

Adverse Outcome Pathway (AOP) informed modelling of aquatic toxicology - QSARs, read-across and inter-species verification of modes of action

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Abstract

Alternative approaches have been promoted to reduce the number of vertebrate and invertebrate animals required for assessment of the potential of compounds to cause harm to the aquatic environment. A key philosophy in the development of alternatives is greater understanding of the relevant adverse outcome pathway (AOP). One alternative method is the fish embryo toxicity (FET) assay. Although the trends in potency have been shown to be equivalent in embryo and adult assays, a detailed mechanistic analysis of the toxicity data has yet to be performed; such analysis is vital for a full understanding of the AOP. The research presented herein used an updated implementation of the Verhaar scheme to categorise compounds into AOP informed categories. These were then used in mechanistic (Quantitative) Structure-Activity Relationship ((Q)SAR) analysis to show that the descriptors governing the distinct mechanisms of acute fish toxicity) are capable of modelling data from the FET assay. The results show that compounds do appear to exhibit the same mechanisms of toxicity across life stages. Thus this mechanistic analysis supports the argument that the FET assay is a suitable alternative testing strategy for the specified mechanisms, and that understanding the AOPs is useful for toxicity prediction across test systems.

1. Introduction

The acute aquatic toxicity assessment of chemicals has traditionally been performed on species representing various trophic levels e.g. algae, invertebrates, and juvenile and adult fish from species such as the fathead minnow (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*) and Japanese medaka (*Oryzias latipes*) amongst others. However, legislative mandates such as the Registration, Evaluation, Authorisation, and restriction of Chemicals (REACH) regulation in the European Union have required alternative, non-animal, models to be sought as a replacement for the expensive, time-consuming and ethically questionable *in vivo* assessment methods. One such alternative assay is the fish embryo toxicity (FET) test¹. The FET has a standardised OECD test guideline (number 236²) for the 96hr assay performed with zebrafish (*Danio rerio*) embryos and, although not explicitly stated in the guideline, can be considered as a suitable assessment method, on its own or as part of a strategy, for

assessing acute aquatic toxicity. For instance, reasonable correlations ($r > 0.85$) have been observed between the 50% effect concentrations (EC_{50}) measured in the FET and the 50% lethality concentrations (LC_{50}) measured in fish³⁻⁶; although a small number of notable mechanisms are poorly predicted in FET (e.g. neurotoxic compounds which require behavioural analysis of the embryos for toxicity to be observed^{6,7}).

One of the key philosophies behind the research into alternative methods to animal testing is that of understanding mode of action. Recently, the assessment of modes of action has been incorporated into adverse outcome pathways (AOPs)⁸. AOPs define a series of key events (KE), and their relationships (key event relationships (KERs)) from an initial exposure, resulting in a molecular initiating event (MIE), through to inducing the adverse outcome (AO)⁹. The MIE may be described in terms of the chemically defining features of a molecule that control the interaction with the biological macromolecule¹⁰, whereas the MIE combined with the required KEs for an AO encompass the biological mode of action⁸. Understanding the AOP can thus aid in the elucidation of the similarities and differences in the mode of action between species by identifying key uncertainties, and corresponding research gaps, in the biological mechanisms of toxicity¹¹. The rationale for the chemical induction of an MIE is a key aspect of understanding the AOP.

The modes of action for acute aquatic toxicity have been established through studies on fish behaviour and physiology^{12, 13}, as well as mechanistic structure-activity relationship (SAR) analysis on the resultant data¹⁴, and more recently on detailed systems biology studies on species from lower taxa such as *Daphnia magna*¹⁵. Fish Acute Toxicity Syndromes (FATS) were derived from measurements of physiological, biochemical and analytical effects separated into discrete mechanisms, or modes, of action¹². Building on such knowledge, Verhaar et al¹⁶ used fish acute toxicity data to identify clear structural rules associated with a variety of modes or mechanisms of action. The Verhaar scheme utilises 2D chemical structure to classify potential environmental pollutants into one of four categories representing one, or more, mechanisms of action: class 1 (narcosis or baseline toxicity), class 2 (less inert compounds), class 3 (unspecific reactivity) and class 4 (compounds and groups of compounds acting by a specific mechanism). In addition, Russom et al¹³ used structural classes to assign mechanisms of action to a range of compounds tested on the fathead minnow (*Pimephales promelas*). This grouping of compounds allows for the development of mechanistically based, local Quantitative Structure Activity Relationship (QSAR) models and also application of the chemical activity principle¹⁷.

Understanding which Verhaar class, or mode/mechanism of action, a compound belongs to is useful for hazard characterisation, not only identifying compounds predicted to act by narcosis, but also identifying those which may elicit excess toxicity (e.g. compounds in Verhaar classes 3 and 4). Within a mechanistic class it is possible to build high quality (Q)SAR models, based on knowledge of the relevant mechanism/mode of action of toxicity, which are able to estimate relative toxic potency¹⁸.

These mechanistic models have been shown to provide more transparency and greater statistical performance than equivalent global models¹⁸⁻²¹. Understanding the mechanism of action also fits well within the AOP framework of toxicity assessment²² and allows inter-species toxicity correlations to be applied within a single mechanism^{23, 24}.

The transparency of the Verhaar classification scheme has assisted in its popularity as a hazard characterisation tool and this popularity has in turn led to automated implementations of the scheme becoming widely available. One such implementation is available in the Toxtree software. Toxtree was developed by Ideaconsult Ltd (Sofia, Bulgaria) under the terms of a contract from the European Commission's Joint Research Centre (JRC). The software encodes several decision trees and classification schemes useful for analysing the potential toxicity hazards of compounds²⁵. The software is freely available (<http://Toxtree.sourceforge.net>) and version (2.6) includes two forms of the Verhaar decision tree: "Verhaar scheme" and "Verhaar scheme (modified)". The "Verhaar scheme" is the original implementation of the decision tree based directly on the scheme as it is described by Verhaar et al¹⁶. Enoch et al²⁶ assessed the performance of this implementation and suggested possible improvements. These improvements form the basis of "Verhaar scheme (modified)" decision tree. Recent work by Ellison et al²⁷ has shown that the implementation of the "Verhaar scheme (modified)" tree, and hence the suitability of resultant categories for modelling, can be improved further. Specifically, with the use of post-processing filters for polar phenols, reactive aromatic compounds, cyclic non-aromatic hydrocarbons and respiratory uncouplers of oxidative phosphorylation, the positive predictivity of each of the categories was increased by an average of 5%. These filters are available from the authors as a KNIME workflow (www.KNIME.org) for use on the output of Toxtree v2.6.

Whilst the Verhaar scheme is accepted for adult fish, it is not known how applicable it may be to other assays such as FET, nor what mechanistic information may be derived from assessing data from such assays. Therefore, the aim of this study was to examine if the mechanistic categories, formed by using the Verhaar scheme classes implemented through the use of Toxtree v2.6 and the Ellison et al KNIME post-processing workflow, are relevant to the AOPs of both juvenile/adult fish and fish embryos. To this end AOP relevant mechanistic (Q)SAR analysis was performed on compounds with measured toxicity in the FET assay and outliers were highlighted. These outliers were of interest as they represent compounds where observed toxicity is in contradiction to that expected according to the mechanism of action for that class, and they may provide useful species specific information. These compounds may either be acting via different mechanisms in the FET assay, have been misclassified by the scheme, or be a misrepresentation of the chemical toxicity due to erroneous data or other effects such as volatility and degradation.

2. Methods

2.1. Dataset

Published experimental results from the FET assay performed using zebrafish (*Danio rerio*) were manually curated from two literature sources^{3, 4} into a single dataset. The names, CAS numbers and EC₅₀ values were extracted. The full range of exposure times (24hr – 120hr) were used, which included specimens in both the embryonic and eleutheroembryonic stages of development. Data from all time points were considered equal as it has been previously established that eleutheroembryo and embryo studies generally provided highly similar results⁴, although some compounds only show significant toxicity at the eleutheroembryo stage and hence it was important to include all data points. The 24hr testing period could provide a lower indication of toxicity because the test duration was insufficient for the compound to reach equilibrium due to the compound's toxicokinetic properties. However, the small number of compounds (n<10) which had data for the 24hr exposure time period had additional comparable data points at longer durations and thus the 24hr data points were included. Inorganic metals and their salts (e.g. cadmium or cadmium chloride) were excluded as they were outside the domain of the Verhaar scheme and substances with ambiguous names (e.g. high solubility alkyl sulphate) were excluded as it was not possible to generate SMILES strings for such compounds. If multiple EC₅₀ values were available for the same compound the mean was calculated and recorded so that each compound was associated with a single EC₅₀ value. After these calculations, a total of 193 compounds remained for analysis (Table S1; supplementary information). In addition the 50% lethality concentration (LC₅₀), covering a variety of durations from 24hr to 96hr, for a range of adult fish species (*Danio rerio* [zebrafish]; *Lepomis macrochirus* [bluegill]; *Oryzias latipes* [Japanese medaka]; *Oncorhynchus mykiss* [rainbow trout]; *Pimephales promelas* [fathead minnow]) were collated from Belanger et al³ and/or Lammer et al⁴ for compounds with FET data (Table S2; supplementary information). These data were collated to enable a comparison of the potency in the two test systems to be undertaken. All toxicity values were converted to molar units and the inverse logarithm ($\log EC_{50}^{-1}$ or $\log LC_{50}^{-1}$) used to allow for comparison of data and model development. The quality of the data were assessed by Belanger et al³ and Lammer et al⁴ as part of their data curation process and thus no further data quality assessments were performed.

2.2. Software

All 193 compounds described above were classified using the “Verhaar scheme (modified)” decision tree through the batch processing functionality of Toxtree v2.6. Structures were entered as SDfiles which were generated from the SMILES strings using MarvinBeans v14 (www.chemaxon.com). The possible outcomes from the scheme are equivalent to those originally published by Verhaar et al¹⁶: class 1 (narcosis or baseline toxicity); class 2 (less inert compounds); class 3 (unspecific reactivity); class 4 (compounds and groups of compounds acting by a specific mechanism); class 5 (not possible to classify according to rules).

After the compounds had been run through Toxtree v2.6 the output file (SDF) was then processed through the KNIME post-processing filter which has been shown to improve the predictive capabilities of the Verhaar (modified) scheme in Toxtree v2.6 as described by Ellison et al²⁷. The filter expands the domain of the Verhaar scheme so that fewer compounds are placed into class 5. The output of the scheme is a table (.csv file) of all compounds with an updated Verhaar classification based on the structural filters within the workflow. The Verhaar (modified) and KNIME post processing classifications (along with FET and acute fish toxicity data and calculated descriptors, see below) for all 193 compounds extracted from the literature are available as supplementary material (Table S1).

Chemical descriptors were calculated for all compounds to enable QSAR analysis to be performed. The KOWWIN module of the EPISuite (ver 4.11) software package²⁸ was used to calculate the logarithm of the octanol:water partition co-efficient (log P) for all compounds. The calculated value was used in data analysis even when an experimental value was available in KOWWIN for consistency. Compounds were processed through KOWWIN using the batch process function with an SDfile as input.

For the compounds classified into class 3 and 4 hydrophobicity alone was not sufficient to model toxicity. The Energy of the Lowest Unoccupied Molecular Orbital (E_{LUMO}) and Energy of the Highest Occupied Molecular Orbital (E_{HOMO}) were calculated using the Gaussian09 package of programs utilizing the B3LYP/6-31G(d) level of theory²⁹. The global electrophilicity index (ω), which has previously been shown to be a good descriptor for predicting toxicity for reactive compounds³⁰, was then calculated for each optimised chemical as shown below:

$$\omega = \mu^2/2\eta$$

Where

$$\mu = (E_{\text{HOMO}} + E_{\text{LUMO}})/2$$

$$\eta = E_{\text{LUMO}} - E_{\text{HOMO}}$$

The statistical analysis required to build the QSAR models linking toxicity to the descriptors described above was performed in Minitab v17.1.

2.3. Data analysis

All 193 compounds were run through the KNIME post-processing filter described above to classify them into one of the four Verhaar classes or out of the domain (class 5). The class 1 and class 2 compounds were used to build baseline and polar narcosis QSAR models respectively. Linear regression analysis of FET ($\log EC_{50}^{-1}$) against hydrophobicity (log P) was performed. It should be possible to model toxicity for these mechanisms using log P alone as it is the toxicokinetics (i.e. ability to distribute and accumulate) of the molecules rather than the toxicodynamics of the system which govern potency. Any outliers of the regression model were investigated to see if they were acting via a

different mechanism or if there were any other reasons why their behaviour did not fit the expected trend. This was performed through expert analysis of the published data (i.e. examining the reliability of the data point and whether the same trends were observed in adult fish (by comparison with a plot of log LC₅₀⁻¹ value(s) against hydrophobicity)) and also examining if the compounds contained structural alerts known to be associated with reactive mechanisms of action (using the alerts published by Enoch et al³¹). If justification based on one or more of the criteria described above could be found, the outliers were removed as they were either misclassified or subject to competing mechanisms. The models were redeveloped and their removal resulted in improved QSAR models.

The baseline and polar narcosis models were used to predict the FET of the compounds classified in classes 3 and 4. This was to highlight any compounds whose toxicity was well predicted by the models. The comparative analysis of mechanisms across species requires compounds within a category to act via one distinct mechanism. Therefore compounds in classes 3 and 4, which could be modelled as either baseline or polar narcotics, were removed from the analysis because of the ambiguity concerning their actual mechanism of action. Also, compounds whose toxicity was significantly less than that predicted by the baseline model (residual greater than 1.5) were investigated to examine why this was the case.

The compounds which remained in classes 3 and 4 after the above stated investigations cannot be modelled as whole classes as they represent a broad range of discrete mechanisms. Therefore the class 3 and 4 compounds were subcategorised. For the class 3 compounds this was achieved by using the mechanistic alerts published by Enoch et al³¹. These provided mechanistic domains (e.g. Michael addition) which were then suitable for trend analysis. For the class 4 compounds expert judgement was required to identify which specific mechanism each compound was likely to act by (e.g. acetylcholinesterase (AChE) inhibition); this process builds, in part, on the approach published by Martin et al [18]. After sub-categorisation trend analysis between the toxicity values (log EC₅₀⁻¹) and chemical descriptors (log P and/or electrophilicity) was performed to examine if the mechanistic rationale was sufficient to describe the observed outcome. If this was not the case it may suggest the compounds are acting via undefined toxicity mechanisms in the FET assay and that the AOPs differ between species.

3. Results and Discussion

It has been documented that the EC₅₀ values obtained from the fish embryo acute toxicity (FET) assay using zebrafish embryos correlate well with the LC₅₀ values from traditional fish toxicity studies, irrespective of fish species³⁻⁵. Thus it has been suggested that the FET assay could act as a replacement for the fish lethality studies. This correlation of toxicity between the species and life stages would suggest that compounds are toxic via the same or similar AOPs irrespective of the test system, with a small number of notable exceptions (neurotoxic compounds and those requiring metabolism)⁶. The aim of this study was to investigate whether compounds tested in the FET can be modelled within their

Verhaar classifications, and thus whether they are acting by the same mechanisms as would be seen in adult fish. To this end 193 compounds were extracted from the literature^{3,4} and run through the Verhaar (modified) decision tree available in Toxtree v2.6 to obtain their classification. Additionally, a further post filter was used to assign a new classification to compounds which may have been misclassified by Toxtree, as this has shown to improve results elsewhere²⁷.

The majority of Toxtree classifications persisted after the application of the post filter suggesting the decision tree had adequate coverage of this dataset (Table 1), with the majority of classified compounds (classes 1-4) showing a narcotic mechanism. However, the categories from the post-processing filter were used as the filter was able to re-classify compounds which may have been classified incorrectly by the Verhaar (modified) decision tree. For example 2,4-dinitrophenol [CAS number 51-28-5] was put into class 3 (unspecific reactivity) by the Verhaar scheme but this was moved to class 4 (specific mechanism) by the post-processing filter because of the likelihood that this compound can act as a weak acid respiratory uncoupler³². Similar issues with misclassifications were also noted by Thomas et al¹⁷. The application of the post-processing filter reduced the number of unclassified compounds (class 5) from 79 to 68; however, this relatively high proportion of class 5 allocation remains an area of investigation for future research with regards to improvements of the scheme.

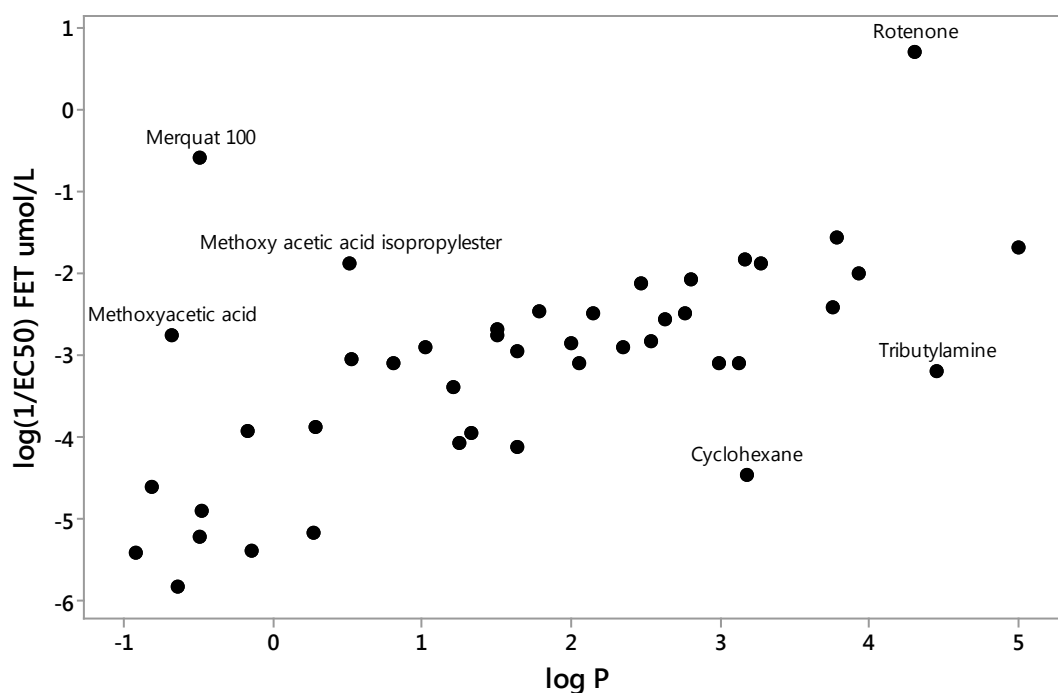
Table 1. The number of compounds classified into each category when using either the Verhaar (modified) scheme as implemented in Toxtree 2.6 or the Verhaar (modified) scheme in combination with the post processing filter published by Ellison et al²⁷.

	Number of compounds				
Classification Method	Class 1 Baseline narcotics	Class 2 Polar narcotics	Class 3 Unspecified reactivity	Class 4 Specified mechanism	Class 5 Unclassified
Verhaar (modified)	44	40	14	16	79
Verhaar (modified) plus post-processing filter	43	47	16	19	68

Regression analysis was performed on data from the 43 class 1, baseline narcotic, compounds. This produced a poor model (Eq.1) with six significant outliers (residual exceeded 1.5 log units). The outliers are identified in Figure 1. Since one of the aims of this work was to examine whether the mechanisms of fish toxicity were also relevant in the FET assay the outliers could not be removed purely because of statistical reasons. The compounds may be poorly predicted because they do not act as narcotics in zebrafish embryos. Thus the original data for the six outliers were examined to see if their removal from the category could be rationalised.

233 $\log EC_{50}^{-1} = 0.49 \log P - 3.95$ Eq. 1.

234 $N=43$; $r^2=0.36$; SE log P Coefficient=0.1 ($p<0.005$); SE Intercept=0.24 ($p<0.005$)



235
236 Figure 1. Relationship between FET ($\log EC_{50}^{-1}$) and hydrophobicity (octanol:water partition coefficient;
237 $\log P$) for the 43 compounds classified as baseline narcotics. The six significant outliers (residual
238 exceeds 1.5 log units) are labelled.

239 Two of the outliers, Rotenone and Merquat 100, also are outliers when the toxicity to adult fish is plotted
240 against hydrophobicity (Figure S1; supplementary information) suggesting the mechanism is the same
241 across assays. Rotenone [83-79-4] is a known toxicant to fish and indeed one of its industrial uses is as
242 a piscicide. The compound's mechanism of action involves disruption of the electron transport chain in
243 mitochondria³³ and thus there is sound mechanistic reasoning for removal of Rotenone from the baseline
244 narcosis category.

245 Merquat 100 [26062-79-3] is widely used in personal care products as a cationic polymer. It has low
246 toxicity with no specific mechanisms of action. Therefore in an aquatic test system it should act as a
247 baseline narcotic. However, the compound is difficult to model as its charged nature means that any
248 predicted $\log P$ value may be unreliable as an ionised species would be expected to have a $\log P$ several
249 orders of magnitude lower. This is suitable justification for removal of Merquat 100 from the model,
250 but in addition it is also worth noting that the rule in the Verhaar (modified) decision tree placing the
251 compound into class 1 is misfiring in this instance. The rule that matches against this compound is rule
252 1.6.1 "Be aliphatic secondary or tertiary amines", and it is clear that Merquat 100 is not a secondary or

tertiary amine. Therefore this compound is in fact outside of the domain of the Verhaar scheme and the outlier was also removed.

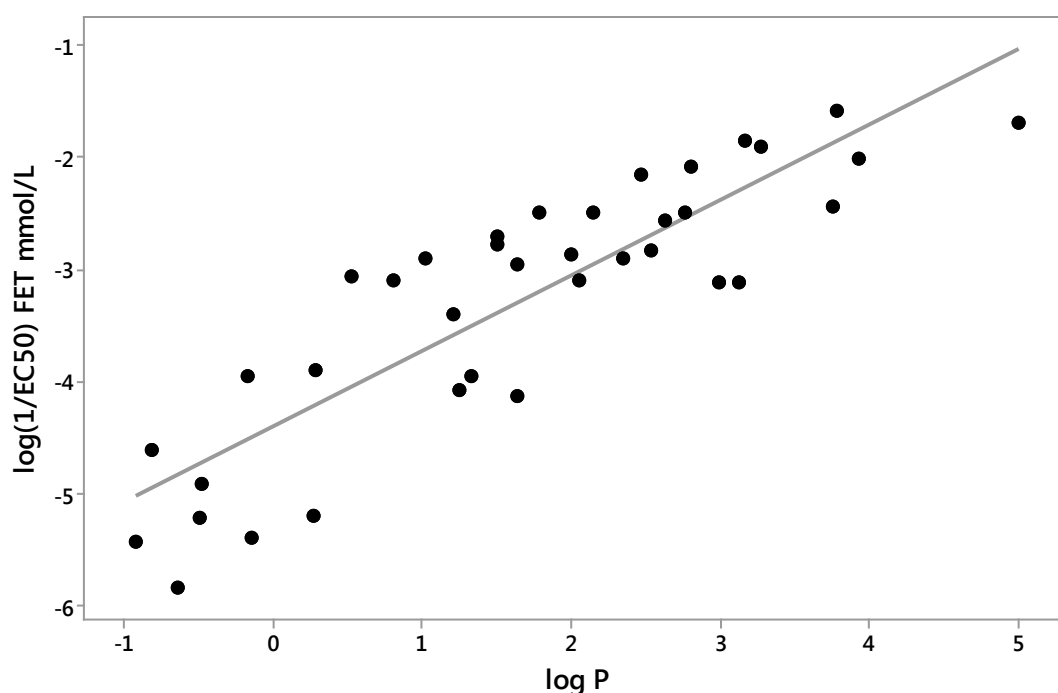
A third outlier, methoxyacetic acid [625-45-6], is ionised at neutral pH. In the original Verhaar publication one of the first rules for inclusion into class 1 is that it does “Not contain ionic groups” and thus this compound should not be within this class. However, if the neutral version of the structure is entered into Toxtree and the KNIME post-processing filter, neither program is able to identify this as an ionised compound. Hence the compound is not identified by rule 1.2 “Not an ionic compound” and is incorrectly placed into this class. When the ionised structure is entered, rule 1.2 correctly fires and the compound is placed into class 5. Thus there is suitable justification for removal of this outlier from the class.

Similar justifications could not be found for the remaining three outliers (tributylamine [102-82-9], methoxyacetic acid isopropylester [17640-21-0] and cyclohexane [110-82-7]); however the data published by Lammer et al⁴ and/or Belanger et al³ only refer to a single datum point for each of these compounds. Therefore it is not possible to assess the accuracy of these toxicity values, resulting in lower confidence and their removal from the model training set can be justified³⁴. This can be exemplified by cyclohexane with a reported EC₅₀ of 2.93x10⁴μM, whereas compounds with log P values in a similar range as cyclohexane (3.18 ± 0.1) have reported EC₅₀ values three orders of magnitude lower. In addition, the adult fish acute toxicity data for cyclohexane are in line with the general hydrophobicity trend (Figure S1; supplementary information). It is likely that the volatility of this compound may have caused problems in establishing an adequate concentration in the FET. The reliability of the FET value for this compound is therefore questionable.

The outliers were removed and regression analysis was repeated which yielded the much improved model described by Equation 2 and Figure 2, with no significant outliers. The regression coefficient (0.67) is lower than other baseline equations (e.g. the Neutral Organics equation in ECOSAR has a regression coefficient of 0.9) indicating that hydrophobicity is having a weaker effect in the FET compared to adult fish assays. This may be caused by the experimental protocol of the FET assay which means the exposure concentrations are rarely maintained⁶ and thus inject variability into the FET data. However, the high coefficient of determination ($r^2=0.75$) would suggest that the relationship between toxicity and hydrophobicity within the FET for baseline narcotics is still strong. Therefore it is possible to conclude that these compounds, which have been categorised using a scheme based on fish data, are all acting as baseline narcotics and thus acting via the same mechanism in both test systems (adult fish and embryos). A trend that is also clearly seen when plotting adult fish toxicity against activity in the FET (Figure S2; supplementary information). The ability to model non-polar narcosis well using log P alone would suggest the MIE is the same membrane disruption effect^{15, 35, 36} in both assays, leading to the same adverse outcome.

288 $\log EC_{50}^{-1} = 0.67 \log P - 4.41$ Eq. 2.

289 $N=37$; $r^2=0.75$; SE log P Coefficient=0.07 ($p<0.005$); SE Intercept=0.15 ($p<0.005$)



290
291 Figure 2. Linear regression between FET ($\log EC_{50}^{-1}$) and hydrophobicity (octanol:water partition
292 coefficient; $\log P$) of 37 compounds remaining within the baseline narcotic category

293 The regression analysis described above was repeated for the class 2 (polar narcotic) compounds, which
294 have less well defined MIE but distinct commonalities in the KEs of the AOP. There has been much
295 discussion into whether polar and baseline narcosis are distinct mechanisms^{37, 38}, but the evidence of
296 potential different MIEs³⁹ would suggest modelling them separately may be beneficial^{27, 40-42}. After an
297 initial regression analysis of the 47 Class 2 compounds, a poor model (Eq. 3) with two significant
298 outliers (2-chloro-5-nitropyridine [4548-45-2], residual = 2.42, and juglone [481-39-0], residual = 2.80)
299 was produced.

300 $\log EC_{50}^{-1} = 0.55 \log P - 3.35$ Eq. 3.

301 $N=47$; $r^2=0.60$; SE log P Coefficient=0.07 ($p<0.005$); SE Intercept=0.19 ($p<0.005$)

302 Justification for removal of these outliers from the model was sought. Juglone (Figure 3) was found to
303 be an outlier when plotting adult fish data against hydrophobicity (Figure S3; supplementary
304 information) suggesting it is a true mechanistic outlier. The compound was found to have the potential
305 to be reactive as it contained one of the electrophilic-chemistry based structural alerts (quinone)
306 published by Enoch et al³¹ and thus has the potential to react with nucleophilic biological
307 macromolecules through Michael addition. In addition, quinones can also cause toxicity through free

radical production⁴³ and disruption of the electron transport chain in mitochondria⁴⁴. There are currently no rules for quinones in the Verhaar scheme (modified) which could place this compound into its correct category, class 3 (unspecified reactivity). Therefore the removal of juglone from this category can be justified.

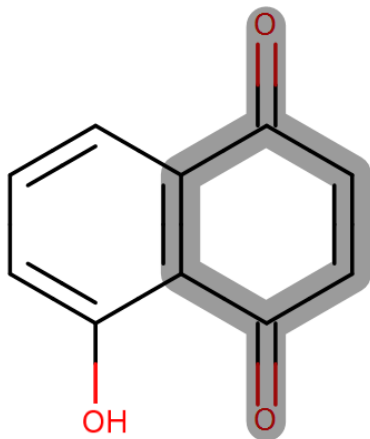


Figure 3. Structure of Juglone [481-39-0] with the alerting substructure highlighted in grey.

The remaining outlier, 2-chloro-5-nitropyridine [4548-45-2] can undergo a nucleophilic substitution reaction via the S_NAr mechanism because of the activating in-ring nitrogen group and chloro leaving group^{45, 46}. Thus, this compound should be in Verhaar class 3 and was removed from class 2.

The outliers were removed and regression analysis was repeated which yielded the much improved model described by Equation 4 and Figure 4. There were still three significant outliers (residual > 0.9) to this model (Figure 4) but no attempts were made to remove these because of the adequate r^2 value and there being no strong mechanistic rationale for their removal. Therefore it is possible to conclude that these compounds, which have been categorised using a scheme based on fish data, are all acting as polar narcotics and thus are acting via the same mechanism in both test systems (adult fish and embryos). A trend that is also seen when plotting adult fish toxicity against activity in the FET (Figure S4; supplementary information).

$$\log EC_{50}^{-1} = 0.59 \log P - 3.57 \quad \text{Eq. 4.}$$

N=45; $r^2=0.84$; SE log P Coefficient=0.04 ($p<0.005$); SE Intercept=0.12 ($p<0.005$)

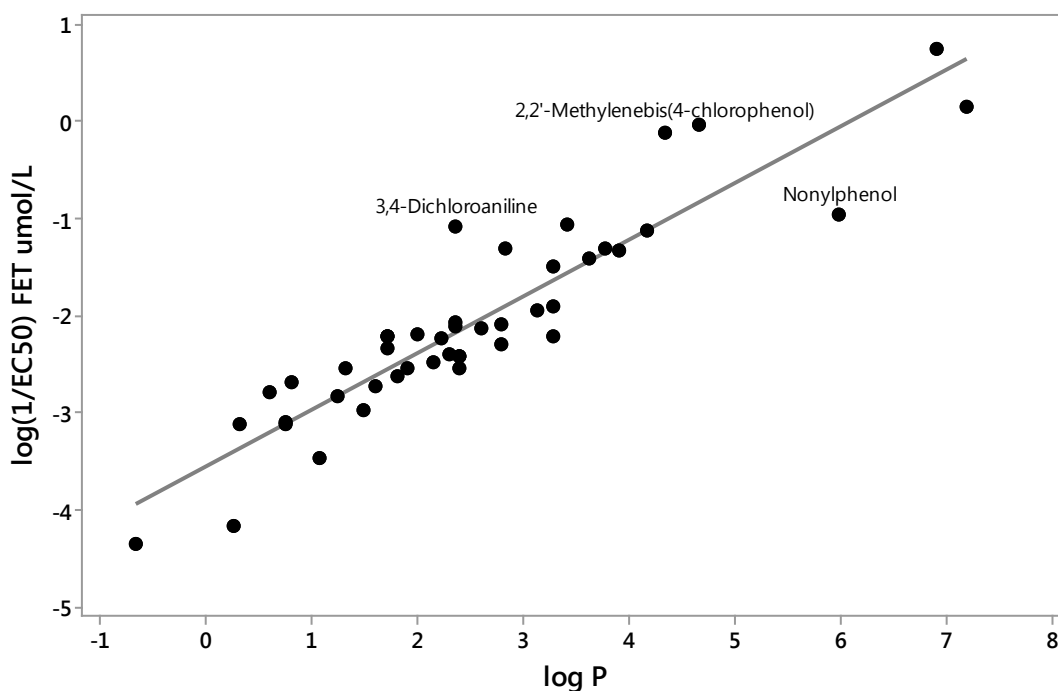
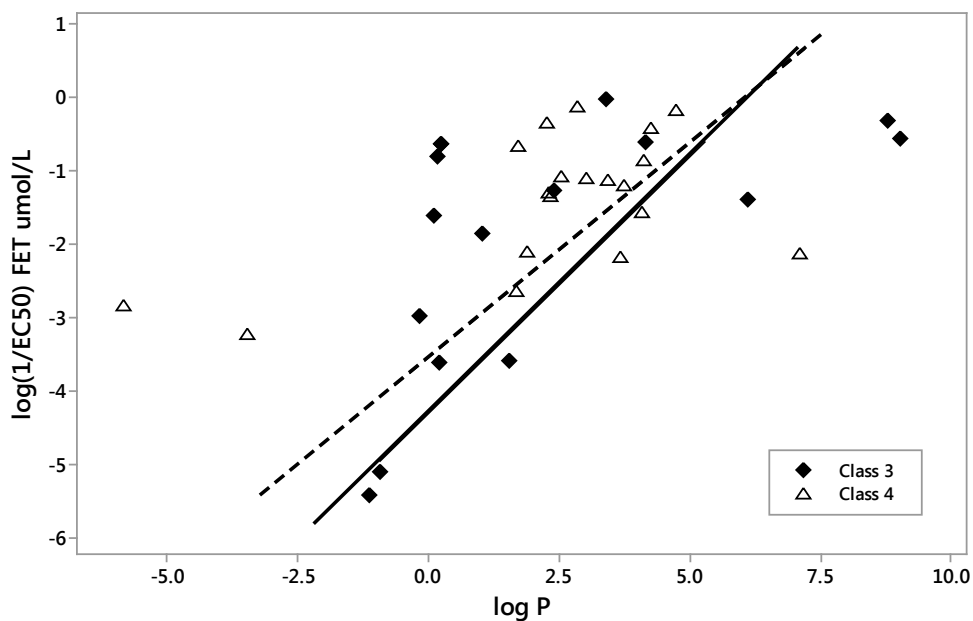


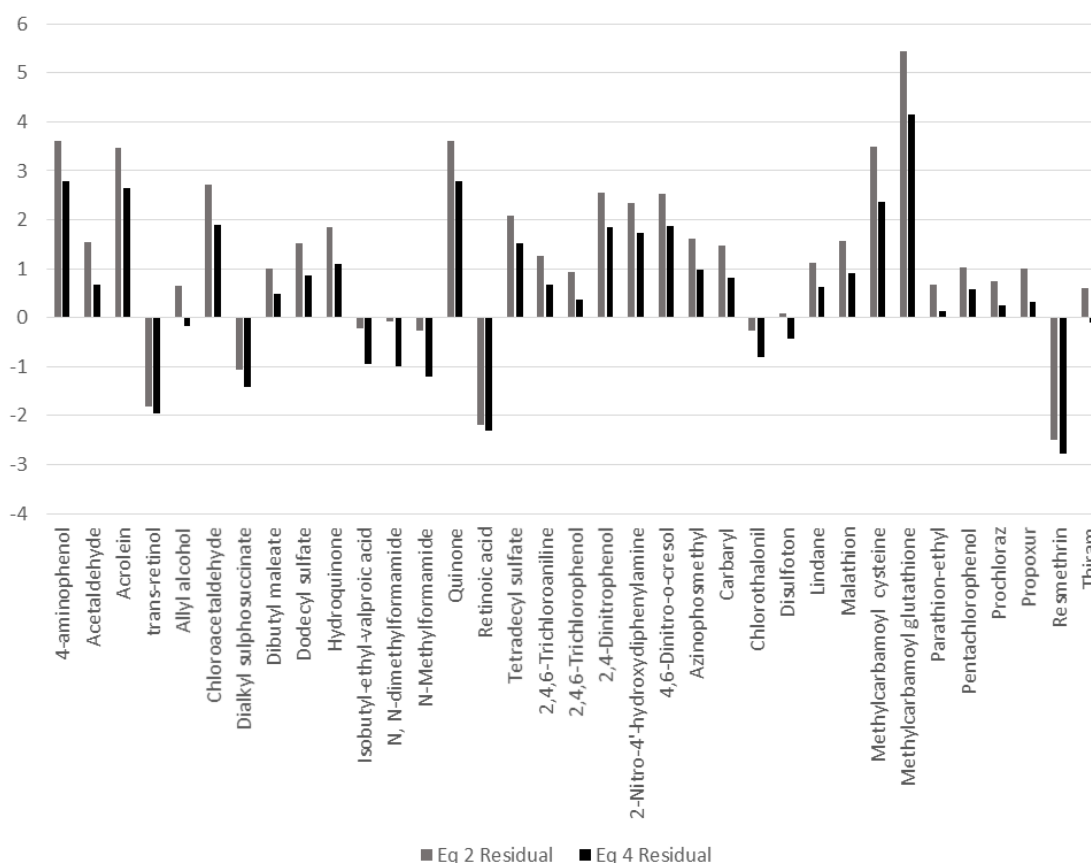
Figure 4. Relationship between FET ($\log EC_{50}^{-1}$) and hydrophobicity (octanol:water partition coefficient; $\log P$) for the 45 compounds classified as polar narcotics, after juglone and 2-chloro-5-nitropyridine had been removed from the category.

Unlike the baseline and polar narcotics the remaining two categories (class 3 (unspecified reactivity) and class 4 (specific mechanism)) cannot each be modelled as whole categories because of the numerous different mechanisms they represent. In addition, the direct correlations between adult fish toxicity and FET activity levels are less clear for these compounds (Figures S5 and S6; supplementary information). Modelling these compounds depends on understanding the specific AOPs involved for each sub-group. The difficulties of modelling across reactive mechanisms has been discussed previously^{18, 47-49}, and ideally models should be built using compounds all acting via the same chemical mechanism or MIE. Thus it is important to create subcategories for these compounds. However, the first step in modelling the mechanisms of these compounds is ensuring that they all exhibit excess toxicity and thus remove compounds which are associated with baseline mechanisms (e.g. narcosis). Some compounds which contain moieties associated with excess toxicity may not produce an observed toxicity in excess of narcosis because the level of specific toxicity is masked by the inherent narcotic effect of the compound. This is especially true for compounds with high $\log P$ values which will remain within the lipid bilayer and hence not undertake the interactions with biological macromolecules required to elicit excess toxicity^{50, 51}. Although the majority of compounds in this class clearly exhibit toxicity above the baseline (Figure 5), to specifically analyse which exhibit toxicity at the narcosis level the baseline and polar narcosis models presented above (Eq. 2 and Eq. 4) were used to predict the toxicity of all 35 compounds classified into class 3 and 4. The difference between this calculated value and the experimental outcome

349 were compared to examine if the residual was significant. The residuals values are presented in Figure
 350 6.



351
 352 Figure 5. Relationship between FET ($\log EC_{50}^{-1}$) and hydrophobicity (octanol:water partition coefficient;
 353 $\log P$) for the 35 compounds classified into class 3 and 4. The regression lines for Equation 2 (—) and
 354 Equation 4 (- -) are also shown.



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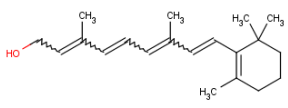
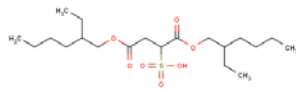
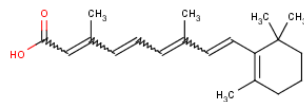
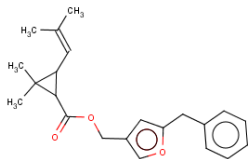
Figure 6. Residual values when embryo toxicity of Class 3 and 4 compounds is predicted using baseline (Eq 2) and polar (Eq 4) narcotic models

There are five compounds, classified as class 3 or 4, with a residual value in the range of -0.5 to 0.5 when using the baseline narcosis model to predict toxicity: isobutyl-ethyl-valproic acid [121-32-4]; N,N-dimethylformamide [68-12-2]; N-Methylformamide [123-39-7]; Chlorothalonil [1897-45-6]; and Disulfoton [298-04-4]. The toxicity values of these compounds are therefore well predicted by the baseline model and although they possess moieties which are attributed to electrophilic or specific mechanisms of action, the level of toxicity is no greater than baseline. This could be caused by the properties of the chemicals affecting the toxicokinetics and thus limiting the amount of compound reaching the site of action. Thus these compounds are not suitable for the mechanistic analysis as the observed toxicity is not representative of compounds containing moieties linked with excess toxicity.

Additionally the toxicity of seven compounds is well predicted by the polar narcosis model: allyl alcohol [107-02-8]; dibutyl maleate [105-76-0]; 2,4,6-trichlorophenol [88-06-2]; parathion-ethyl [56-38-2]; prochloraz [67747-09-5]; propoxur [114-26-1]; and thiram [137-26-8]. One compound which stands out from this list is allyl alcohol which is known to cause excess toxicity in fish⁵². However, its toxicity is dependent on metabolic activation⁵³ which does not occur in the 48hr FET assay from which this datum point originates³. Thus, the analysis presented here agrees with previous research suggesting the FET may not be suitable for compounds where metabolic activation is required⁶. Therefore, as above, although these compounds possess moieties which are attributed to electrophilic or specific mechanisms of action in adult fish, the level of toxicity is no greater than polar narcosis in the FET assay.

The four compounds where the observed toxicity is significantly less than that predicted from the baseline narcosis model (residual less than -0.5; Table 2) need to be examined before subcategories can be formed. The baseline model should represent the lowest level possible for toxicity and therefore the validity of these experimental outcomes must be questioned. The baseline and polar narcosis lines merge at a log P value of approximately 6 (figure 5), suggesting that this is the toxicokinetic cut-off for the assay and thus all compounds with a log P greater than 6 will model as narcotics. Three of the four compounds with high log P values, exhibiting toxicity below the baseline (trans-retinol [68-26-8], dialkyl sulphosuccinate [577-11-7] and resmethrin [10453-86-8]) were removed from their analysis by Belanger et al³ because of questionable experimental validity, and the remaining compound (retinoic acid [302-79-4]) also has special considerations reported in relation to its observed toxicity (Table 2). The data from these compounds suggest that at extreme values of log P it is not possible to achieve a 50% lethal response because of poor solubility. Thus these compounds cannot be modelled because their properties exceed the experimental limits of the assay.

Table 2. Reactive and specifically reactive compounds which exhibit observed toxicity less than that predicted from the baseline narcosis model.

Name	Structure	log P	Observed log EC ₅₀ ⁻¹ mmol/L	Predicted (Eq 2) log EC ₅₀ ⁻¹ mmol/L	Experimental consideration(s) ³
trans-Retinol		8.80	-0.33	1.84	- low solubility - single datum point
Dialkyl sulphosuccinate		6.10	-1.40	-0.05	- tests highly exceed solubility limit
Retinoic acid		9.03	-0.56	2.00	- low solubility - single datum point
Resmethrin		7.11	-2.15	0.66	- single datum point - low solubility

391

392 The remaining 19 compounds identified as class 3 or 4 all exhibit toxicity at levels above that expected
393 from narcosis (residual greater than 0.5; Figure 6); whether that be via an electrophilic or receptor based
394 mechanism. For most compounds their experimental toxicity is far greater than that predicted by either
395 the baseline or polar narcosis models, which is the pattern of excess toxicity which has been well
396 documented in fish⁵⁴. Therefore for these compounds it may be possible to sub-categorise according to
397 mechanistic trends in toxicity.

398 The Verhaar classification rules themselves cannot be used as a means of sub-classifying compounds
399 into specific mechanisms; i.e. compounds which fire the same rule do not necessarily act via the same
400 mechanism (Table 3). For example, Rule 3.8 “Contain a specific substructure” covers a wide range of
401 compounds containing electrophilic substructures, and for the class 4 compounds, only a single rule is
402 used for the whole category and no information is provided on the potential mechanism of action of the
403 compound. Thus further mechanistic analysis was required to subcategorise these compounds.

404 The eight remaining class 3 compounds were subcategorised into four mechanistic domains based on
405 whether the compounds contained one of the electrophilic structural alerts published by Enoch et al³¹.
406 Further expert analysis was required in the case of the two surfactants (dodecyl sulphate [151-21-3] and
407 tetradecyl sulphate [1191-50-0]). These two compounds did not contain any alerts associated with
408 electrophilic mechanisms, and it has been suggested that surfactants act via narcosis⁵⁵⁻⁵⁷. The poor

predictive ability of equations 2 and 4 for these compounds could be related to the difficulty in calculating, and indeed measuring, log P for surfactant compounds⁵⁸. However, even after correcting the KOWWIN log P calculations following the method described by Roberts⁵⁶ (manually calculated values were 0.65 for dodecyl sulphate and 1.64 for tetradecyl sulphate), the potency of these compounds is still under predicted by equations 2 and 4. An alternative mechanism may be for the compounds to act as alkylating agents via a bimolecular nucleophilic substitution (S_N2)⁵⁹. The four resultant mechanistic categories for the eight remaining class 3 compounds were thus Michael addition, pre-Michael addition, Schiff base and S_N2 (Table 3).

The eleven remaining class 4 compounds were subcategorised into three mechanistic domains based on expert analysis of the chemical structures and consideration of possible MIEs. Respiratory uncouplers were defined as those compounds which contain a weak acid assemblage (i.e. an amino or hydroxyl group), a hydrophobic aromatic moiety, and multiple electronegative groups (i.e. nitro and/or halogen substituents); a total of five compounds. Acetylcholinesterase (AChE) inhibitors comprised all of the organophosphothionate esters and (thio)carbamates; a total of five compounds. Both of these mechanisms have well described AOPs and predicting the MIE gives a suitable indication of toxicity which can be explained through biological reasoning⁶⁰. Lindane [58-89-9] was a unique compound as it does not act by any of the above mechanisms but instead interacts with the GABA receptor chlorine channel complex⁶¹.

Table 3. Compounds classified into Class 3 and 4 with their corresponding Verhaar rule as detailed in Toxtree ver. 2.6 and mechanistic subcategory

Name	CAS	Verhaar Rule (as stated in Toxtree)	Mechanistic domain
Class 3			
4-aminophenol	123-30-8	None: classified as potential reactive phenol by post-processor	Pre-Michael addition
Acetaldehyde	75-07-0	Rule 3.8 "Contain a specific substructure; aldehyde"	Schiff base
Acrolein	107-02-8	Rule 3.5 "Possess activated C-C double/triple bond"	Michael addition
Chloroacetaldehyde	107-20-0	Rule 3.8 "Contain a specific substructure; aldehyde"	Schiff base
Dodecyl sulphate	151-21-3	Rule 3.8 "Contain a specific substructure; sulphuric ester"	S _N 2

Hydroquinone	123-31-9	None: classified as potential reactive phenol by post-processor	Pre-Michael addition
Quinone	106-51-4	Rule 3.5 “Possess activated C-C double/triple bond”	Michael addition
Tetradecyl sulphate	1191-50-0	Rule 3.8 Rule 3.8 “Contain a specific substructure; sulphuric ester”	S _N 2
Class 4			
2,4,6-Trichloroaniline	634-93-5	Rule 4	Respiratory uncoupler
2,4-Dinitrophenol	51-28-5	None: classified as potential uncoupler by post-processor	Respiratory uncoupler
2-Nitro-4'-hydroxydiphenylamine	54381-08-7	None: classified as potential uncoupler by post-processor	Respiratory uncoupler
4,6-Dinitro-o-cresol	534-52-1	None: classified as potential uncoupler by post-processor	Respiratory uncoupler
Azinophosmethyl	86-50-0	Rule 4	AChE Inhibitor
Carbaryl	63-25-2	Rule 4	AChE Inhibitor
Lindane	58-89-9	Rule 4	GABA receptor chloride channel interaction
Malathion	121-75-5	Rule 4	AChE Inhibitor
Methylcarbamoyl cysteine	7324-17-6	Rule 4	AChE Inhibitor
Methylcarbamoyl glutathione	38126-73-7	Rule 4	AChE Inhibitor
Pentachlorophenol	87-86-5	Rule 4	Respiratory uncoupler

429

430 All of the subcategories contain too few data to build models. However, it is possible to observe the
431 trends between toxicity and physicochemical descriptors. The toxicity of the direct acting electrophiles
432 should be proportional to their electrophilicity if their ability to react with nucleophiles is the rate
433 limiting step in the toxic pathway. Enoch et al³⁰ have shown that the electrophilicity index, ω , can be
434 used to model toxicity of direct acting electrophiles. The electrophilicity index was thus calculated for
435 the Michael acceptors, Schiff base formers and S_N2 compounds (Table 4). The Michael acceptors and
436 Schiff base formers show the expected trend with the more toxic compounds having a higher
437 electrophilicity index. However, the same relationship was not present for the S_N2 compounds; the
438 electrophilic descriptors for the S_N2 surfactants are very similar due to their identical sulphate leaving

groups and do not fully explain the large difference in observed toxicity. Thus it would appear the hydrophobicity of the compounds is having a large effect on toxicity which is to be expected considering the only difference between the compounds is the length of their carbon chain. The effect of increasing chain length of anionic surfactants on toxicity has been well documented for ecotoxicity⁶². Therefore it is possible that the MIE is a membrane disruption effect such as narcosis as previously discussed. An expansion in the number of anionic surfactants tested in the FET is required before the AOP can be successfully modelled.

Table 4. Electrophilicity index (ω) values for the six compounds which are direct acting electrophiles

Compound name	CAS	Mechanistic subcategory	log EC ₅₀ ⁻¹ mmol/L	Electrophilicity (ω)
Acrolein	107-02-8	Michael addition	-0.82	4.77
Quinone	106-51-4	Michael addition	-0.64	8.70
Acetaldehyde	75-07-0	Schiff base	-2.99	2.93
Chloroacetaldehyde	107-20-0	Schiff base	-1.63	3.67
Dodecyl sulphate	151-21-3	S _N 2	-1.28	2.55
Tetradecyl sulphate	1191-50-0	S _N 2	-0.04	2.53

Unlike the direct acting electrophiles, the rate limiting step for the pre-Michael acceptors is their conversion into a reactive product, which is not reflected in their toxicity values⁴³. Also, there are two competing toxicity mechanisms for these compounds: conversion to the reactive quinone but also formation of free-radicals. Nitrogen is better than oxygen at stabilising a radical centre and therefore 4-aminophenol [123-30-8] may be more likely to exhibit toxicity through a radical mechanism than hydroquinone [123-31-9] where the resultant quinone would likely act as a Michael acceptor⁶³. This may explain why the toxicity of 4-aminophenol is far in excess of that shown by hydroquinone (log EC₅₀⁻¹, 4-aminophenol = -0.63, hydroquinone = -1.86). Modelling this complex mechanism, or finding any trends in toxicity through chemical read across, is impossible with only two compounds and hence the relationships discussed above cannot be replicated for this subcategory.

The respiratory uncouplers show clear trends with hydrophobicity, especially when split according to their electronegative groups (halogens or nitro; Figure 7). This is the same trend as modelled by Schultz and Cronin³² in several species, and shows that although this mechanism is clearly distinct from the AOP for narcosis, the AOP can be modelled using toxicokinetic parameters. Thus it is clear this mechanism of action is valid across species and can be tested in the FET assay.

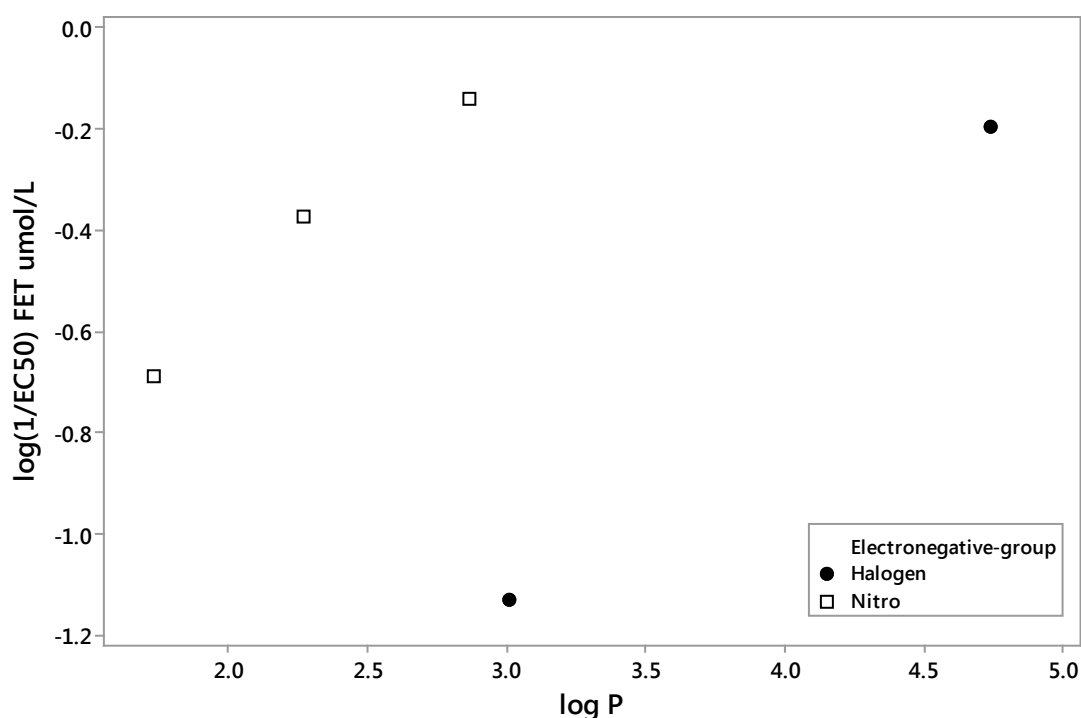


Figure 7. Relationship between FET ($\log EC_{50}^{-1}$) and hydrophobicity (octanol:water partition coefficient; $\log P$) for the five respiratory uncouplers, categorised according to the electronegative groups which they contain.

The final sub-category of compounds is the AChE inhibitors, comprising two structural groups (carbamates and organophosphates). The mechanism of action in this instance is governed by the ability of a compound to react with the acetylcholine esterase enzyme and form a covalent bond with the active site, specifically forming a covalent bond with the hydroxy group on the serine residue⁵⁴. The difference between the inhibition initiated by the carbamates and the organophosphates is caused by the stability of the AChE-organophosphate/carbamate complex. The carbamylated serine residue is less stable and the carbamyl structure can be split from the enzyme by spontaneous hydrolysis, whereas the phosphorylation of the serine residues is considered non-reversible as dephosphorylation is very slow (in the order of days)⁶⁴. This mechanism, like the direct acting electrophiles, depends on the electronic properties of the compounds. However, the compounds within this class are too diverse to observe trends using simple ground state calculations such as the electrophilicity index. Bermudez-Saldana and Cronin⁶⁵ found that modelling heterogeneous groups of AChE inhibitors was difficult with 2D and 3D descriptors; calculations performed on the transition states of congeneric series were required to model activity effectively. Unfortunately that was not possible for the AChE inhibitors which have been tested in the FET assay.

The subcategories of class 3 and 4 compounds have demonstrated that the relationships between toxicity and physicochemical descriptors for compounds tested in the FET assay are similar to those seen in adult fish; i.e. toxicity is related to the properties which best describe the specific interactions of the

mechanism. However, it is important to note that all of these subclasses are small ($n < 5$) and creating true predictive models has been impossible because of this and also the structural diversity within the data. An extensive dataset of class 3 and 4 compounds tested in the FET would be required for a comprehensive mechanistic analysis of compounds exhibiting excess toxicity. These data would preferably be for compounds within the mechanistic classes for which some data are available, e.g. Michael addition, to enable full mechanistic modelling to be completed, before expanding into the other unknown mechanistic domains. To this end it would be important to formulate an intelligent testing strategy with regard to which other compounds should be tested, concentrating on specific series of excess toxicants. This focussed approach to modelling has previously been shown to be effective for modelling compounds which exhibit excess toxicity to *Tetrahymena pyriformis* using a specific reactivity assay^{43, 66-69}. The outcomes of the FET when applied to specially selected compounds would allow for mechanistic modelling and chemical read across to elucidate the physicochemical descriptors which best describe the AOP. These descriptors could then be used to assess toxicity of compounds tested in other assays such as the traditional *in vivo* fish assays to assess the interspecies compatibility of the AOP^{70, 71}.

In conclusion, the four mechanistic categories built on toxicity data from fish proposed by Verhaar et al have been shown to be applicable to the FET assay using zebrafish. The majority of industrial compounds within this dataset can be modelled as narcotics. The baseline narcosis effect is well modelled by log P alone which suggests the AOP in FET is governed by membrane disruption as part of, if not the complete, MIE. The polar narcosis effect has a less well defined MIE, but the AOP shares common key events and hence the effect can also be well modelled by log P. Both mechanisms produce high quality hydrophobicity dependent QSAR models which are based on relatively well understood, if not yet documented, AOPs and those compounds acting via reactive or specific mechanisms exhibit toxicity in excess of that predicted from these models. For these AOPs representing relatively unspecific mechanisms of action, it is noted that a single parameter is able to model the response even when the precise MIE has yet to be established. The outliers of the narcosis models provide useful information relating to interspecies differences and/or highlight the limitations of the assay. For example, allyl alcohol does not exhibit excess toxicity in the 48hr FET assay. Modelling within the reactive and specific mechanistic categories is theoretically possible, but more data are required to fully investigate the mechanisms; an understanding of which descriptors are driving the observed toxicity is required. For these classes the AOP should be more easily defined as the mechanisms of action are more specific; due to the increased complexity and greater specificity of the AOP it is probable that more terms are required to capture the effects. It is an understanding of the AOPs involved that will suitably inform future sub-categorisation, and suggest where interspecies differences will become important. However, currently, there are not enough examples of excess toxicants which have been tested in the FET assay and focussed toxicity testing is required to clearly define the domains of the reactive and specifically

acting compounds. Such testing would assist in understanding the descriptors required to model the mechanisms and AOPs of these reactive or specifically acting compounds. Overall the FET data shows that with increasing log P, compounds are less inclined to enter the aqueous media of cells and therefore they tend to exhibit toxicity through the narcosis mechanism. The log P cut-off for this effect appears to approximately 6 for this assay. This cut-off will vary according to species because of their varying membrane properties and the design of the test system; longer tests are more likely to allow for quantification than shorter tests. These issues will be important as development of quantitative acute aquatic AOPs progresses.

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Supporting Information

Tables of all collated FET and adult fish data are available as supplementary information along with figures of relevant plots of this data. This information is available free of charge via the Internet at <http://pubs.acs.org>

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