WSF and other compounds (Table 1); however, we cannot rule out more specific acting mechanisms.

It is possible that the reduction in heart rate described by Brette et al.[7,9] for myocytes from juvenile and adult fish may be a specific response to PAHs and/or other components in a WSF elicited by channel blockage. To our knowledge, this level of detail for the mechanistic process of cardiac abnormalities described by these authors has not been examined for baseline toxicity. Phenanthrene was tested in Brette et al.[7], but at concentrations expected to cause baseline toxicity. A critical experiment would entail similar studies with myocytes exposed to known baseline toxicants. Additional studies with PAH mixtures at environmentally relevant levels in relation to plasma and ambient water concentrations would also be necessary.

4.2. Other Chemicals Cause Similar Responses in ELS Fish

There is strong support that this suite of abnormalities for larval fish exposed to high concentrations of a variety of compounds is not unique for PAHs and is more indicative of a baseline response. Noted chemicals include alkylphenols (mixture)[98], bisphenol A[69], benzo[a]pyrene and fluoranthene[99], aniline and 6 chlorinated anilines[71], triclosan[67], acrylamide[66], and diketone antibiotics[100]. Horie et al.[65] tested 20 chemicals (metals and organic compounds, comprising of pesticides, pharmaceuticals, aromatics, and chlorinated anilines) using the OECD short-term toxicity test for fish embryo and sac-fry stages. For most organic compounds and one metal at high concentration the heart rate for zebrafish was significantly reduced. Most of the organic compounds in this study caused yolk-sac and pericardial edema, inhibition of swim bladder inflation, and body curvature at high concentration indicating that these are common effects for all organic compounds at high

exposure concentrations. As noted by Roush et al.[101], pericardial edema is commonly observed response in fish embryo toxicity testing.

Even nanoparticles are known to disrupt membrane potential resulting in altered intracellular potassium and calcium levels through depolarization of the membrane[97,102], causing similar responses to that observed for baseline toxicity. Related to that is the observation of yolk sac and pericardial edema and fin malformations in zebrafish embryos exposed to a variety of nanoparticles[103]. Additionally, Wu et al.[104] observed pericardial edema, spinal curvature, and a variety of morphological abnormalities in medaka embryos exposed to silver nanoparticles and one study[105] recommends assessing intracellular calcium as a screening tool for nanoparticle toxicity.

As noted by McCarty et al.[64] an evaluation of 161 neutral compounds causing baseline toxicity for small aquatic organisms exhibited a geometric mean lethal residue (LR50) (also known as the critical body residue (CBR)) of 1.80 mmol/kg (mM) wet weight (95% CI 0.18–18.0). Table 1 lists the LC50 values for several compounds, which was translated to a LR50 for comparison to the range noted by several authors for baseline toxicity[64,85]. This was accomplished with a standard bioconcentration factor (BCF) prediction equation using the Kow[106]. The BCF equation was rearranged to predict the LR50 based on the LC50[63]. The LC50 was not available for the Turcotte et al.[44] data so the EC50 data were used for these calculations. The data in Table 1 for 30 organic compounds, 6 WAF mixtures, and 2 metals (excluding nanoparticles) also demonstrate that the syndrome of effects for ELS fish, including lowered heart rate, edemas, body curvature, and impaired swim bladder inflation is a result of baseline toxicity.

The LR50 for 29 of the organic compounds in Table 1 were very close to this mean baseline LR50 value (CBR) of 1.8 mM (within a factor of 3), or exceeded it. This was also true for the crude oil WAFs assuming a mean

log10 Kow of 4. The LR50 for one compound (lovastatin) was less than 3x below the mean LR50, but within the 95% CI and one compound (cymoxanil) was far below the expected baseline LR50. In some cases an LR50 may be lower than expected as a result of a pH specific Kow, rapid half-life, or the presence of toxic metabolites. Pharmaceuticals such as propranolol and diclofenac can cause specific effects at low doses and are also known to cause baseline toxicity at high doses as demonstrated by Escher et al.[88], therefore the observed syndrome of abnormalities noted for fish exposed to these compounds is likely consistent with baseline toxicity.

All compounds (except cymoxanil and lovastatin) from Table 1 where plotted against logKow and the results clearly show a strong relationship between lethal and sublethal responses and this physicochemical parameter (**Figure** 1). The high coefficient of determination (r^2 >0.77) for these regressions indicates baseline toxicity for all plotted compounds. The slopes for these regressions (0.63 – 0.71) are lower than the universal narcosis slope of 0.94[91]; however they are very similar to the slope coefficient (0.67) shown by Ellison et al.[107] for baseline toxicants from fish embryo toxicity testing. The observed variability among data points is likely a result of the variable lipid content for ELS fish[50], a mix of polar and nonpolar compounds, variable exposure concentrations[107], evaluation of responses at different time points, estimated Kow values, and variable toxicity metrics (LOEC versus ECp).

In terms of TPH in the WSF, ELS fish toxicity occurs at aqueous concentrations in the range of 0.1 – 5 mg/L[76,77,80] resulting in pericardial edema, skeletal and jaw abnormalities. Bioaccumulation modeling predicts whole-body concentrations of 2,500 mg/Kg in fish exposed to a WSF of 5 mg/L assuming an average $\log_{10}$Kow of 4 for compounds, which is approximately 6 – 7 times the level needed for a baseline lethal response[63]. Also noteworthy are the data from Hawkins et al.[51] and Vergauwen et al.[70] who observed the crude oil toxicity syndrome for ELS fish at phenanthrene

whole-body tissue concentrations of 0.2 – 3 mM (Table 1) after exposure to 0.05 – 0.5 mg/L, which is consistent with the data from Turcotte et al.[44] and within the expected range for baseline toxicity.

Table 1 also highlights the separation between lethal and sublethal effect concentrations (LC50/LOEC or LC50/ECp) and the likelihood that these exposure concentrations fall within the range noted for baseline toxicity. For almost all compounds in Table 1, the lethal to sublethal ratio (ACR) falls within the expected range of 1 - 10 for baseline toxicity[91,93,108], and most are less than 5. Lower ACR values indicate the relative closeness of lethal values to sublethal responses for the ELS toxicity syndrome, which is noted for baseline toxicants and less so for specific acting toxicants[108,109]. Also noteworthy are the Esbaugh et al.[40] data for LC50 and pericardial edema exhibiting ACRs ranging from 0.7 to 3.5 for all WAF preparations, which are based on dissolved ∑PAHs. Based on the predicted LR50 values, ACR data, and correlations between Kow and toxic effects, most of the compounds in Table 1 appear to be acting as baseline toxicants at these high concentrations, which is also noted for the WAF results and tricyclic PAHs in this table.

5. OTHER MODES AND MECHANISMS OF ACTION FOR PAHS

The baseline mode of action mediated via calcium and potassium imbalance is not the only possible response for PAHs. Numerous studies indicate that PAHs can affect growth, lipid metabolism, immune dysfunction, and related physiological responses in fish[110–114]. Many of these studies were conducted with older life stages; however, these responses may also occur for ELS fish. Suspected receptors for these responses include peroxisome proliferator-activated receptors (PPARs), early growth response protein-1 (ERG-1)[115], and other unidentified receptors. For example, receptor based changes via

PPARs may play a role in lipid metabolism, growth, and other related physiological responses[116].

Additionally, some PAHs (especially high molecular weight (HMW) compounds) are known to act via the aryl hydrocarbon receptor (AhR) to produce a similar suite of developmental abnormalities in ELS fish, (blue-sac disease)[43]. This response for ELS fish, although similar, is considered separate from the AhR-independent action of the tricyclic PAHs that are assumed to be the main toxic agents in a crude oil WSF. Even though dioxin-like toxicity is considered to be receptor based and responses occur at very low doses, the specific molecular events causing such toxicity are not well established. It is interesting to note that AhR-dependent toxicity for PAHs is known to affect calcium levels and the heart is considered to be the target organ[117]. Calcium metabolism has also been highlighted as an important altered process for dioxin toxicity to zebrafish[118]. As noted by Incardona[37], AhR activation for some HMW PAHs will down-regulate genes associated with calcium homeostasis, such as SERCA. Perhaps it is this commonality in molecular events that results in the similar syndrome of abnormalities for ELS fish resulting from AhR dependent and independent action, one specific (receptor based) and the other non-specific (baseline toxicity).

6. TOXIC UNITS AND THE FALLACY OF ASSUMING HIGHLY TOXIC UNKNOWN COMPOUNDS

One of the best ways to understand the contribution of various components within a mixture is to evaluate the toxic responses with a toxic unit approach. The base equation is as follows:

$$\text{Sum of toxic units } (\Sigma TU) = \sum_{i=1}^{n} \frac{[\text{water}]i}{ECp\ i}$$

Where ∑TU is the sum of toxic units (TU), [water] is the water concentration for the individual PAH, ECp is the effective concentration for a given endpoint

(e.g., spinal curvature or edema) and p is proportion responding. For an example, we can look at the 6 EC50s for phenanthrene and alkylphenanthrenes (excluding retene) from Turcotte et al.[44], which range from 39 – 116 µg/L. If each compound was represented equally (0.166) in the mixture, the sum of those PAHs would equal 75 µg/L and the sum of toxic units would equal 1.0. Consider that the sum of concentrations for individual compounds in a mixture cannot be lower than the EC50 of the most toxic component when the ∑TU=1. For the TU analysis, it does not matter if you have 6 or 100 PAHs; the results will be the same. The sum all these PAHs cannot be lower than the EC50 for the most toxic compound in the mixture when ∑TU=1. This TU approach also assumes additivity and there are no data to indicate that the toxicity of a mixture of tricyclic PAHs is more than (or less than) additive. To achieve the low total tricyclic PAH concentrations (often below 1 µg/L) proposed by several authors, the potency for the PAHs found in the WSF would have to be at least 2 or 3 orders of magnitude greater than what was reported in Turcotte et al.[44] and Geier et al.[45]. There are no data to the contrary and we suspect that these 6 phenanthrenes (excluding retene) are representative of the toxicity expected for this group of tricyclic PAHs covering a wide range in Kow. Based on this, we propose that a more reasonable explanation for the observed WSF toxicity includes the contribution of other unidentified hydrocarbons and polar compounds causing baseline toxicity or a receptor-mediated response at far higher concentrations than reported using a limited set of PAHs.

As noted above for a toxic unit approach, a mixture of these compounds will not result in a comparable response at concentrations below that for the most toxic of alkylated phenanthrenes. It is also highly unlikely that there are unknown alkylated phenanthrenes that can produce a toxic response in ELS fish at concentrations far below those on the Turcotte et al.[44] list, which may be an assumption for those concluding sub ppb concentrations for PAHs

causing larval fish abnormalities. The review paper by Hodson[13] (supplemental data) also supports toxic responses for tricyclic PAHs in the mid- to high ppb range, not sub-ppb aqueous concentrations as proposed by many authors.

7. CONCLUSIONS AND RECOMMENDATIONS

We would fully embrace the concept of PAHs as the toxic component of weathered crude oil WSFs if the data supported that conclusion. Such a framework would make comparing doses across WSFs and WAFs in the lab and field far easier for chemists and toxicologists. At this time, the critical experiment(s) to support that claim have not been performed or reported. This would entail exposing fish embryos to a mixture of the most toxic PAHs and eliciting responses at the claimed concentrations noted for the WSF from crude oil. A large number of alkylated phenanthrenes and other tricyclic PAHs are available commercially, so this is not an impossible task. Additionally, categorical identification of key receptor(s) and data on compound potency, including binding affinity, ligand efficacy, and IC50 would be needed to define the MIE, which to date has not been achieved.

The only reasonable conclusion is that there are additional compounds in the WSF that are likely contributing to the toxic response. In light of that, the most acceptable conclusion is that numerous component(s) of the WSF (PAHs, aliphatics, heterocyclics, polar compounds, and others) are contributing to the dose causing the observed syndrome of abnormalities. There is simply no proof that PAHs alone can cause adverse effects at the concentrations claimed by multiple authors. There is no doubt that relatively low levels of total petroleum hydrocarbons (mg/L) result in cardiac abnormalities leading to a cascade of abnormalities in ELS fish. What is remarkable about this response is the apparently permanent impairment to cardiac function when exposure occurs at a critical point during

development. Recent studies have demonstrated that fish embryos exposed to crude oil exhibited decreased swimming performance one year after exposure[8,119], which is an important response having population-level consequences. This is also the case for impaired metabolism and growth at this critical life stage due to oil exposure[112], which will affect vitality and the likelihood of a successful life cycle.

Is it a coincidence that a large number of compounds, including nanoparticles, act by non-specific membrane disruption causing a very similar suite of responses for ELS fish as that claimed for exceedingly low levels of tricyclic PAHs via a specific receptor? Perhaps, however this cannot be confirmed until critical experiments are performed, the receptor is characterized, and tricyclic PAH potency is quantified.

Based on the disparate data presented above, we conclude that the ELS fish toxicity syndrome elicited by suspected tricyclic PAHs is in fact a result of a much larger suite of compounds, perhaps some acting by a specific mechanism of action. We believe that the prevailing studies support a baseline toxic response for a common syndrome of effects for ELS fish exposed to crude oil WSF because of the lack of an identified receptor, similarity in the phenotypic response for other organic compounds and nanoparticles, and the lack of a well-characterized exposure dose, both external and internal. This is not to say that specific receptors for PAHs do not exist and that specific toxic responses, such as metabolic disruption and cardiac abnormalities are not evident. We would like to add that this synopsis does not negate any previous research, but strives to encourage a more scientifically defensible inference of the observed results.

The most overriding recommendation we have to offer researchers is to suspend characterizing the toxicity of the WSF of oil in terms of the ∑PAHs, or at least include sufficient caveats highlighting the uncertainty. Toxicity for

these compounds must be evaluated on an individual basis and in terms of mixtures with all components known. Only then can we conduct field studies and provide reasonable levels of total petroleum hydrocarbons or a known suite of the most toxic compounds that can be used to predict toxic effects and set protective levels. We also recommend continued research on the UCM, especially the polar compounds, and their role in ELS fish toxicity. There is no doubt that the WSF from crude oil causes severe effects to larval fish and we believe the most appropriate, but not necessarily the best dose metric at this time would be water or tissue concentrations of total petroleum hydrocarbons (mg/L or mg/Kg TPH). As noted by Landrum et al.[56], the absorbed dose along with information on toxicodynamics for individual components or classes (if available) is crucial for a more accurate toxicity assessment of complex mixtures.

Additionally, because the ELS fish toxicity syndrome consisting of edemas, heart rate abnormalities, and morphological abnormalities is a generalized response to high concentrations of organic compounds acting by a baseline mechanism, we recommend searching for other genomic, physiological, or apical endpoints that may be more indicative of crude oil toxicity. Longer exposures at lower concentrations may also help to reveal specific responses that can be used to characterize crude oil toxicity.

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Figure caption

Figure 1. Data from Table 1 plotted to show relationship between Kow and toxicity in early life stage fish exposed to high concentrations of organic compounds. Lines show the fitted regression and upper and lower 95% confidence bands (blue). All values for compounds with a $\log_{10}$Kow >0 and baseline toxicity predicted were plotted. a. Mortality, b. Reduce heart rate, c. Edema, d. Morphological abnormalities. Edema includes yolk sac and cardiac edema and morphological abnormalities includes spinal (mostly), eye, and jaw deformities. A few compounds of interest are labeled. Some points indistinguishable and designated as (2 or 3). Two additional LC50s included for phenanthrene from Gündel et al.[62] and Butler et al.[49]. Data from Turcotte et al.[44] included one or more of these sublethal endpoints and are were plotted under morphological abnormalities (Figure 1d). Acry = acrylamide, DCA = 2,4-dicloroanailine, NP = 4-nonylphenol, BPA = bisphenol A, Phen = phenanthrene, Alkyl-Phen = alkylated phenanthrenes, TPH = total petroleum hydrocarbons. See Table 1 for details.

6

Figure 1a

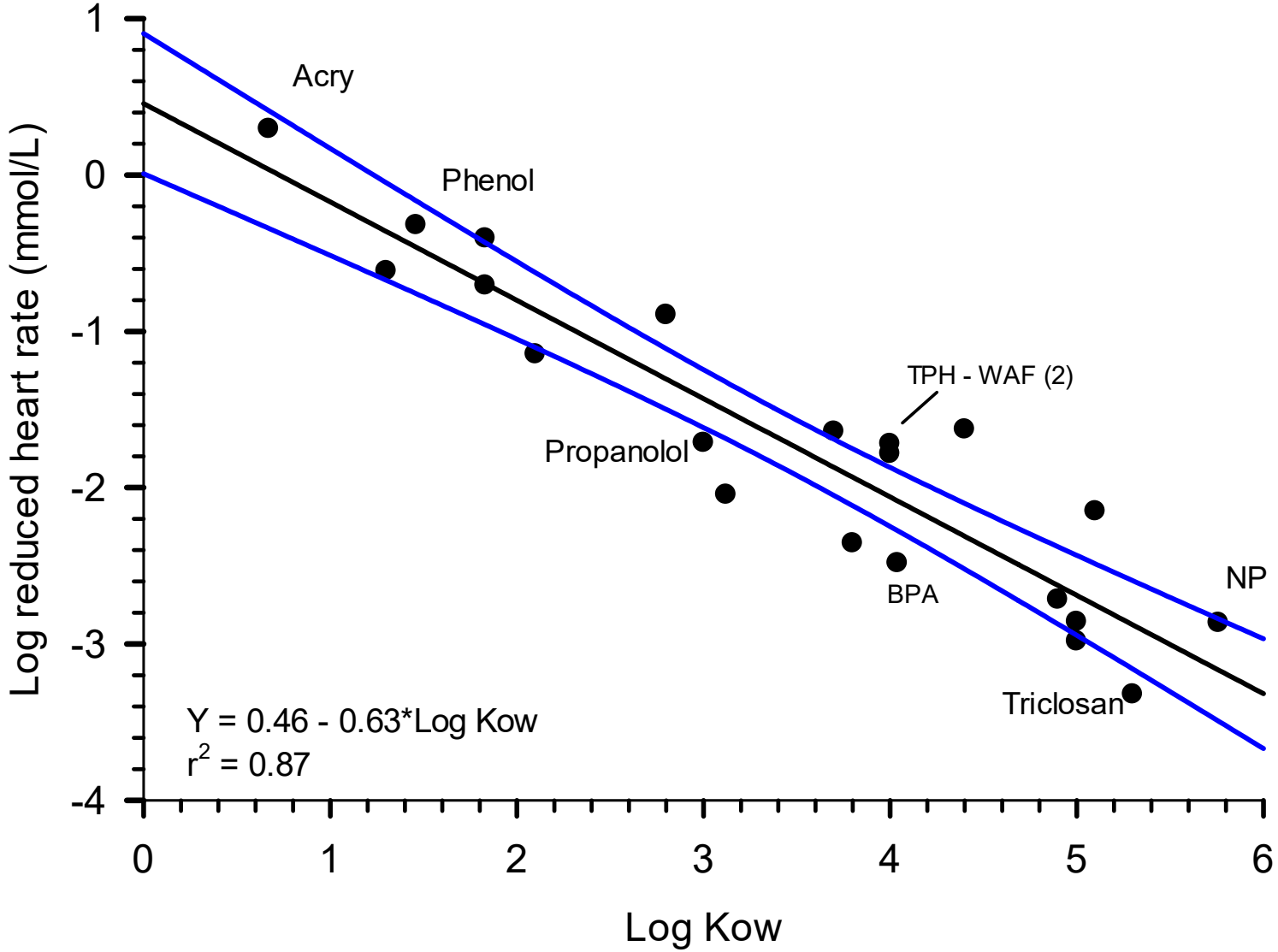
Log reduced heart rate (mmol/L)
Log Kow
Acry
Phenol
Propanolol
TPH - WAF (2)
BPA
NP
Triclosan
Y = 0.46 - 0.63*Log Kow
r2 = 0.87
1
0
-1
-2
-3
-4
0
1
2
3
4
5
6

Figure 1b.

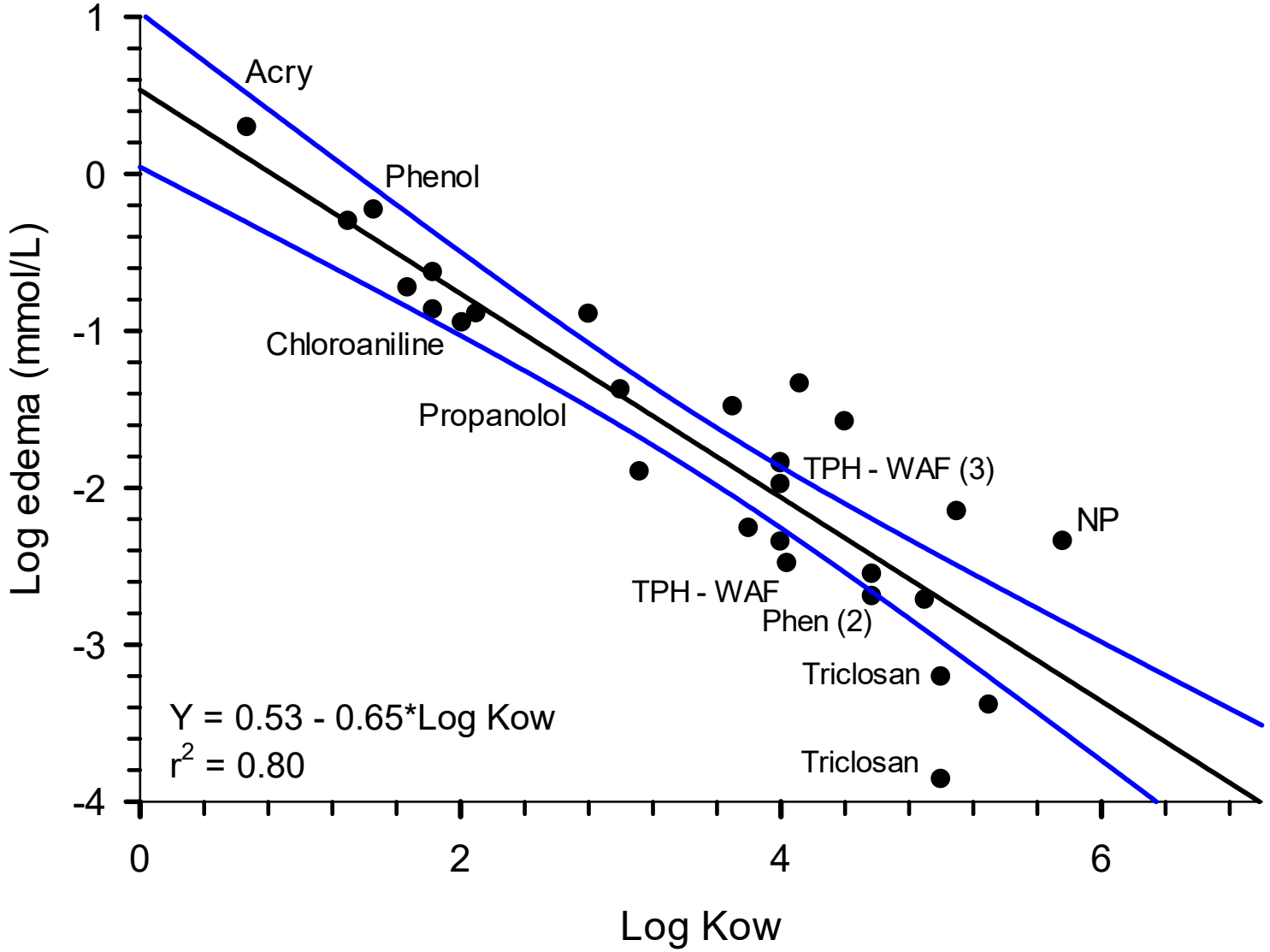
Log edema (mmol/L)
1
0
-1
-2
-3
-4
0
2
4
6
Log Kow
Acry
Phenol
Chloroaniline
Propanolol
TPH - WAF (3)
NP
TPH - WAF
Phen (2)
Triclosan
Triclosan
Y = 0.53 - 0.65*Log Kow
r2 = 0.80

Figure 1c.

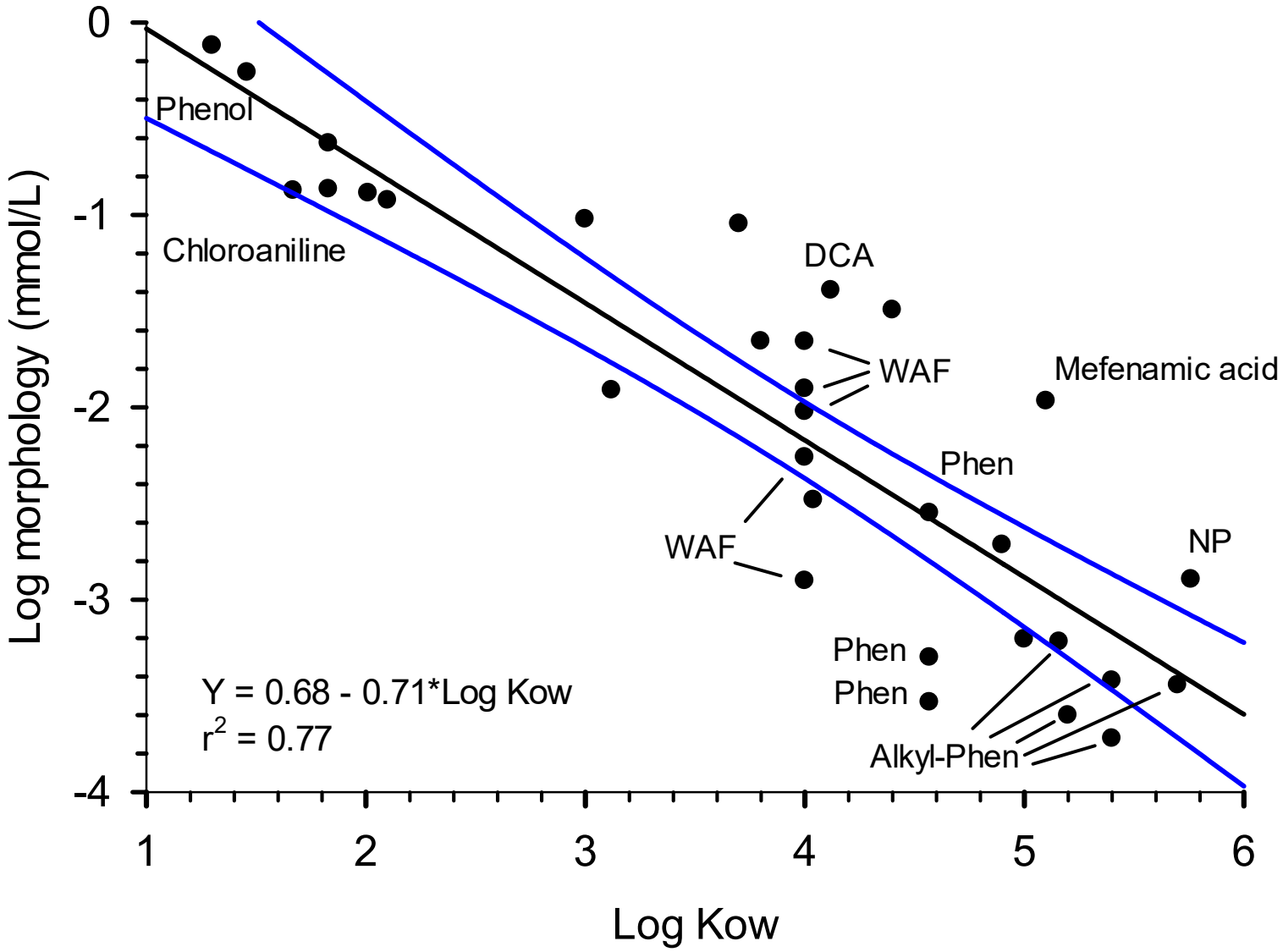
Log morphology (mmol/L)
0
-1
-2
-3
-4
1
2
3
4
5
6
Log Kow
Phenol
Chloroaniline
DCA
WAF
Mefenamic acid
Phen
WAF
NP
Phen
Phen
Alkyl-Phen
Y = 0.68 - 0.71*Log Kow
r2 = 0.77

Figure 1d.

Table 1. Toxicity metrics for syndrome of effects observed for early-life stage fish.

		LOEC or EC50 (mg/L)				LC50 (mg/L)	LR50 pred mg/kg (mM)	Baseline tox[1]	ACR	Ref
Chemical	Log_{10} Kow	Heart rate reduced	Edema	Morph abnormality	Impaired swim bladder inflation					
2,6 Dinitrotoulene	2.1	13	23.4	21.7	12.1	21.8	265 (1.5)	Y	0.9 – 1.8	1
3-Chloroaniline	1.83	50	29.9	29.9	17.7	30.2	217 (1.7)	Y	0.6 - 1.7	1
4-Chloroaniline	1.83	> 25	> 17.3	> 17.3	9.9	18.9	136 (1.1)	Y	0.8 – 1.9	1
4-nonylphenol	5.76	0.3	> 1	0.28	0.23	0.29	4554 (20.7)	Y	0.3 - 1.3	1
Cymoxanil	0.67	5	1.6*	< 7.2	< 5.2	4.3	6.1 (0.03)	?	0.6 – 2.7	1
Diclofenac	4.4	7	7.8*	9.5*	7.9	6.9	7565 (25.6)	Y	0.7 - 1.0	1
Fenitrothion	3.12	2.5	3.5	3.4	2.25	3.2	286 (1.0)	Y	0.9 – 1.4	1
Lovastatin	4.31	0.01	0.008	0.008	0.006	0.12	110 (0.27)	?	15 - 20	1
Mefenamic acid	5.1	1.7	1.7	2.6	1.7	1.6	6904 (28.6)	Y	0.6 – 0.9	1
Mercury (II)	–	NR	26.4	20.7	11.0	32.4	–		1.2 - 2.9	1
Phenol	1.46	45	55.4	51.8	33.0	60.4	210 (2.23)	Y	1.1 - 1.8	1

		LOEC or EC50 (mg/L)				LC50 (mg/L)	LR50 pred mg/kg (mM)	Baseline tox[1]	ACR	Ref
Chemical	Log_{10} Kow	Heart rate reduced	Edema	Morph abnormality	Impaired swim bladder inflation					
Propanolol	3.0	5	10.9	24.6	5.3	8.8	623 (2.4)	Y	0.4 – 1.8	1
p-Toluidine	1.3	26	53.4	81.4	39.9	64.6	164 (1.5)	Y	0.8 – 2.5	1
Silver nitrate	–	0.05	> 0.23	> 0.23	> 0.23	0.08	–		0.3 – 1.6	1
Tebuconazole	3.7	7	10.1	27.6*	11.0	10.8	3010 (9.80)	Y	0.4 - 1.5	1
Triclosan	5	0.3	> 0.18	> 0.18	0.14	0.10	231 (0.80)	Y	0.7 – 3.3	1
Acrylamide @	0.67	140	140	NR	NR	140	197 (2.8)	Y	1.0	2
Triclosan	5	0.4	0.04 -0.4	NR	NR	NR	887 (3.1)	Y	1.0 - 10	3
Verapamil	3.8	2	1 – 5	10.0	NR	16.4	436 (0.96)	Y	1.6 – 8.2	4
Bisphenol A	4.04	0.75 – 3.0	0.75 – 3.0	0.75 – 3.0	0.75 – 3.0	3.96	2146 (9.4)	Y	5.3	5
Phenanthrene	4.57	NR	0.36 (3.2 mM wb)71%^	0.052 (0.2 mM wb)^	0.059 (0.3 mM wb)^	0.31	382 (2.7)^	Y	0.9 – 6.0	6
2-Nitroaniline	1.67	NR	26	18.5	9.6	40.1	210 (1.5)	Y	1.5 – 4.2	7
2,4-Dichloroaniline	4.12	NR	9.5	8.4	3.5	12.3	7797 (37)	Y	1.3 – 3.5	7
2-Chloroaniline	2.01	NR	14.3	> 16.5	4.4	6.9	70.4 (0.6)	Y	0.4 – 1.6	7

		LOEC or EC50 (mg/L)				LC50 (mg/L)	LR50 pred mg/kg (mM)	Baseline tox[1]	ACR	Ref
Chemical	Log_{10} Kow	Heart rate reduced	Edema	Morph abnormality	Impaired swim bladder inflation					
Phenanthrene	4.57	NR	NR	0.089	NR	–	136 (0.76)	Y		8
1-Methylphenanthrene	5.16	NR	NR	0.116	NR	–	563 (2.9)	Y		8
2-Ethylphenanthrene	5.2	NR	NR	0.048	NR	–	252 (1.3)	Y		8
2,7- Dimethyl phenanthrene	5.4	NR	NR	0.039	NR	–	303 (1.5)	Y		8
1,7- Dimethyl phenanthrene	5.4	NR	NR	0.078	NR	–	605 (2.9)	Y		8
7-ethyl, 1-Methyl phenanthrene	5.7	NR	NR	0.079	NR	–	1103 (5.0)	Y		8
Butyl benzyl phthalate	4.9	0.6	0.6	0.6	0.6	1.2[#]	3500 (11.2)	Y	2	9
Triadimefon	2.8	37.4	37.4	NR	NR	47.2	2259 (7.7)	Y	1.3	10
Triclocarban	5.3	0.15	0.13[&]	NR	0.13	0.22	1372 (4.0)	Y	1.5 – 1.7	11
Phenanthrene @	4.57	NR	0.5 (1.8 mM wb)^ 9%=signf	0.5 (1.8 mM wb)^ 100%	NR	0.5 (44%)	316 (1.8)^	Y	1.0	12
Mono & diaromatics	–	NR	1.5 – 2	1.5 – 2	NR	2.2 (10 dph)	–	Y	1.1 - 1.5	13

		LOEC or EC50 (mg/L)				LC50 (mg/L)	LR50 pred mg/kg (mM)	Baseline tox[1]	ACR	Ref
Chemical	Log_{10} Kow	Heart rate reduced	Edema	Morph abnormality	Impaired swim bladder inflation					
TPH in WAF £	4	NR	2.85ϒ	NR	NR	7.1	3,558 (17.8)	Y	2.5	14
TPH in WAF	4	NR	0.9	1.9	NR	1.3 (larvae)	652 (3.3)	Y	0.7 – 1.4	15
TPH in WAF	4	NR	NR	0.25 – 0.97 (morphological and edema)	NR	NR	–	Y		16
TPH in WAF	4	NR	NR	1.1	NR	1.1	551 (2.8)	Y	1.0	17
TPH in WAF	4	3.3	2.9 – 3.5	> 4.4	NR	2.6 – 3.4	1,504 (7.5)	Y	0.7 - 1.0	18
TPH in WAF	4	3.6 – 3.9	0.32 – 3.3	1.5 – 3.5	0.32 – 1.0	3.6 – 4.3	2,005 (10.0)	Y	1.1 – 6.0	19

Most Log_{10} Kow values obtained from ChEMBL and PubChem. Edema includes yolk sac and pericardial edema and morphological abnormalities include spinal curvature, jaw, and eye. All studies conducted with embryos and larvae ranging from 1 – 17 dpf, unless noted. LR50pred for Turcotte et al.[45] based on EC50, which combined all edemas and morphological abnormalities into one EC50 value. # is LC20 and & = yolk sac absorption delay. All sublethal values as LOEC or ECp, where p=percentage and most were 50%. LR50pred is the predicted tissue concentration causing mortality using a rearranged standard BCF equation[64]. NR = not reported. @= one concentration tested. ^ data from study (sac fry or embryo concentrations measured). Wb = whole body. £ includes dispersant (e.g., Corexit), ϒ = blue-sac index (includes edemas and morphological deformities). Baseline tox[1] indicates if the predicted tissue concentration associated with the LC50 or ECp falls within the range observed for baseline toxicity (mean= 1.8 mM; 95% CI = 0.18 – 18.0 mM[65]. Y=yes. Comparison of concentrations for LC50 and sublethal responses expressed as acute-to-chronic ratio (ACR) (lethal/sublethal), which is LC50/LOEC or ECp. ACR and LR50 used to determine if baseline toxicity possible. TPH = total petroleum hydrocarbons (not all compounds in WAF measured). Studies that reported TPH using

fluorescence were not included because of under reporting. Baseline tox prediction for TPH assumes an average $\log_{10}$Kow=4 and molecular weight of 200 (see text). Weathered oil used in study 14 and unweathered oil for 15 - 19.

1. Horie et al.[66], 2. Huang et al.[67], 3. Saley et al.[68], 4. Steinbach et al.[69], 5. Kankaya et al.[70], 6. Vergauwen et al.[71], 7. Horie et al.[72], 8. Turcotte et al.[45], 9. Sun and Liu[73], 10. Liu et al.[74], 11. Dong et al.[75], 12. Hawkins et al.[52], 13. Carls and Rice[76], 14. Greer et al.[77], 15. Pollino and Holdway[78], 16. Kocan et al.[79], 17. Karam et al.[80], 18. Philibert et al.[81], 19. Li et al.[82]. Fish species studied in refs 1-3, 6-7, 9-11, and 18-19 was *Danio rerio.* 4, *Cyprinus carpio. 5, Chalcalburnus tarichi.* 8, *Oryzias latipes*. 12, *Oncorhynchus mykiss.* 13, *Theragara Chalcogramma*. 14, *Clupea harengus.* 15, *Melanotaenia fluviatilis.* 16, *Clupea pallasi.* 17, *Epinephelus coicoides*.