

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

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8 Abstract

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

23 1. Introduction

24 The acute aquatic toxicity assessment of chemicals has traditionally been performed on species
25 representing various trophic levels e.g. algae, invertebrates, and juvenile and adult fish from species
26 such as the fathead minnow (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*) and Japanese
27 medaka (*Oryzias latipes*) amongst others. However, legislative mandates such as the Registration,
28 Evaluation, Authorisation, and restriction of Chemicals (REACH) regulation in the European Union
29 have required alternative, non-animal, models to be sought as a replacement for the expensive, time-
30 consuming and ethically questionable *in vivo* assessment methods. One such alternative assay is the fish
31 embryo toxicity (FET) test¹. The FET has a standardised OECD test guideline (number 236²) for the
32 96hr assay performed with zebrafish (*Danio rerio*) embryos and, although not explicitly stated in the
33 guideline, can be considered as a suitable assessment method, on its own or as part of a strategy, for

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

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

50 The modes of action for acute aquatic toxicity have been established through studies on fish behaviour
51 and physiology^{12,13}, as well as mechanistic structure-activity relationship (SAR) analysis on the
52 resultant data¹⁴, and more recently on detailed systems biology studies on species from lower taxa such
53 as *Daphnia magna*¹⁵. Fish Acute Toxicity Syndromes (FATS) were derived from measurements of
54 physiological, biochemical and analytical effects separated into discrete mechanisms, or modes, of
55 action¹². Building on such knowledge, Verhaar et al¹⁶ used fish acute toxicity data to identify clear
56 structural rules associated with a variety of modes or mechanisms of action. The Verhaar scheme utilises
57 2D chemical structure to classify potential environmental pollutants into one of four categories
58 representing one, or more, mechanisms of action: class 1 (narcosis or baseline toxicity), class 2 (less
59 inert compounds), class 3 (unspecific reactivity) and class 4 (compounds and groups of compounds
60 acting by a specific mechanism). In addition, Russom et al¹³ used structural classes to assign
61 mechanisms of action to a range of compounds tested on the fathead minnow (*Pimephales promelas*).
62 This grouping of compounds allows for the development of mechanistically based, local Quantitative
63 Structure Activity Relationship (QSAR) models and also application of the chemical activity principle¹⁷.

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

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

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

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

102 2. Methods

103 2.1. Dataset

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

130 2.2. Software

131 All 193 compounds described above were classified using the “Verhaar scheme (modified)” decision
132 tree through the batch processing functionality of Toxtree v2.6. Structures were entered as SDfiles
133 which were generated from the SMILES strings using MarvinBeans v14 (www.chemaxon.com). The
134 possible outcomes from the scheme are equivalent to those originally published by Verhaar et al¹⁶: class
135 1 (narcosis or baseline toxicity); class 2 (less inert compounds); class 3 (unspecific reactivity); class 4
136 (compounds and groups of compounds acting by a specific mechanism); class 5 (not possible to classify
137 according to rules).

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

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

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

$$157 \quad \omega = \mu^2/2\eta$$

158 Where

$$159 \quad \mu = (E_{HOMO} + E_{LUMO})/2$$

$$160 \quad \eta = E_{LUMO} - E_{HOMO}$$

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

163 2.3. Data analysis

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

171 different mechanism or if there were any other reasons why their behaviour did not fit the expected
172 trend. This was performed through expert analysis of the published data (i.e. examining the reliability
173 of the data point and whether the same trends were observed in adult fish (by comparison with a plot of
174 $\log LC_{50}^{-1}$ value(s) against hydrophobicity)) and also examining if the compounds contained structural
175 alerts known to be associated with reactive mechanisms of action (using the alerts published by Enoch
176 et al³¹). If justification based on one or more of the criteria described above could be found, the outliers
177 were removed as they were either misclassified or subject to competing mechanisms. The models were
178 redeveloped and their removal resulted in improved QSAR models.

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

186 The compounds which remained in classes 3 and 4 after the above stated investigations cannot be
187 modelled as whole classes as they represent a broad range of discrete mechanisms. Therefore the class
188 3 and 4 compounds were subcategorised. For the class 3 compounds this was achieved by using the
189 mechanistic alerts published by Enoch et al³¹. These provided mechanistic domains (e.g. Michael
190 addition) which were then suitable for trend analysis. For the class 4 compounds expert judgement was
191 required to identify which specific mechanism each compound was likely to act by (e.g.
192 acetylcholinesterase (AChE) inhibition); this process builds, in part, on the approach published by
193 Martin et al [18]. After sub-categorisation trend analysis between the toxicity values ($\log EC_{50}^{-1}$) and
194 chemical descriptors ($\log P$ and/or electrophilicity) was performed to examine if the mechanistic
195 rationale was sufficient to describe the observed outcome. If this was not the case it may suggest the
196 compounds are acting via undefined toxicity mechanisms in the FET assay and that the AOPs differ
197 between species.

198 3. Results and Discussion

199 It has been documented that the EC_{50} values obtained from the fish embryo acute toxicity (FET) assay
200 using zebrafish embryos correlate well with the LC_{50} values from traditional fish toxicity studies,
201 irrespective of fish species³⁻⁵. Thus it has been suggested that the FET assay could act as a replacement
202 for the fish lethality studies. This correlation of toxicity between the species and life stages would
203 suggest that compounds are toxic via the same or similar AOPs irrespective of the test system, with a
204 small number of notable exceptions (neurotoxic compounds and those requiring metabolism)⁶. The aim
205 of this study was to investigate whether compounds tested in the FET can be modelled within their

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

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

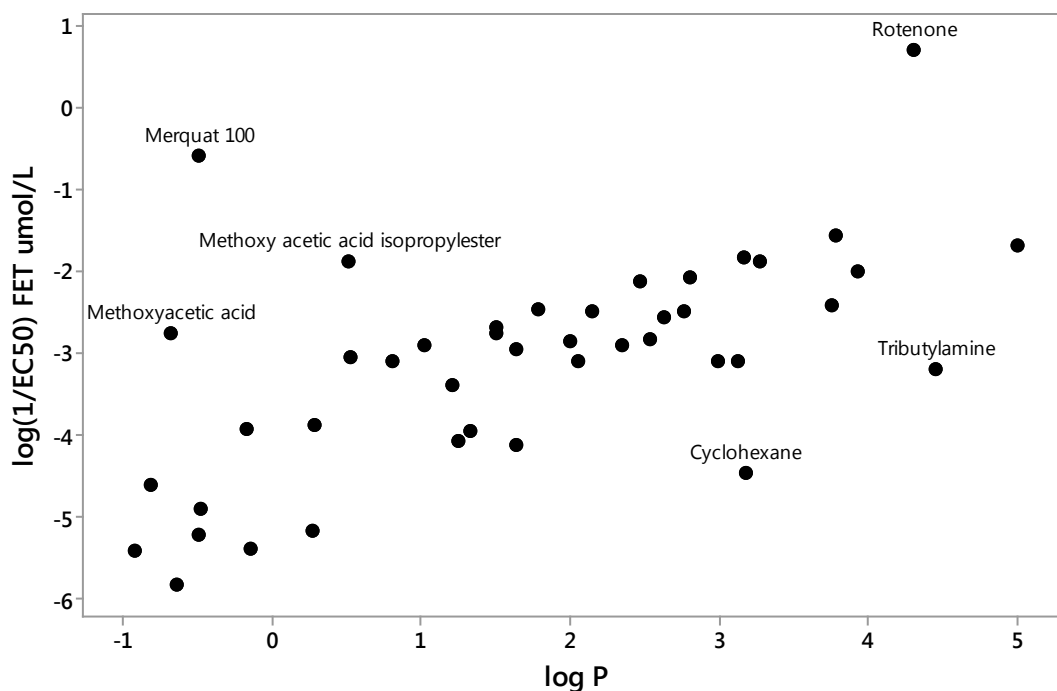
222 Table 1. The number of compounds classified into each category when using either the Verhaar
 223 (modified) scheme as implemented in Toxtree 2.6 or the Verhaar (modified) scheme in combination
 224 with the post processing filter published by Ellison et al²⁷.

Classification Method	Number of compounds				
	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

225
 226 Regression analysis was performed on data from the 43 class 1, baseline narcotic, compounds. This
 227 produced a poor model (Eq.1) with six significant outliers (residual exceeded 1.5 log units). The outliers
 228 are identified in Figure 1. Since one of the aims of this work was to examine whether the mechanisms
 229 of fish toxicity were also relevant in the FET assay the outliers could not be removed purely because of
 230 statistical reasons. The compounds may be poorly predicted because they do not act as narcotics in
 231 zebrafish embryos. Thus the original data for the six outliers were examined to see if their removal from
 232 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

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

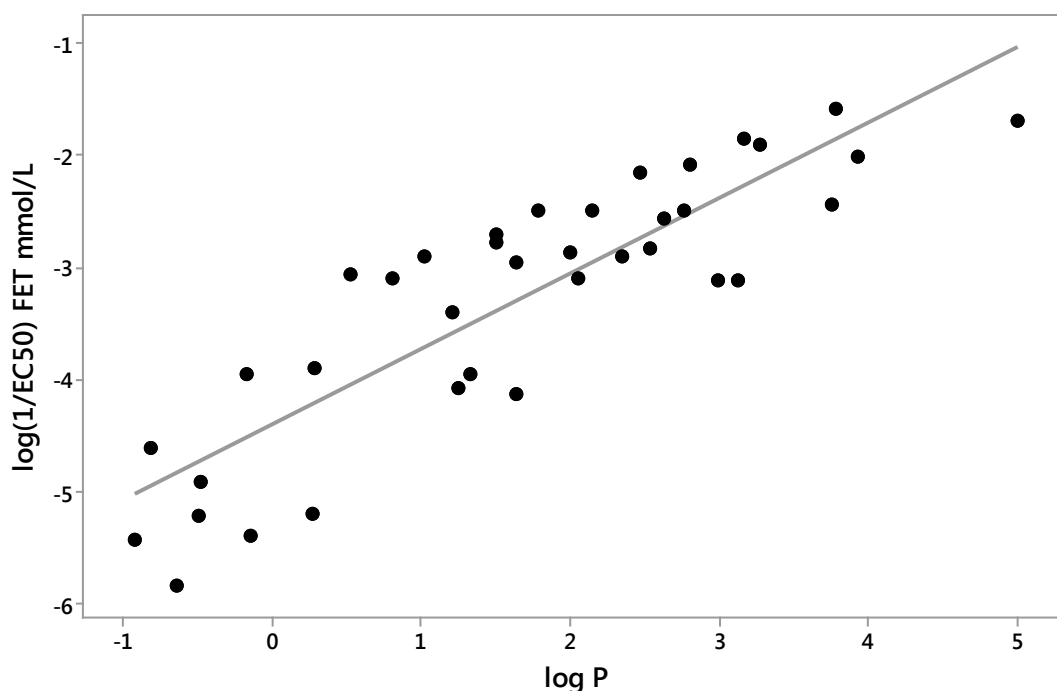
255 A third outlier, methoxyacetic acid [625-45-6], is ionised at neutral pH. In the original Verhaar
256 publication one of the first rules for inclusion into class 1 is that it does “Not contain ionic groups” and
257 thus this compound should not be within this class. However, if the neutral version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

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

274 The outliers were removed and regression analysis was repeated which yielded the much improved
275 model described by Equation 2 and Figure 2, with no significant outliers. The regression coefficient
276 (0.67) is lower than other baseline equations (e.g. the Neutral Organics equation in ECOSAR has a
277 regression coefficient of 0.9) indicating that hydrophobicity is having a weaker effect in the FET
278 compared to adult fish assays. This may be caused by the experimental protocol of the FET assay which
279 means the exposure concentrations are rarely maintained⁶ and thus inject variability into the FET data.
280 However, the high coefficient of determination ($r^2=0.75$) would suggest that the relationship between
281 toxicity and hydrophobicity within the FET for baseline narcotics is still strong. Therefore it is possible
282 to conclude that these compounds, which have been categorised using a scheme based on fish data, are
283 all acting as baseline narcotics and thus acting via the same mechanism in both test systems (adult fish
284 and embryos). A trend that is also clearly seen when plotting adult fish toxicity against activity in the
285 FET (Figure S2; supplementary information). The ability to model non-polar narcosis well using log P
286 alone would suggest the MIE is the same membrane disruption effect^{15, 35, 36} in both assays, leading to
287 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

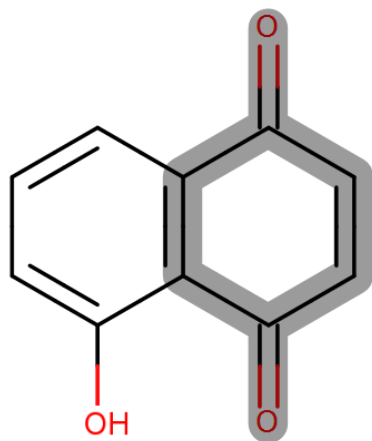
293 The regression analysis described above was repeated for the class 2 (polar narcotic) compounds, which
294 have less well defined MIE but distinct commonalities in the KEs of the AOP. There has been much
295 discussion into whether polar and baseline narcosis are distinct mechanisms^{37, 38}, but 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

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



312

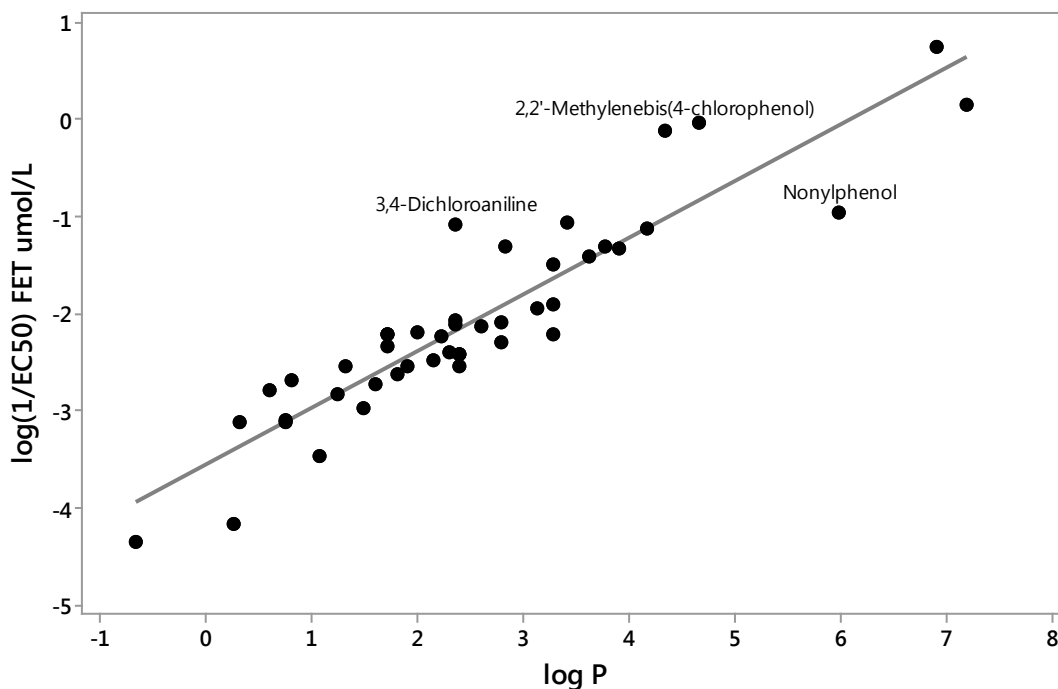
313 Figure 3. Structure of Juglone [481-39-0] with the alerting substructure highlighted in grey.

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

317 The outliers were removed and regression analysis was repeated which yielded the much improved
318 model described by Equation 4 and Figure 4. There were still three significant outliers (residual > 0.9)
319 to this model (Figure 4) but no attempts were made to remove these because of the adequate r² value
320 and there being no strong mechanistic rationale for their removal. Therefore it is possible to conclude
321 that these compounds, which have been categorised using a scheme based on fish data, are all acting as
322 polar narcotics and thus are acting via the same mechanism in both test systems (adult fish and embryos).
323 A trend that is also seen when plotting adult fish toxicity against activity in the FET (Figure S4;
324 supplementary information).

325 $\log EC_{50}^{-1} = 0.59 \log P - 3.57$ Eq. 4.

326 N=45; r²=0.84; SE log P Coefficient=0.04 (p<0.005); SE Intercept=0.12 (p<0.005)

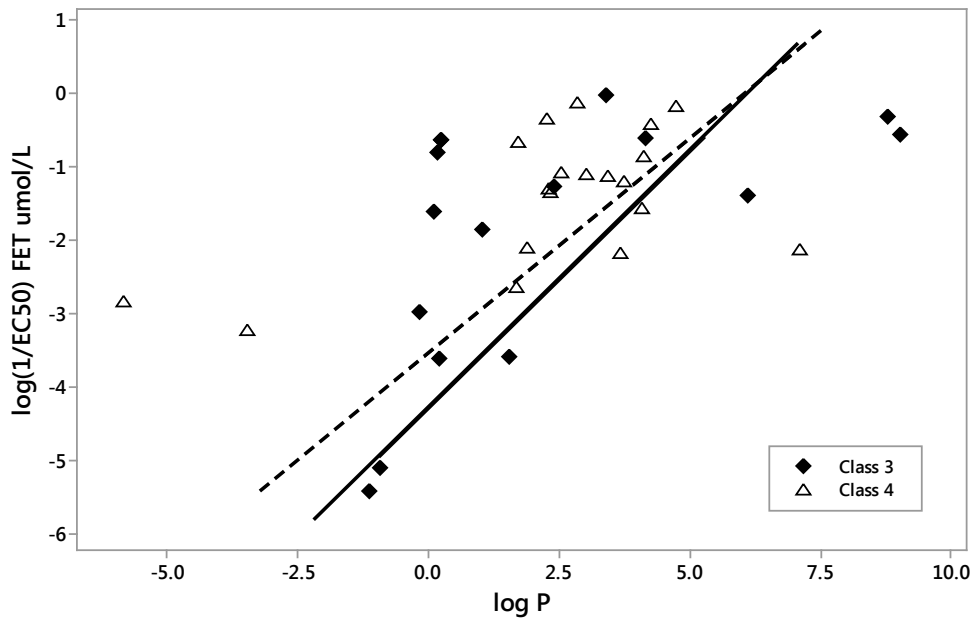


327

328 Figure 4. Relationship between FET ($\log EC_{50}^{-1}$) and hydrophobicity (octanol:water partition coefficient;
 329 $\log P$) for the 45 compounds classified as polar narcotics, after juglone and 2-chloro-5-nitropyridine
 330 had been removed from the category.

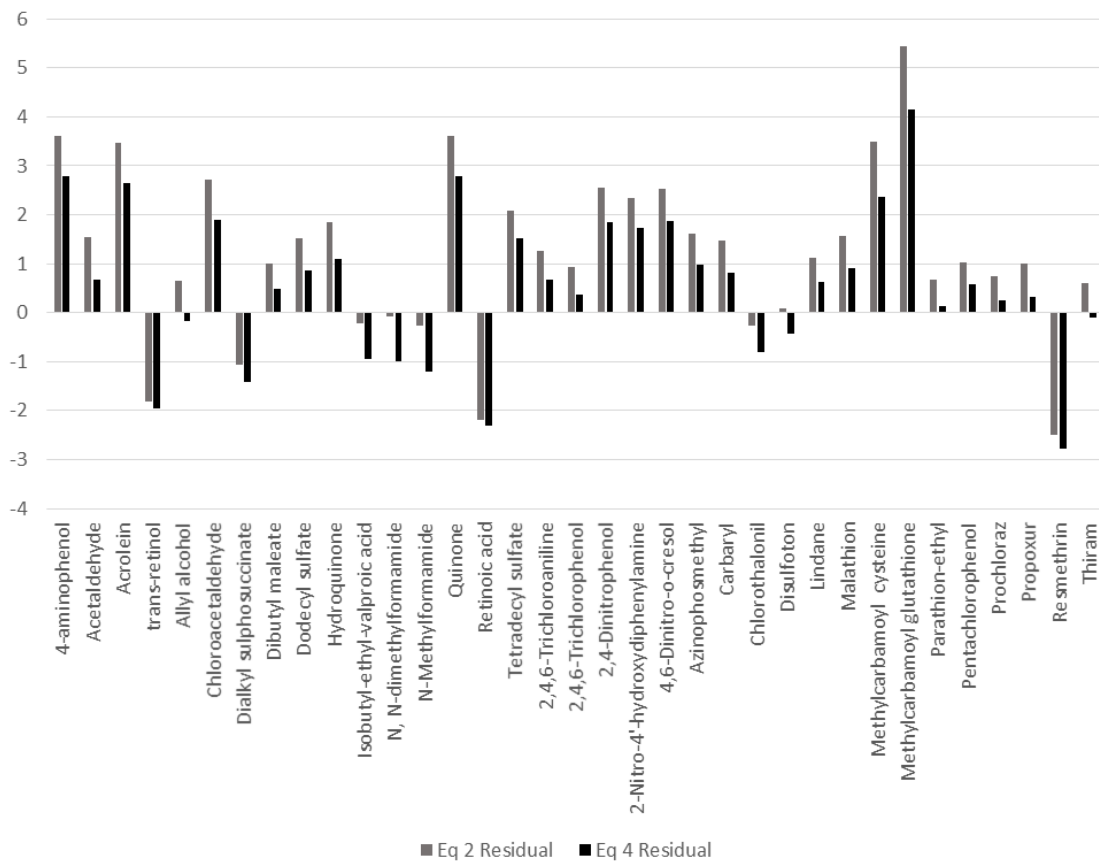
331 Unlike the baseline and polar narcotics the remaining two categories (class 3 (unspecified reactivity)
 332 and class 4 (specific mechanism)) cannot each be modelled as whole categories because of the numerous
 333 different mechanisms they represent. In addition, the direct correlations between adult fish toxicity and
 334 FET activity levels are less clear for these compounds (Figures S5 and S6; supplementary information).
 335 Modelling these compounds depends on understanding the specific AOPs involved for each sub-group.
 336 The difficulties of modelling across reactive mechanisms has been discussed previously^{18, 47-49}, and
 337 ideally models should be built using compounds all acting via the same chemical mechanism or MIE.
 338 Thus it is important to create subcategories for these compounds. However, the first step in modelling
 339 the mechanisms of these compounds is ensuring that they all exhibit excess toxicity and thus remove
 340 compounds which are associated with baseline mechanisms (e.g. narcosis). Some compounds which
 341 contain moieties associated with excess toxicity may not produce an observed toxicity in excess of
 342 narcosis because the level of specific toxicity is masked by the inherent narcotic effect of the compound.
 343 This is especially true for compounds with high $\log P$ values which will remain within the lipid bilayer
 344 and hence not undertake the interactions with biological macromolecules required to elicit excess
 345 toxicity^{50, 51}. Although the majority of compounds in this class clearly exhibit toxicity above the baseline
 346 (Figure 5), to specifically analyse which exhibit toxicity at the narcosis level the baseline and polar
 347 narcosis models presented above (Eq. 2 and Eq. 4) were used to predict the toxicity of all 35 compounds
 348 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.



355

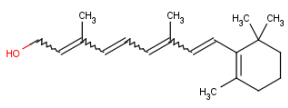
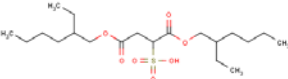
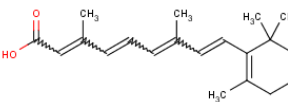
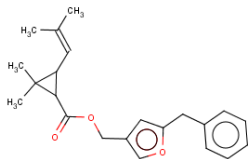
356 Figure 6. Residual values when embryo toxicity of Class 3 and 4 compounds is predicted using baseline
357 (Eq 2) and polar (Eq 4) narcotic models

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

367 Additionally the toxicity of seven compounds is well predicted by the polar narcosis model: allyl alcohol
368 [107-02-8]; dibutyl maleate [105-76-0]; 2,4,6-trichlorophenol [88-06-2]; parathion-ethyl [56-38-2];
369 prochloraz [67747-09-5]; propoxur [114-26-1]; and thiram [137-26-8]. One compound which stands
370 out from this list is ally alcohol which is known to cause excess toxicity in fish⁵². However, its toxicity
371 is dependent on metabolic activation⁵³ which does not occur in the 48hr FET assay from which this
372 datum point originates³. Thus, the analysis presented here agrees with previous research suggesting the
373 FET may not be suitable for compounds where metabolic activation is required⁶. Therefore, as above,
374 although these compounds possess moieties which are attributed to electrophilic or specific mechanisms
375 of action in adult fish, the level of toxicity is no greater than polar narcosis in the FET assay.

376 The four compounds where the observed toxicity is significantly less than that predicted from the
377 baseline narcosis model (residual less than -0.5; Table 2) need to be examined before subcategories can
378 be formed. The baseline model should represent the lowest level possible for toxicity and therefore the
379 validity of these experimental outcomes must be questioned. The baseline and polar narcosis lines
380 merge at a log P value of approximately 6 (figure 5), suggesting that this is the toxicokinetic cut-off for
381 the assay and thus all compounds with a log P greater than 6 will model as narcotics. Three of the four
382 compounds with high log P values, exhibiting toxicity below the baseline (trans-retinol [68-26-8],
383 dialkyl sulphosuccinate [577-11-7] and resmethrin [10453-86-8]) were removed from their analysis by
384 Belanger et al³ because of questionable experimental validity, and the remaining compound (retinoic
385 acid [302-79-4]) also has special considerations reported in relation to its observed toxicity (Table 2).
386 The data from these compounds suggest that at extreme values of log P it is not possible to achieve a
387 50% lethal response because of poor solubility. Thus these compounds cannot be modelled because
388 their properties exceed the experimental limits of the assay.

389 Table 2. Reactive and specifically reactive compounds which exhibit observed toxicity less than that
390 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

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

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

427 Table 3. Compounds classified into Class 3 and 4 with their corresponding Verhaar rule as detailed in
 428 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

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

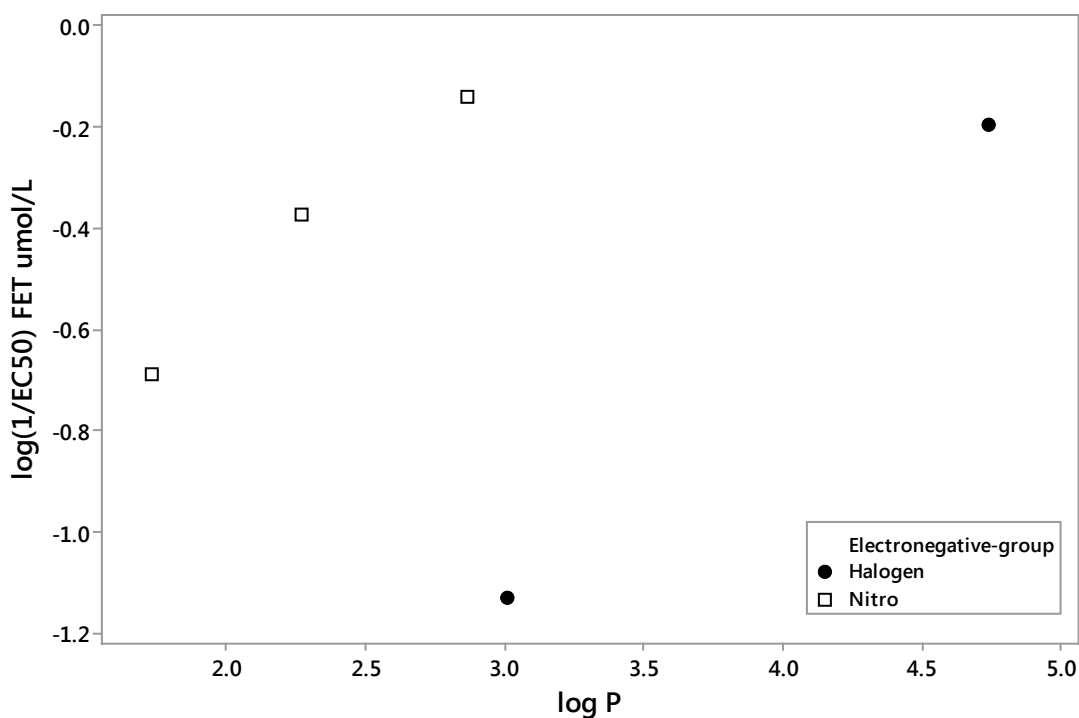
446 Table 4. Electrophilicity index (ω) values for the six compounds which are direct acting electrophiles

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

447

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

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



463

464 Figure 7. Relationship between FET ($\log EC_{50}^{-1}$) and hydrophobicity (octanol:water partition coefficient;
 465 $\log P$) for the five respiratory uncouplers, categorised according to the electronegative groups which
 466 they contain.

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

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

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

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

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

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532 Supporting Information

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

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