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Predicted and measured acute toxicity and developmental abnormalities in zebrafish embryos produced by exposure to individual aromatic acids

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Title: **Predicted and measured acute toxicity and developmental abnormalities in zebrafish embryos produced by exposure to individual aromatic acids**

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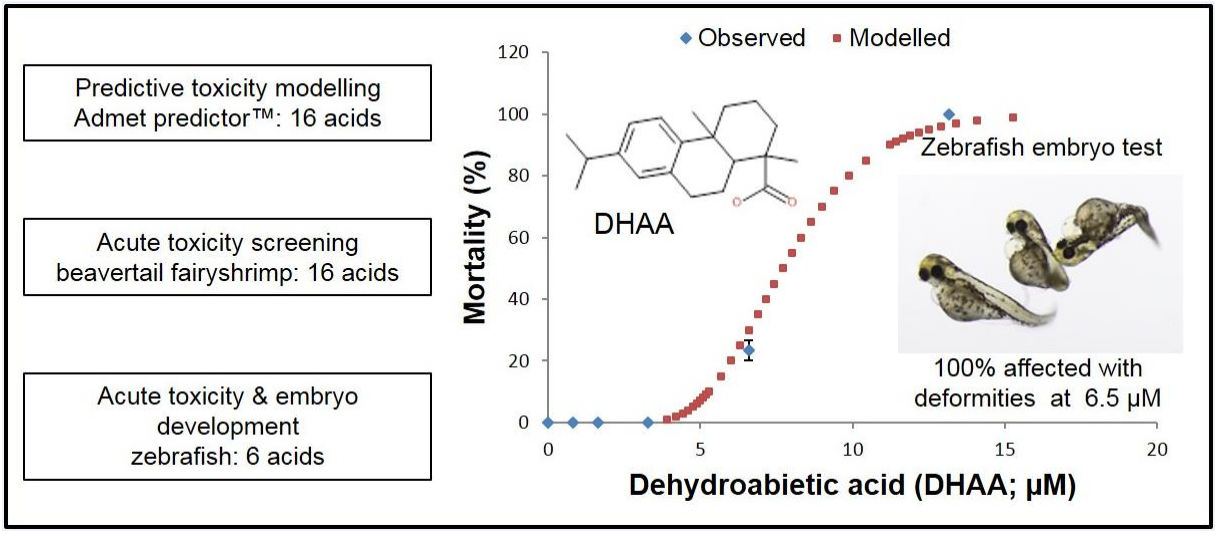
Highlights

- Acute toxicity of 16 individual aromatic ‘naphthenic’ acids assayed (invertebrate).
- Lethality and developmental abnormalities of 6 individual acids assayed (fish).
- Toxicities modelled using commercially-available Admet predictor™ software.
- Significant relationship found between predicted and experimental fish LC_{50}s.
- Embryo abnormality EC_{50}s <7µM in 4 of 6 acids; 4-(p-tolyl)benzoic acid most toxic.

Graphical Abstract



Abstract

Petroleum acids, often called ‘Naphthenic Acids’ (NA), enter the environment in complex mixtures from numerous sources. These include from Produced and Process-Affected waters discharged from some oil industry activities, and from the environmental weathering of spilled crude oil hydrocarbons. Here, we test the hypothesis that individual NA within the complex mixtures can induce developmental abnormalities in fish, by screening a range of individual acids, with known chemical structures.

Sixteen aromatic NA were tested using a *Thamnocephalus platyrus* (beavertail fairyshrimp) assay, to establish acute toxicity. Toxicities ranged from 568 to 8 µM, with the methylbiphenyl acid, 4-(*p*-tolyl)benzoic acid, most toxic.

Next, five of the most toxic monoacids and, for comparison, a diacid were assayed using *Danio reri*o (zebrafish) embryos to test for lethality and developmental abnormalities. The toxicities were also predicted using Admet predictor™ software. Exposure to the five monoacids produced deformities in zebrafish embryos in a dose-dependent manner. Thus, exposure to 4-(*p*-tolyl)benzoic acid produced abnormalities in >90% of the embryos at concentrations of <1µM; exposure to dehydroabietic acid caused pericardial edema and stunted growth in 100% of the embryos at 6 µM and exposure to pyrene-1-carboxylic acid caused 80% of embryos to be affected at 3 µM.

The findings of this preliminary study therefore suggest that some aromatic acids are targets for more detailed mechanistic studies of mode of action. The results should help to focus on those NA, which may be important for monitoring in oil industry wastewaters and polluted environmental samples.

1. Introduction

Crude oil and its refined fractions make major contributions to contamination of some aquatic environments, especially in the vicinities of urban and industrial areas (NRC, 2003; GESAMP, 2007; Jernelov, 2010; White et al., 2013). However, since some deleterious effects on aquatic organisms could not be explained by exposure to the most abundant petroleum contaminants, such as polycyclic aromatic hydrocarbons (PAHs) and alkyl PAHs, studies have also focused occasionally on more 'polar' compounds (e.g. Knag et al., 2013). The so-called 'naphthenic acids' (NA) are often the most abundant polar compounds in petroleum (e.g. Melbye et al., 2009; Thomas et al., 2009; Knag et al., 2013; Ruddy et al., 2014). The term NA describes broadly those carboxylic acids in biotransformed petroleum, including in oil sands and in biodegraded, spilled and reservoired, crude oils (Clemente and Fedorak, 2005; West et al., 2014; Brown and Ulrich, 2015; Headley et al., 2015). Following releases of crude oil into the environment, NA and other polar components may be detected, either because they were already present, or because of subsequent physico-chemical 'weathering' including photo-oxidation and/or microbiological oxidation. For instance, after the Deepwater Horizon, Macondo well spill in 2010 released an estimated 4.9 million barrels of crude oil (780000 m^3) into the Gulf of Mexico; analyses of beached oil revealed a diverse range of polar compounds, including NA (Ruddy et al., 2014). The undegraded spilled Macondo well oil already comprised ~10% polar constituents (Reddy et al., 2012) and therefore around 80000 m^3 of these substances were possibly released directly into the environment initially and this was likely increased by subsequent natural transformations of the hydrocarbons (Aeppli et al., 2012). Indeed, NA metabolites for anaerobic oxidation of the Macondo well oil were reported in Gulf of Mexico sediments close to Deepwater Horizon following the spill

(Kimes et al., 2013; Valentine et al., 2014). Wan et al., (2014) also measured increased concentrations of NA in sediments following the Hebei Spirit oil spill.

In addition to microbial processes, in which NA are produced from the hydrocarbons either in-reservoir or in the environment (e.g. reviewed by Agrawal and Gieg, 2013), hydrocarbon metabolism by sediment-dwelling organisms such as worms also produces the corresponding acids (Malmquist et al., 2015). Thus, there are a number of pathways by which NA may enter the environment and by which organisms may become exposed to petroleum-derived NA.

Various toxic effects have been attributed to NA (reviewed by Clemente and Fedorak, 2005; Kannel and Gan, 2012; Brown and Ulrich, 2015; Mahaffey and Dube, 2017). Several individual NA were tested using the bioluminescence inhibition assay Microtox™ and *Daphnia magna* acute lethality test; toxicities were found to be in the range 0.01 – 29 mM (Frank et al., 2009). Jones et al., (2011) tested the acute toxicities of a range of individual NA using Microtox™ and reported that only a few NA had EC_{50} values <50 µM. Interestingly, the concentration curves reported by Jones et al., (2011) were very steep, a phenomenon also reported for exposure to NA fractions when tested on larvae of zebrafish, prompting the hypothesis that the surfactant properties of NA were having a significant influence on toxicity (Scarlett et al., 2013). Tollefsen et al., (2012) tested the toxicity of some synthetic NA to rainbow trout liver cells. The combined toxicity of multicomponent mixtures of the NA was also assessed using the concept of concentration addition and independent action prediction. All of the acids tested had EC_{50} values in the range 108–405 µM (24–89 mg L^{-1}) and 188–656 µM (43–148 mg L^{-1}) when assessed by effects on metabolic inhibition or loss of membrane integrity, respectively. Binary and 6-compound mixtures of NA caused combined toxicity according to the concept of additivity, although slight deviations from

additivity were observed at a few mixture concentrations. More recently, Petersen et al., (2017) reported the results from *in vitro* screening on fish cells, which indicated that of the endpoints tested, the predominant toxic mode of action (MoA) was cytotoxicity. EC_{50} values for cytotoxicity were obtained for 16 compounds and ranged from 77 µM–24 mM, whereof aliphatic monocyclic acids, monoaromatic acids, polycyclic monoaromatic acids were amongst the most toxic. The observed cytotoxicity of the chemicals correlated well with the hydrophobicity (Log K_{OW}) suggesting that the toxicity was predominantly due to a non-specific MoA. Scarlett et al., (2012) studied the estrogenic and androgenic activity of individual adamantane acids using a panel of human cell-derived nuclear receptor reporter gene bioassays (CALUX®) but found no significant activity. However, genotoxic activity was observed in hepatocytes of marine mussels following *in vivo* exposures to adamantane carboxylic acids (Dissanayake et al., 2016).

Toxicity models (ADMET predictor™, Lancaster, CA, USA) have also been applied to prediction of the toxicities of individual NA and thus far, at least, have generally been in good agreement with results obtained from the small number of assays made on individual NA (Scarlett et al., 2012).

The early life stages (ELS) of fish appear to be particularly sensitive to some petroleum components (e.g. Incardona, 2017; Sorensen et al., 2017) and some studies strongly implicate NA as causative agents for developmental abnormalities in fish. For example, Marentette et al., (2015a) reported that exposure of fathead minnow *Pimephales promelas* embryos to NA fractions derived from fresh and aged oil sands process-affected water (OSPW) produced significant dose-response relationships of cardiovascular edemas and/or haemorrhages. Exposure to commercially-available NA mixtures, did not result in such relationships, but did produce an increase in finfold

deformities (Marentette et al., 2015b). This implies that development of abnormalities in fish embryos may be dependent on particular NA. Reinardy et al., (2013) noted that a fraction of NA classified as broadly alicyclic, produced no estrogenic response in embryonic fish, whilst an 'aromatic' NA fraction isolated by argentation chromatography, did produce a (albeit weak) response.

Knowledge of the toxic effects of individual NA is therefore still extremely limited. Until relatively recently, even methods for the identification of the individual acids in the complex NA mixtures from petroleum and oil sands, did not exist. However, some acids have now been identified (Rowland et al., 2011a; Rowland et al., 2011b; Rowland et al., 2011c; Jones et al., 2012; Lengger et al., 2013; Bowman et al., 2014; Wilde and Rowland, 2015; Wilde et al., 2015; Wilde and Rowland, 2018).

Given the paucity of studies of the toxic effects of individual NA and the observations that fish exposed to NA fractions derived from OSPW suffered developmental abnormalities (Marentette et al., 2015a), the present preliminary study was designed to test the hypothesis that exposure to individual aromatic acids might result in developmental abnormalities consistent with those observed for more complex mixtures of NA. A range of aromatic acids found previously to be associated with OSPW and in biodegraded crude oils and petroleum-contaminated aquifers (Annweiler et al., 2001; Elshahed et al., 2001; Boll et al., 2002; Gieg and Suflita, 2002; Aitken et al., 2004; Safinowski et al., 2006), or as the products of PAH biotransformation (e.g. Malmquist et al., 2015), were screened. The chemicals were chosen so that greater insight could be gained into any structural activity relationships. In particular, the hypothesis that toxicity might be independent of structure and similar for isomeric chemicals with identical elemental formulae was tested. The results were compared to modelled data for the waterflea *D. magna* and fathead minnow *P.*

*prome*las to examine whether prediction software, which takes account of the physicochemical properties, could predict toxicities more accurately. Zebrafish embryos were then exposed to a subset of six of these chemicals; 96h LC_{50} values were calculated and any deformities recorded and used to calculate 96h EC_{50} values.

2. Materials and Methods

2.1 Selection of test chemicals

Sixteen acids were chosen for initial lethality screening (Table 1). All test chemicals were purchased from Sigma-Aldrich (Poole, UK) with the exception of Dehydroabietic acid (DHAA, Orchid Cellmark, New Westminster BC, Canada) and had minimum stated purities ≥97%. Thamnotoxkit FTM was from MicroBioTests Inc. (Gent, Belgium).

2.2 Preparation of test solutions

The preparation of test solutions was similar to that previously reported for zebrafish larvae exposures to NA (Reinardy et al., 2013; Scarlett et al., 2013). Stock solutions of test chemicals were made prior to the exposure in amber glass vials by diluting in 1 M NaOH until fully dissolved and a known volume of exposure water. The pH of the stock was then adjusted to pH 7.4 ± 0.1 using 1 M HCl and stored at 4 ºC in amber vials until use. A negative control solution was prepared comprising of the highest NaOH concentration used and pH adjusted to match test solutions. Compared to water controls, no significant effects were observed following exposure to this NaOH control solution and all test results presented are relative to this. The concentration range

tested for each chemical was based on the predicted lethality of *D. magna* and *P. promelas* (ADMET predictor™, Lancaster, CA, USA).

2.3 Fairyshrimp acute lethality assay

The acute toxicity of the test materials was evaluated using the freshwater beavertail fairyshrimp. Exposure water was made as specified by ISO 7346-1 (ISO, 1996). The test was performed according to manufacturer's instructions (available online: http://www.microbiotests.be/SOPs/Thamnotoxkit%20F%20SOP%20-%20A5.pdf accessed 25.01.2018). Briefly, the test 24 h LC_{50} test was performed in a 24-well test plate using instars II–III larvae of the shrimp, which were hatched from cysts. Hatching was initiated 24 h prior to the start of the test. Upon hatching, 10 shrimp were exposed to various concentrations (n = 3) of the compounds and incubated at 26°C for 24 h in the dark. The test endpoint was mortality. The numbers of dead shrimps for each concentration were recorded and the respective LC_{50} values were determined using Probit or Logit analysis in SPSS 21.

2.4 Zebrafish embryo test

Wild-type WIK (Wild-Type India Calcutta) strain zebrafish embryos were obtained from the Max Plank Institute, Tubingen, Germany, and maintained at the University of Exeter as described in the supplementary material (SI).

The test was initiated as soon as possible after fertilization of the embryos and not later than 3 h post-fertilization (128-cell stage). The exposures used 300 mL crystalline dish test vessels with 100 mL of test solutions containing 20 embryos with 3 replicates

per concentration and employed a semi-static renewal technique. Temperature was maintained at 26±1 °C, and fish were kept under a constant artificial dark/light cycle of 8/16 h. Every 24 hrs, up to four apical observations were recorded as indicators of lethality (i) coagulation of fertilised eggs, (ii) lack of somite formation, (iii) lack of detachment of the tail-bud from the yolk sac, and (iv) lack of heartbeat. At the end of the 96 h exposure period, acute toxicity was determined based on a positive outcome in any of the four apical observations recorded, and LC_{50} calculated. Embryos were exposed for 96 h, with viability being checked every 24 h and dead embryos removed.

The results were deemed acceptable if: fertilisation rate of the eggs ≥70%, dissolved O_2 concentration in the negative control and highest test concentration ≥80 % of saturation at test commencement, water temperature maintained at 26±1 °C in test chambers at all times, overall survival of embryos in the negative/NaOH control ≥90% and hatching rate in the negative control ≥80 % at test termination. The number of dead fish and abnormally developed embryos for each concentration were recorded and the respective LC_{50} and half-maximal effect (EC_{50}) determined using Probit or Logit analysis in SPSS 21 based on Pearson Goodness-of-Fit test.

2.5 Predictive toxicity

Commercially available software, ADMET predictor™ (Simulations Plus Inc., Lancaster, CA) was used to predict physiochemical properties of the test chemicals plus lethal and sublethal toxicities. ADMET predictor™ uses chemical structures and experimental data to create the QSAR models which are then used to predict properties of the molecules. The models provide a range of physicochemical outputs, including log P (i.e. predicted log Kow) and water solubility provided in Table 1, plus

predictive toxicity to organisms including *D. magna* (48 h exposure lethality (LC_{50}) and *P. promelas* (96 h exposure lethality (LC_{50}). Predictive models that generate EC_{50} values for developmental abnormalities were not available but a model that predicts a binary yes/no outcome for reproductive/ developmental toxicity that relates to anything that disturbs the reproductive process of organisms, including adverse effects to sexual organs, behaviour, ease of conception, and developmental toxicity of offspring both before and after birth was available (ADMET predictor™). Full detail of the model's training/verification sets are available online http://www.simulations-plus.com/software/admetpredictor/toxicity/, accessed 25.01.2018. The LC_{50} values used to build the models were for species not routinely tested in our labs. As this is also likely for many other lab studies we wished to test if, despite these differences, there was reasonable agreement between experimental and predicted toxicities. If so, there can be greater confidence in the predictions as they will not be specific to a certain species. Regression analyses for experimental versus predicted LC_{50} values were calculated using Statgraphics Online (Warrenton, Virginia, US).

3. Results

3.1 Acute lethal toxicity to fairyshrimp

A large range of acute LC_{50} values was observed for the chemicals tested (Table 2). 4-(p-tolyl)benzoic acid (A10; Table 2), which has a biphenyl structure, was the most toxic NA with a LC_{50} of 8 µM. Phenanthrene-4-carboxylic acid was the least toxic with a LC_{50} of 568 µM. Comparing toxicities of NA with the same chemical formula, some, such as the tetralin acids (tetralin 1- and 5-carboxylic acids, A2 and A3; $C_{11}H_{12}O_2$) had reasonably similar LC_{50} values (i.e. within a factor of two), whilst others were very

different (e.g. one of the diaromatic acids 4-(p-tolyl)benzoic acid (A10; $C_{14}H_{12}O_2$) was about 30× more toxic than another (2-benzylbenzoic acid, A11; Table 2). Comparing the experimental and predicted toxicities, the experimental data obtained for fairyshrimp were generally in reasonable agreement with the predicted LC_{50} values (albeit for another aquatic crustacean species, the waterflea *D. magna*; Table 2). Five of the chemicals had LC_{50}s less than those predicted by a factor of 2, but all were within an order of magnitude. However, linear regression of experimental versus predicted LC_{50} values produced an r^2 value of 0.066 which was not statistically significant (p >0.05).

3.2 Acute lethal toxicity to zebrafish

Concentration response curves for exposures of zebrafish were generally very steep for the five chemicals for which LC_{50} values could be determined (Fig. 1). A value could not be generated for naphthalene-2,6-dicarboxylic acid as no mortality occurred. For the exposures to acids for which LC_{50} values were generated, the values ranged from 4.9 µM for 4-(p-tolyl)benzoic acid (A10;Table 2) to 151µM for tetralin-5-carboxylic acid (A3; Table 2). Pyrene-1-carboxylic acid (A 15) and DHAA (A16) had LC_{50} values of 7-8 µM. Experimental LC_{50} values for zebrafish were mainly within an order of magnitude of the predicted values for fathead minnow, despite the fact that the model was based on a different fish species and was not specific to early life stages (Table 2). Linear regression produced the relationship: experimental LC_{50} (µM) = 0.302 × predicted LC_{50} (µM) - 5.4 (r^2 = 0.934, p < 0.01, n = 5).

3.3 Developmental abnormalities in zebrafish

Abnormalities (>10%) in zebrafish embryos were observed in five of the six chemicals tested. Deformities were no greater than controls in naphthalene-2,6-dicarboxylic acid, but exposure to all of the monoacids produced concentration-dependent abnormalities including yolk-sac and pericardial edema, fin abnormalities, tail flexure and truncation, and reduced growth (illustrated for pyrene-1-carboxylic acid, A15 in Fig. 2). In addition, head and ocular edema were also observed in embryos exposed to naphthalene-2-carboxylic acid (A1; Tables 1 & 2), but not for exposures to the other chemicals (Fig. 3). Effects were only observed at relatively high concentrations for some compounds (e.g. tetralin-5-carboxylic acid, A3) produced about 80% deformities, mainly yolk-sac edema, at 96 hours post fertilisation (hpf) at 32 µM (Fig S1) produced an EC_{50} of 32 µM (Table 2). However, abnormalities were observed earlier and at much lower concentrations, for other acids. For example, following exposure to pyrene-1-carboxylic acid (A15), around 80% of embryos were affected at 3 µM and virtually all by 6 µM at 72 hpf (Fig. 2; Table 2). The diaromatic NA 4-(p-tolyl)benzoic acid (A10), produced around 90% deformities at <1 µM with an EC_{50} of 0.8 µM (Table 2). Abnormalities were predominantly pericardial edema and were observed at 24 hpf (Table 2; Fig S2). DHAA produced the same range of deformities in 100% of embryos at a concentration of 6 µM at 96 hpf (Fig. S3). Reproductive/developmental toxicity was predicted for two of the six chemicals, but developmental abnormalities in embryos were observed for only one of these: pyrene-1-carboxylic acid (A15). This tetra-aromatic acid produced an EC_{50} of 2.3 µM (Table 2). Four chemicals predicted not to cause reproductive/developmental toxicity did produce abnormalities (Table 2).

4. Discussion

4.1. Fairyshrimp screening assay

The acids tested in the initial screening assay (Table 1) were chosen on the basis of their occurrence in various petroleum-related scenarios. Thus, naphthoic-type acids (A1,4,5,12; Table 1) are known biodegradation products of naphthalene and alkyl naphthalenes in petroleum (e.g. reviewed by West et al., 2014) and tetralin carboxylic acids (A2,3; Table 1) are known constituents of OSPW (Bowman et al., 2014). Phenanthrene (A13; Table 1) and pyrene (A15; Table 1) carboxylic acids are known products of petroleum or alkylpyrene biotransformation (e.g. Malmquist et al., 2013; Aitken et al., 2018). DHAA and isomeric acids have been reported in OSPW (Jones et al., 2012) and toxicity data to fish exists so this acid also acts as a useful cross reference for comparison with data from previous studies and as a check on the validity of the present methods (Straus et al., 1997; Jones et al., 2012; Scarlett et al., 2013). A diacid, naphthalene-2,6-dicarboxylic acid, was included as diacids are known products of anaerobic biodegradation of petroleum hydrocarbons (e.g. Agrawal and Gieg, 2013; Kimes et al., 2013). In order to better understand the importance of chemical structure with regard to NA toxicity, this study included tests on several pairs of chemicals with the same empirical formulae (Table 1).

For the tetralin acids (A2, 3; $C_{11}H_{12}O_2$) there was relatively little difference in toxicity (factor ~2) regardless of whether the carboxylic acid group was on the aromatic or cyclohexyl ring. Also, very similar acute toxicities to fairyshrimp were observed for two compounds with the formula $C_{13}H_{10}O_2$ (A6,7; Table 2).

The naphthoic acids (A1, 4, 5), which possessed either a methyl group or an ethanoic (acetic) acid group, again showed similar LC_{50} values (i.e. within a factor of ~2; Table

2). These examples would imply that chemical formula alone was a reasonable predictor of toxicity in such cases.

However, other NA with identical formulae showed very different toxicities. One of the diaromatic acids (4-(p-tolylbenzoic acid A10; $C_{14}H_{12}O_2$) was about 30× more toxic than another (2-benzylbenzoic acid, A11; Table 2). Clearly, knowledge of exact chemical structure is required to assess the potential toxicity of such NA. This is particularly important for monitoring NA in the environment.

4.2 Predictive models

The use of predictive models of toxicity offers a means of assessing the toxicities of specific chemicals, but is of most value if the numbers of false negatives and false positives, are acceptable. Pyrene-1-carboxylic acid (A15) was considerably more toxic than predicted (Table 2). On the other hand, there was a serious over estimation of the toxicity of phenanthrene-4-carboxylic acid (A13; Table 2). Carbazole-3-carboxylic acid (A9; Table 1) was included in the tests as nitrogen-containing acids of unknown structure have been reported in OSPW (e.g. Nyakas et al., 2013) and parent alkylcarbazoles are common environmental contaminants associated with diesel (Bennett and Larter, 2000). The LC_{50} was slightly below that predicted for the latter chemical (Table 2).

Experimental LC_{50} values for zebrafish showed generally good correlation with predicted values (for fathead minnow; Table 2; r^2 = 0.940; p < 0.01), although experimental values were ~3× lower (i.e. more toxic) than predicted. This is perhaps to be expected as embryos tend to be more sensitive than adults (e.g. Incardona, 2017; Sorensen et al., 2017) and the training set for the model was for fathead minnow rather than for zebrafish. Large scale multispecies studies of fish embryos and adults

show good correlation in acute toxicity LC_{50} values, both between species and life stages, with embryos being most sensitive (Braunbeck et al., 2005). DHAA (A16, Table 1) was included in the study mainly for the purposes of providing a cross reference to other studies. The LC_{50} value of 7.7 µM DHAA for zebrafish embryos (Table 2) was similar to that reported previously (4 µM) for zebrafish larvae (Scarlett et al., 2013) and rainbow trout (Straus et al., 1997) and was also very close to the predicted LC_{50} of 5 µM (Table 2).

Taken as a whole, the use of the Admet predictor™ models shows promise for the ability to correctly identify acutely toxic compounds. However, the occurrence of both serious over- and especially under-estimation of toxicity suggests that polar compounds are at present insufficiently represented in the training sets of the present models.

4.3 Potential mechanisms of toxicity

Both petroleum-derived NA mixtures and OSPWs containing NA, have been shown to cause a concentration-dependent increase in craniofacial and spinal deformities, reduced yolk utilization, time to hatch, hemorrhages, edemas and incomplete hatching in embryos of yellow perch (*Perca flavescens*) and Japanese medaka (*Oryzias latipes*) (Peters et al., 2007). Exposure to OSPW was also found to produce increased rates of cardiovascular and spinal deformities, premature hatching and greater rates of spontaneous activity in embryos of fathead minnow (He et al., 2012). Such embryological effects are said to be associated with exposure to ‘dioxin-like’ compounds and mediated via the aryl hydrocarbon receptor (AhR) binding and CYP1A induction. He et al., (2012) found that the abundance of transcripts for *cyp1a* was not

significantly greater in embryos exposed to OSPW, so it was postulated that for NA, the effects may instead be due to increased oxidative stress and abnormally high rates of apoptosis (He et al., 2012). In another study, Marentette et al., (2015a) found that a fraction of OSPW containing NA also produced a range of deformities in embryos, thus strongly implicating NA as causative agents for producing developmental abnormalities in fish.

Our results also showed that some NA produce developmental abnormalities in fish embryos in a concentration-dependent manner (Fig. S1). However, an important difference is that our studies were made on individual acids (Tables 1, 2), not on complex NA mixtures (cf Peters et al., 2007; Marentette et al., 2015a; Marentette et al., 2015b). In this way the causative agents may be better constrained. Abnormalities (>10%) in zebrafish embryos were observed in five of the six chemicals tested i.e. all of the monoacids. Three acids were most toxic (A10, 15, 16).

The monoaromatic tricyclic DHAA (A16) produced a range of deformities in 100% of embryos at a concentration of 6 µM at 96 hpf (Fig. S3). Although DHAA has been linked to embryo toxicity (Sepulveda et al., 2003), no previous reports concerned with the effects of DHAA on fish embryo development were found. DHAA is a common contaminant in wood pulp and paper mill effluent (e.g. Rissanen et al., 2003). ‘ The diaromatic, 4-(p-tolyl)benzoic acid (A10) produced around 90% deformities at <1 µM and tetraaromatic, pyrene-1-carboxylic acid was found to cause developmental abnormalities in 80% of embryos at 3 µM and virtually all by 6 µM at 72 hpf (Fig. 2). Although the present study showed that higher concentrations of naphthalene-2-carboxylic acid (A1) were required to produce developmental abnormalities, unusual head and ocular edemas were noted (Fig. 3). Hydroxynaphthoic acid isomers have also been found to cause a range of developmental abnormalities in fish embryos

(*Oryzias latipes*), in the range 10-70 µM with variable sensitivities dependent upon chemical structure (Carney et al., 2008); it is perhaps likely that a wider range of oxygenated organic compounds produce such effects. It is unclear what, if any, are the common structural characteristics of these acids and more work will be needed to elucidate the mechanisms and mode(s) of action (MoA). Recent work on the MoA of petroleum contaminants causing heart defects in ELS of fish have suggested that individual polycyclic aromatic compounds (PACs) produce a diversity of cardiotoxic mechanisms (Incardona, 2017). However, most work to date has been conducted on individual aromatic hydrocarbons (Brette et al., 2014). These studies have revealed multiple mechanisms linking cardiomyocyte physiology to heart development, and abnormal development that turn to latent impacts on physiology at later life stages (Incardona and Scholz, 2017). Incardona (2017) noted that "For some PACs that are strong agonists of the aryl hydrocarbon receptor (AHR), defects in heart development arise in an AHR-dependent manner, which has been shown for potent organochlorine agonists, such as dioxins. However, crude oil contains a much larger fraction of compounds that have been found to interfere directly with cardiomyocyte physiology in an AHR-independent manner". Whether the active components are the hydrocarbons or common metabolites, such as the acids tested herein, remains to be better elucidated. Our study indicates one potential avenue for further study.

4.4 Relative importance of aromatic acids

As deformities were found to occur following exposure to all the aromatic acids tested (other than the diacid A12), and that this class of chemicals has been reported to contribute about a third of the NA fraction of some OSPW (Jones et al., 2012), it seems

likely that such acids contribute to the developmental abnormalities observed in fish exposed to OSPW (e.g. Peters et al., 2007; Marentette et al., 2015a). Of course this will also depend on the concentrations of individual acids present, amongst other factors. The tailings ponds of Alberta, Canada probably represent the most concentrated aqueous NA but much larger volumes are generated by offshore oil industries. The large dilution factors that occur when oil industry produced waters are released offshore would likely reduce concentrations below those which deformities were observed to occur herein, quite rapidly, but this may depend on specific geographical locations. Metabolism of hydrocarbons from spilled oil (or natural oil seeps) to their corresponding acids may also result in rapid dilution, and reduced potential for toxic effects. However, in terrestrial oil spills this could also permit mobilisation of the toxic acids through sediment pore waters leading to contamination of aquifers or groundwaters. Naphthoic acids and their methyl isomers were detected at concentrations of *ca*30 nM and 300 nM respectively in contaminated groundwater in a former gas plant area (Testfeld Su¨d) located in southwestern Germany (Annweiler et al., 2001; Safinowski et al., 2006). Naphthoic and putative methylnaphthoic and dimethylnaphthoic acids plus tetralin acids were also detected with estimated concentrations in the range ca5-30 nM in hydrocarbon-impacted aquifers in Sedgwick County, Kansas and southwest Alberta (Safinowski et al., 2006). Unlike the other acids tested in this study, the resin acid DHAA is more likely to present in the environment not as a result of metabolic activity. Concentrations of DHAA have been reported in wood pulp mill effluent (Makris and Banerjee, 2002) similar those observed to cause deformities in embryos reported herein. Overall, the environmental significance of this study is difficult to assess due to the paucity of data concerned with

aquatic concentrations of individual acids, rather than NA mixtures. Future targeted environmental studies could now be conducted based on the results presented herein.

5. Conclusions

This preliminary study demonstrates the possible toxicological importance of certain aromatic petroleum acids, particularly to the ELS of fish. The experiments need to be repeated for a wider range of aromatic acids with, albeit difficult, measurement of the exposure concentrations in the embryonic and larval fish. The results of such studies might allow focus on identification of particular individual isomeric acids in the complex NA mixtures derived from degraded petroleum. Some chemicals with isobaric molecular masses tested herein, demonstrated very different toxicities, indicating the importance of the measurement of targeted individual pollutants, rather than mixtures.

Our results also show that although a commercial predictive model generally produced reasonably good correspondence with experimentally derived LC_{50} values, serious under- and over-estimations also occurred. Predictions may currently be insufficiently reliable for screening purposes. The observed developmental abnormalities in zebrafish embryos exposed to aromatic NA strongly suggest that this class of chemical contributes to the reproductive toxicity reported in OSPW and certainly merits further attention.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version.

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Table and Figure legends

Table 1 Physical and predicted properties of test chemicals

Table 2 Predicted and measured lethal toxicities of test chemicals to invertebrates and fish

Figure 1 Concentration response relationships for *D. rerio* exposed to aromatic carboxylic acids. Codes, top left of each panel refer to chemical names provided in Table 1 and structures in Table 2. Curves are predicted by Probit analyses. Error bars for observed mortality are standard errors of the mean.

Figure 2 *D. rerio* embryos 72 h post fertilisation exposed to (A) 0 µM, (B) 0.8 µM (C) 1.5 µM, (D) 3.0 µM, (E) 6.1 µM and (F) 12.2 µM 1-pyrene carboxylic acid. Arrows indicate (1) yolk-sac and (2) pericardial edema

Figure 3 Arrows indicate head and ocular edemas in *D. rerio* embryos exposed to 25.5 µM naphthalene-2-carboxylic acid.

Supplementary Material

Title: **Predicted and measured acute toxicity and developmental abnormalities in zebrafish embryos produced by exposure to individual aromatic acids**

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Fish source, husbandry and egg collection

Wild-type WIK strain zebrafish embryos were obtained from the Max Planck Institute, Tubingen, Germany and maintained at University of Exeter. Fry from approximately 2 days post-hatch (dph) were fed on a microencapsulated diet (ZM Advanced Fry feed; ZM Ltd., Hampshire, U.K.). This was supplemented from approximately 7 dph with freshly hatched *Artemia nauplii* (ZM Premium Grade Artemia; ZM Ltd.). From approximately 21 dph onwards to adulthood fish were fed with freshly hatched *Artemia nauplii* to satiation twice daily. As adults, fish were fed daily on both freshly hatched *Artemia nauplii* and TetraMin tropical flake food (TetraMin, Tetra Werke, Melle, Germany).

A breeding stock of unexposed, wild-type zebrafish with a well-documented fertilisation rate of eggs was used for egg production. Fish were free of macroscopically discernible symptoms of infection and disease and had not undergone any pharmaceutical (acute or prophylactic) treatment for 2 months before spawning. Breeding fish were maintained in aquaria with a loading capacity of a minimum of 1 L water per fish. Standardized dilution water as specified in ISO 7346-1 and 7346-2 with ≥60% oxygen saturation was used for keeping and breeding. Temperature was maintained at 26±1 °C, and fish were kept under a constant artificial dark/light cycle of 8/16 h. Constant filtering and permanent flow-through conditions guaranteed that ammonia, nitrite, and nitrate were kept below detection limits (0–5, 0.025–1 and 0–140 mg/L, respectively). Spawning chambers were put in either in the evening before collection. Since zebrafish are known to feed upon their own offspring, the bottom of the spawning tanks were covered with a grid of stainless steel, thus allowing the embryos to be sampled without interference by the adults. As a spawning stimulus, artificial plants made of green plastic was fixed to the grid covering the spawning dishes. About 30–60 min after spawning, the spawning dishes were removed, and the embryos were transferred to a temperature-controlled dissecting microscope to check for viability.

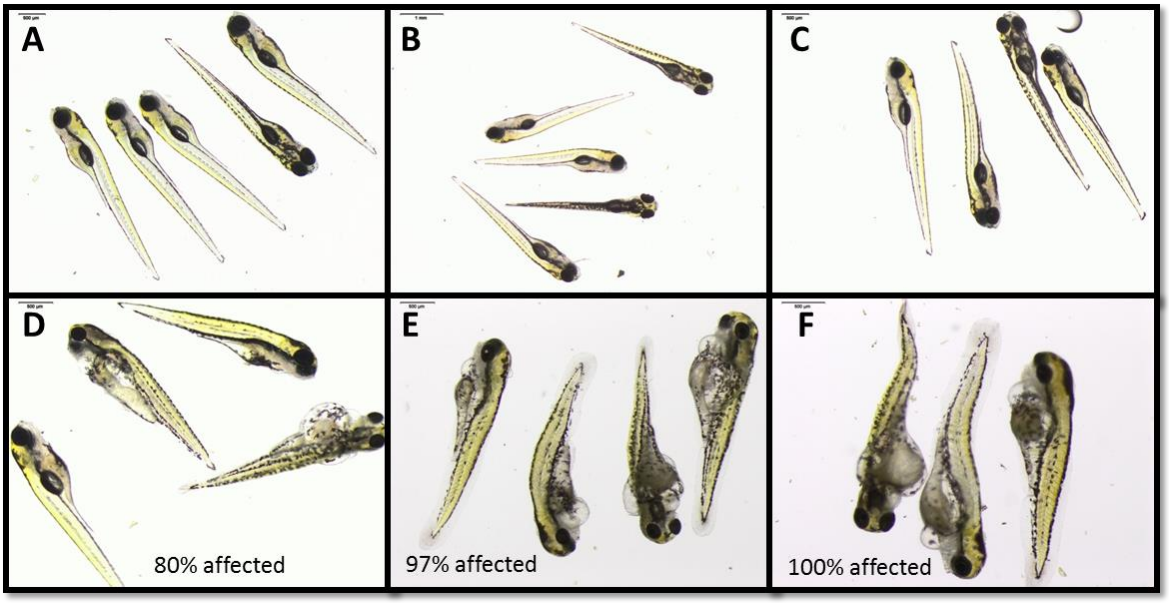


Figure S1. Zebrafish embryos 96 h post fertilisation exposed to (A) 0 µM, (B) 7.9 µM (C) 15.8 µM, (D) 31.6 µM, (E) 63.1 µM and (F) 126.3 µM tetralin-5-carboxylic acid (aka 5,6,7,8-tetrahydronaphthalene-1-carboxylic acid) . Abnormalities included yolk-sac edema (most predominate), pericardial edema, tail flexure and truncation and stunted growth.

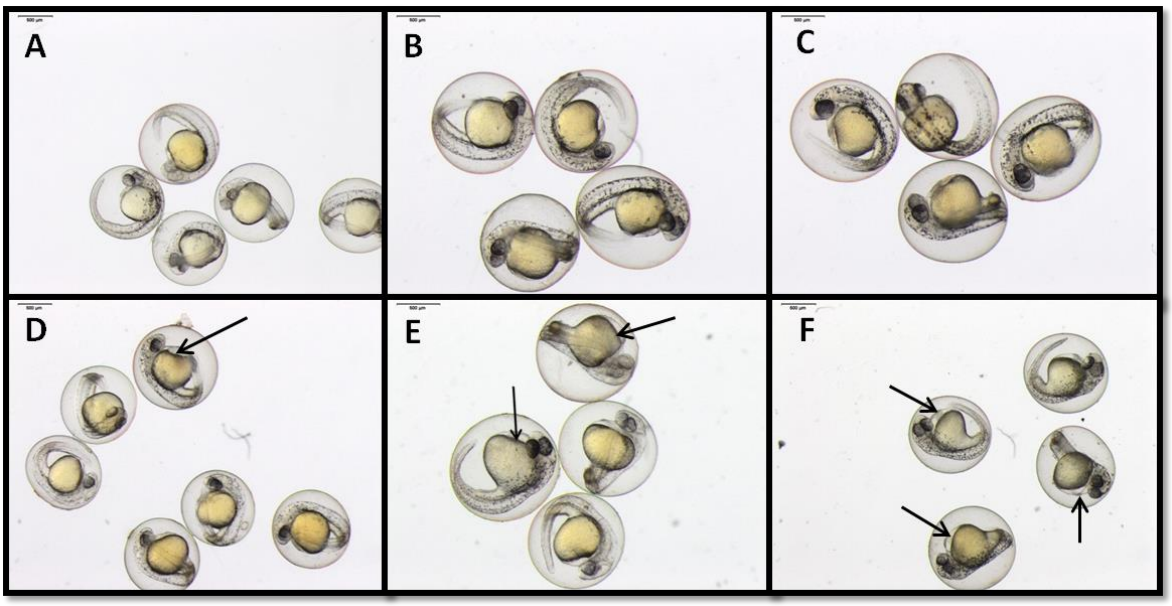


Figure S2. Zebrafish embryos 24 h post fertilisation to (A) 0 µM, (B) 0.5 µM (C) 0.9 µM, (D) 1.9 µM, (E) 3.8 µM and (F) 7.5 µM 4-(p-tolyl)benzoic acid (aka 4'-methyl-4-biphenylcarboxylic acid). Arrows indicate early signs of pericardial edema.

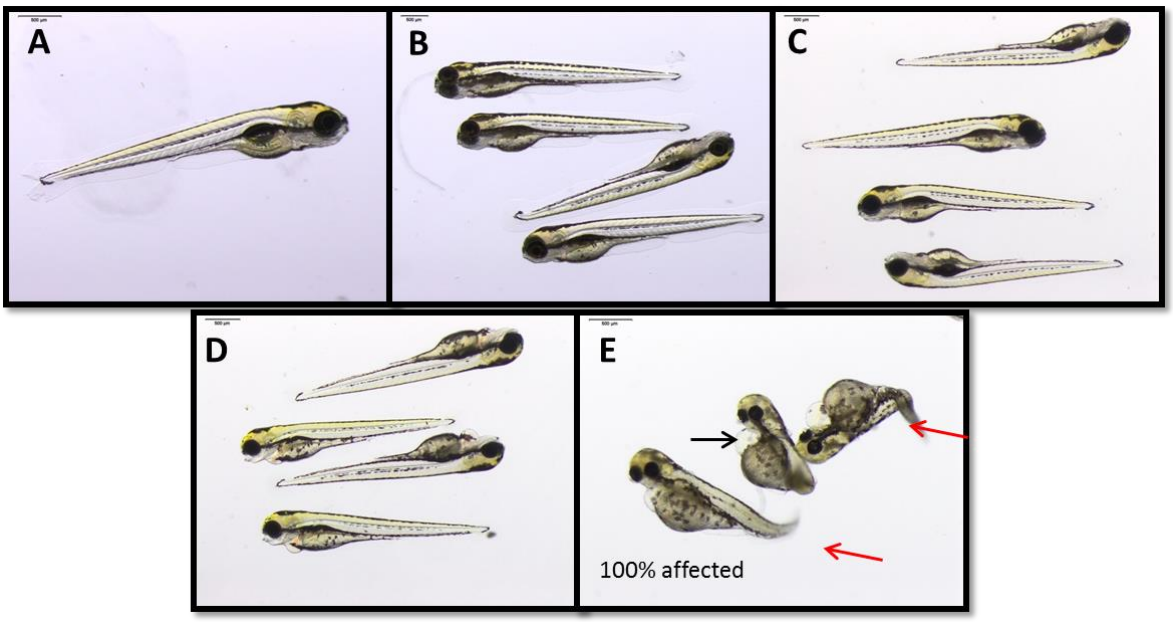


Figure S3. Zebrafish embryos 24 h post fertilisation to (A) 0 µM,(B) 0.8 µM (C) 1.6 µM, (D) 3.2 µM and (E) 6.5 µM dehydroabietic acid. Arrows indicate signs of pericardial edema and tail flexure and truncation (red). Early signs of pericardial edema observed at 24 hpf. Mortality was 100% at 13 µM.

46 Table 1 Physical and predicted properties of test chemicals

Code	CAS Number	IUPAC name^	Alternative name	Formula	Mol Wt (Da)	LogP *	Solubility (mg/L)*
A1 #	93-09-4	naphthalene-2-carboxylic acid	2-naphthoic acid	$C_{11}H_8O_2$	172.18	3.1	129.0
A2	1914-65-4	tetralin-1-carboxylic acid	1,2,3,4-tetrahydronaphthalene-1- carboxylic acid	$C_{11}H_{12}O_2$	176.22	2.3	627.0
A3 #	4242-18-6	tetralin-5-carboxylic acid	5,6,7,8-tetrahydronaphthalene-1- carboxylic acid	$C_{11}H_{12}O_2$	176.22	3.0	176.0
A4	86-87-3	2-(1-naphthyl)acetic acid	1-naphthaleneacetic acid	$C_{12}H_{10}O_2$	186.21	2.5	208.0
A5	1575-96-8	2-methylnaphthalene-1-carboxylic acid	2-methyl-1-naphthoic acid	$C_{12}H_{10}O_2$	186.21	3.1	115.0
A6	55720-22-4	1,2-dihydroacenaphthylene-5-carboxylic acid	5-acenaphthenecarboxylic acid	$C_{13}H_{10}O_2$	198.22	3.6	42.5
A7	947-84-2	2-phenylbenzoic acid	2-biphenylcarboxylic acid	$C_{13}H_{10}O_2$	198.22	3.3	152.0
A8	1989-33-9	9H-fluorene-9-carboxylic acid	fluorene-9-carboxylic acid	$C_{14}H_{10}O_2$	210.23	3.0	15.8

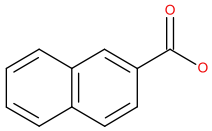
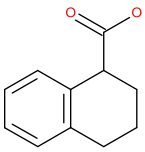
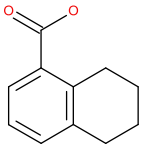
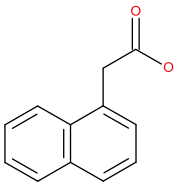
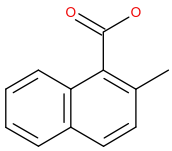
A9	51035-17-7	9H-carbazole-3-carboxylic acid	carbazole-3-carboxylic acid	$C_{13}H_9NO_2$	211.22	3.5	16.4
A10#	720-73-0	4-(p-tolyl)benzoic acid	4'-Methyl-4-biphenylcarboxylic acid	$C_{14}H_{12}O_2$	212.25	4.2	34.7
A11	612-35-1	2-benzylbenzoic acid	α-phenyl-o-toluic acid	$C_{14}H_{12}O_2$	212.25	3.5	137.0
A12#	1141-38-4	naphthalene-2,6-dicarboxylic acid	2,6-Naphthalenedicarboxylic acid	$C_{12}H_8O_4$	216.19	2.5	423.0
A13	42156-92-3	phenanthrene-4-carboxylic acid	4-Phenanthroic acid	$C_{15}H_{10}O_2$	222.24	4.0	22.1
A14	606-83-7	3,3-Diphenylpropionic acid	benzenepropanoic acid, β-phenyl-	$C_{15}H_{14}O_2$	226.28	3.0	113.0
A15#	19694-02-1	pyrene-1-carboxylic acid	1-Pyrenecarboxylic acid	$C_{17}H_{10}O_2$	246.27	4.6	2.7
A16#	1740-19-8	abieta-8(14),9(11),12-trien-18-oic acid	dehydroabietic acid	$C_{20}H_{28}O_2$	300.44	5.4	1.9

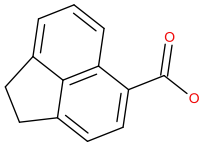
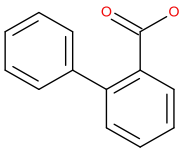
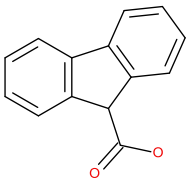
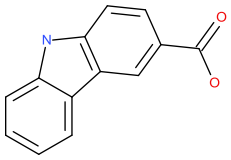
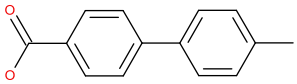
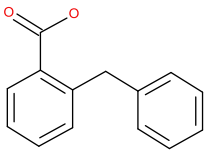
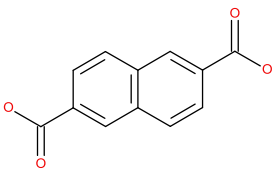
*Properties generated by ADMET predictor™. Sol = predicted solubility in water # Chemicals tested using zebrafish embryo assay as well as fairyshrimp acute lethality test. Log P = predicted Log Kow.

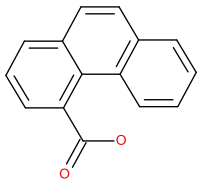
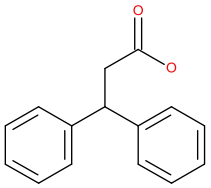
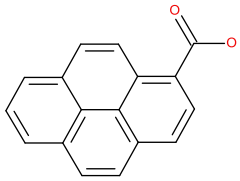
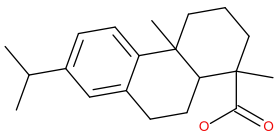
^IUPAC names generated by Accelrys Draw (Biovia, Cambridge, UK) if not stated by supplier.

53 Table 2 Predicted and experimental lethal and sublethal toxicities of test chemicals to

54 invertebrates and fish

		Predicted (grey columns) and experimental LC_{50} values for invertebrates and fish (µM)					Exper Abn em
Code^	Structure	*D. magna*	*T. platyurus*	*P. promelas*	*D. rerio*	P-Rep*	
A1 #		123	**51 (43, 61)**	215	42.3	N	
A2		202	217 (174, 270)	543	*n/a*	N	
A3 #		261	126 (102, 152)	483	151.0	N	
A4		57	71 (71, 71)	331	*n/a*	N	
A5		139	143 (108, 191)	223	*n/a*	N	

A6		341	212 (188, 338)	113	*n/a*	Y	
A7		113	235 (235, 235)	128	*n/a*	N	
A8		60	97 (77, 126)	80	*n/a*	N	
A9		53	**24 (20, 27)**	24	*n/a*	Y	
A10 #		36	**8 (4, 9)**	88	**4.9**	N	
A11		56	225	103	*n/a*	N	
A12 #		142	177 (177, 177)	852	ND	Y	

A13		96	568	16	*n/a*	Y	
A14		47	93 (72, 129)	53	*n/a*	N	
A15 #		99	**12 (12, 12)**	3	7.1	Y	
A16 #		42	**13 (11, 15)**	5	7.7	N	

^ Names of chemicals provided in Table 1. Lower and upper 95% confidence limits for *T. platyurus* LC_{50} in parentheses if calculable. Errors marginal for *D. rerio*. *P-Rep = predicted by ADMET predictor™ to cause reproductive/developmental toxicity and relates to anything that disturbs the reproductive process of organisms, including adverse effects to sexual organs, behavior, ease of conception, and developmental toxicity of offspring both before and after birth. *n/a* = not assayed in fish test. ND = Not determined as 50% lethality or abnormal embryo development not reached during test. For invertebrates, measured (24h) LC_{50} less than predicted by a factor of 2 in bold. For fish, measured LC_{50} less than predicted by a factor of 10 in bold. Note that predictions for *P. promelas* were for not for early life stage toxicity.

Figure 1

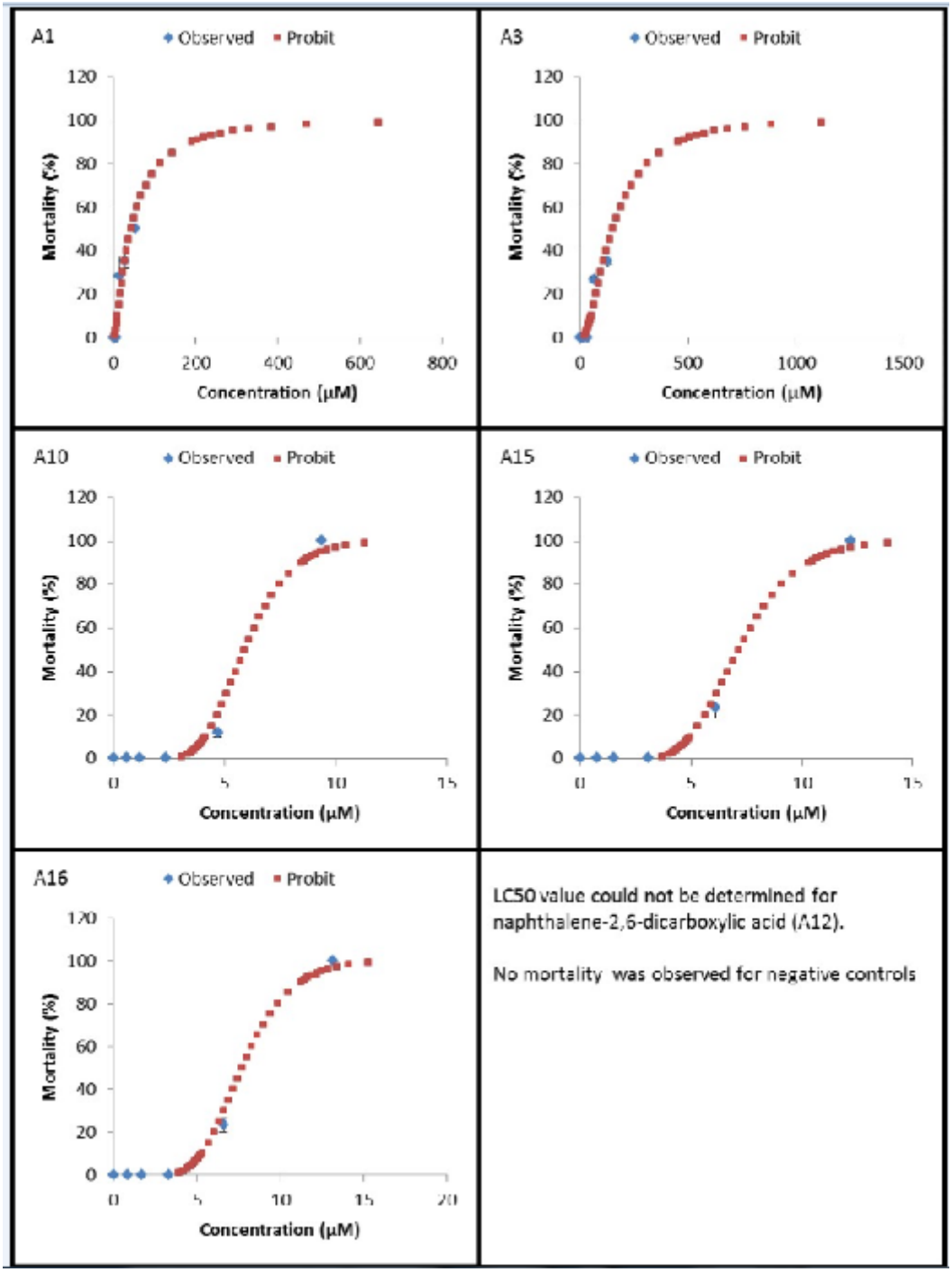
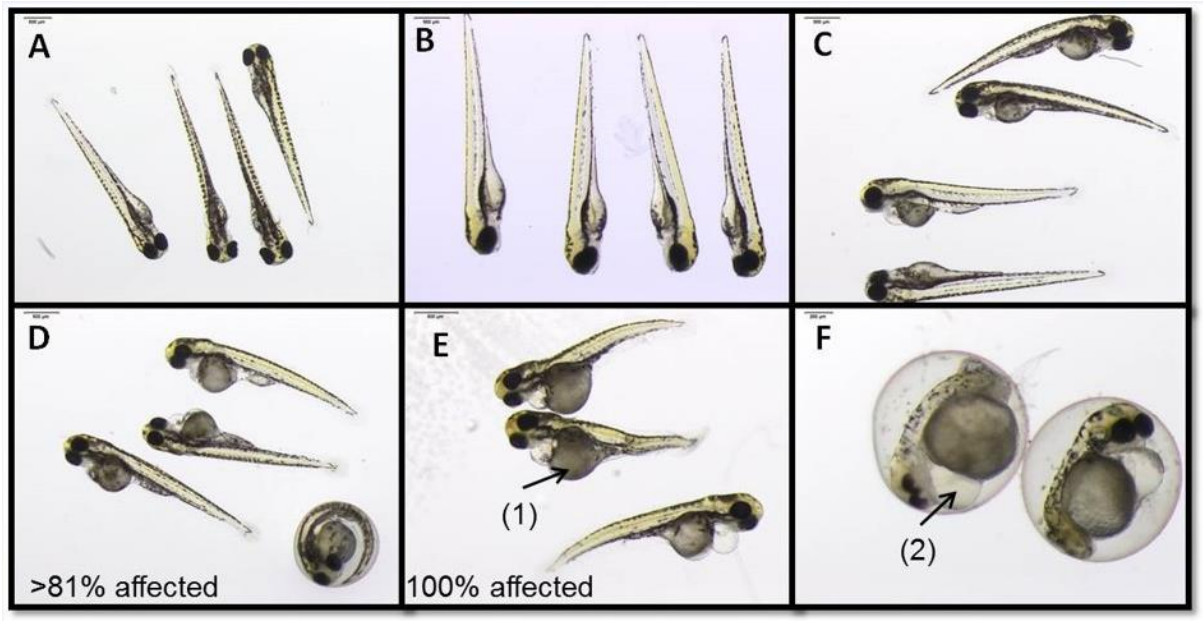
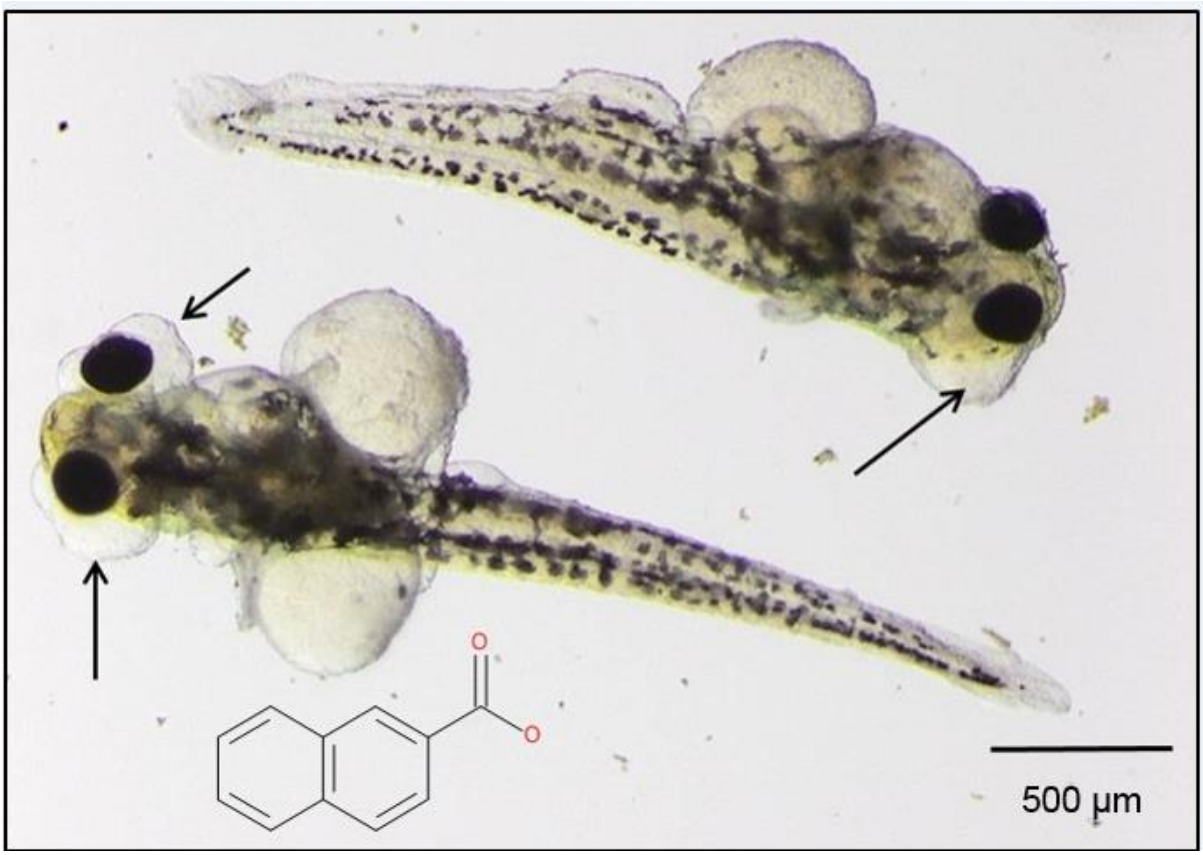


Figure 2.



98 Figure 3.



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