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## Assessment of predictive models for estimating the acute aquatic toxicity of organic chemicals†

Fjodor Melnikov,<sup>a</sup> Jakub Kostal,<sup>b,c</sup> Adelina Voutchkova-Kostal,<sup>c</sup> Julie B. Zimmerman<sup>a,d</sup> and Paul T. Anastas<sup>\*a,d</sup>

*In silico* toxicity models are critical in addressing experimental aquatic toxicity data gaps and prioritizing chemicals for further assessment. Currently, a number of predictive *in silico* models for aquatic toxicity are available, but most models are challenged to produce accurate predictions across a wide variety of functional chemical classes. Appropriate model selection must be informed by the models' applicability domain and performance within the chemical space of interest. Herein we assess five predictive models for acute aquatic toxicity to fish (ADMET Predictor™, Computer-Aided Discovery and REdesign for Aquatic Toxicity (CADRE-AT), Ecological Structure Activity Relationships (ECOSAR) v1.11, KAshinhou Tool for Ecotoxicity (KATE) on PAS 2011, and Toxicity Estimation Software Tool (TEST) v.4). The test data set was carefully constructed to include 83 structurally diverse chemicals distinct from the training data sets of the assessed models. The acute aquatic toxicity models that rely on properties related to chemicals' bioavailability or reactivity performed better than purely statistical algorithms trained on large sets of chemical properties and structural descriptors. Most models showed a marked decrease in performance when assessing insoluble and ionized chemicals. In addition to comparing tool accuracy and, this analysis provides insights that can guide selection of modeling tools for specific chemical classes and help inform future model development for improved accuracy.

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## Introduction

To protect human health and the environment from exposure to anthropogenic chemicals, global regulations have called for systematic testing of potential environmental contaminants, such as industrial chemicals and pharmaceuticals.<sup>1–3</sup> The rapidly growing number of chemicals in commerce presents challenges to traditional ecotoxicity testing strategies, which are expensive, time-consuming and reliant on large number of animal subjects,<sup>4</sup> especially in light of new EU legislation to phase out animal testing.<sup>5</sup> Furthermore, existing ecotoxicity assessment methods may be inadequate when assessing novel compounds, such as flame retardants, pharmaceuticals, and nanomaterials.<sup>6</sup>

To mitigate the challenges associated with *in vitro* and *in vivo* toxicity testing, global regulations, including European Chemical Agency (ECHA) REACH initiative, U.S. Toxic Substances Control Act (TSCA) and Canadian Environmental Protection Act (CEPA), encourage increased reliance on *in silico* approaches.<sup>1,7</sup> Similarly, the 2014 National Research Council (NRC) alternatives assessment framework advocates for increased use of *in silico* methods.<sup>8</sup> While not necessary definitive, *in silico* models can also inform prioritization of chemicals for further testing.<sup>5,7,9–12</sup> The development of reliable *in silico* models for aquatic toxicity relies on availability of high-quality toxicity data for a range of fish species. Office of Chemical Safety and Pollution Prevention (OCSPP) outlined a list of fish species approved for measuring or estimating toxicity to specific organisms and aquatic systems as a whole.<sup>13–15</sup>

The cost-benefit advantages and regulatory support of *in silico* methods,<sup>16,17</sup> have led to the development of a number of tools for ecotoxicity assessments. Specifically, several Quantitative Structure–Activity Relationships (QSARs), which relate chemical's structural features and physicochemical properties to biological activity, and read-across models, which estimate the toxicity of chemical by comparison to structurally similar compounds have been developed for chemical toxicity to fish and are widely used for ecological risk assessment.<sup>7,8</sup> Such

<sup>a</sup>School of Forestry and Environmental Studies, Yale University, New Haven, CT 06520, USA. E-mail: paul.anastas@yale.edu

<sup>b</sup>Computational Biology Institute, the George Washington University, Washington DC, USA

<sup>c</sup>Department of Chemistry, the George Washington University, Washington, DC, USA

<sup>d</sup>Department of Chemical and Environmental Engineering, Yale University, New Haven, CT 06520, USA

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tools include the Ecological Structure Activity Relationships (ECOSAR), Kashinhou Tool for Ecotoxicity (KATE) and Toxicity Estimation Software Tool (TEST), which are freely available standalone packages. Ecological Structure Activity Relationships (ECOSAR) and Toxicity Estimation Software Tool (TEST) were developed by the US EPA and the Syracuse Research Corporation,<sup>13</sup> while KATE is a product of The Japanese Ministry of the Environment and the Japanese National Institute for Environmental Studies (NIES).<sup>18</sup> ECOSAR and KATE rely on octanol-water partitioning coefficient to estimate fish toxicity *via* series of linear regression models, while TEST uses a large number of structural and electrotopological properties to estimate acute fish toxicity *via* a number of statistical algorithms.<sup>19</sup> Another tool of interest, ADMET, developed by Simulation Plus,<sup>20</sup> relies on a range of chemical properties to estimate acute aquatic toxicity using neural networks. CADRE-Aquatic Toxicity (CADRE-AT) uses a small number of mechanistically-relevant reactivity and bioavailability parameters to predict a category of concern for both acute and chronic aquatic toxicity. CADRE-AT is an extension of a set of heuristic rules for molecular design of chemicals with minimal aquatic toxicity that are based on physicochemical properties and reactivity parameters.<sup>21–23</sup> In addition to assessment, CADRE-AT is aimed at helping chemists design (or re-design) compounds in order to minimize likelihood of high concern for aquatic toxicity. Unlike other tools in this evaluation, CADRE-AT is computationally intensive, requiring the use of high-performance computing clusters.<sup>23</sup>

To ensure model quality and regulatory relevance, the Organization for Economic Cooperation and Development (OECD) created a set of guidelines for model development that require external validation metrics, clear applicability domains, and mechanistic relevance to the modeled biochemical processes.<sup>24</sup> Despite these guidelines, lack of external validations and model performance outside the training sets remain a major concern.<sup>4,25,26</sup> While clear applicability domain definition ensures that the model assumptions are met and provides a measure of prediction confidence,<sup>24,27–29</sup> model overfitting and poor applicability domain definitions may lead to low external prediction accuracy in spite of the high accuracy in the model training set.<sup>29–32</sup> Previous validation efforts have suggested that model accuracy for a range of aquatic toxicity endpoints decreases during validation.<sup>18,33–37</sup> However, these studies either did not conduct a strictly external validation, relied on small data sets, or evaluated one tool at a time.

This study presents a systematic assessment of the widely used and recently developed software tools to predict acute aquatic toxicity to fish and provides insights into the applicability, accuracy and ease of use (*e.g.*, speed, convenience, and the level of expert knowledge required) of these models. Unlike prior research in the area, the test set used in this evaluation is distinct from the training sets of all evaluated tools. Thus, the assessment gives a common benchmark for model performance and further development. Since best practices in model development dictate that independent variables should

be empirically relevant to target endpoints,<sup>29</sup> special attention is given to chemical properties considered by each program and their relevance to the current understanding of fish toxicity modes of action (MOAs).

## Material and methods

### Validation dataset

Systematic and rigorous model evaluation requires reliable experimental data that was not used for model training.<sup>31</sup> As such, acute aquatic toxicity experimental thresholds (LC<sub>50</sub>) were extracted from the EPA ECOTOX database.<sup>38</sup> The data was filtered to eliminate compounds that were used in the training sets of the five *in silico* models being analyzed. Studies that did not meet the OECD testing guidelines were eliminated; data from OECD accepted freshwater fish species and any of the 4 accepted time points (48 h, 72 h, 96 h, 120 h) were considered. The 83 chemicals carried forward for analysis included aldehydes, halides, phenols, alcohols, ketones, aliphatic amines, amides, anilines, esters, and neutral organics functional chemical classes (Tables 1 and S1†). Chemical classes were identified by intrinsic, structural properties and subunits. The resulting classifications aims to reflect the types of chemical interaction and chemical function exerted from the structure.<sup>13</sup> Overall, the data showed high correlations between LC<sub>50</sub> measurements at different time intervals regardless of the test species (Fig. S1†). This observation is aligned with the most recent US EPA approach to aquatic toxicity prediction, which uses a single time interval and does not distinguish among freshwater fish species when predicting toxicity.<sup>13</sup>

Chemical categories were defined based on LC<sub>50</sub> values and EPA acute aquatic toxicity categories of concern.<sup>39</sup> Category 4 was added to distinguish chemicals with very low hazard potential. The four regulatory categories are: Category 1 – High hazard (LC<sub>50</sub> < 1 mg L<sup>-1</sup>), Category 2 – Moderate hazard (1 < LC<sub>50</sub> < 100 mg L<sup>-1</sup>), Category 3 – Low hazard (100 < LC<sub>50</sub> < 500 mg L<sup>-1</sup>) and Category 4 – No hazard (LC<sub>50</sub> > 500 mg L<sup>-1</sup>). Multiple LC<sub>50</sub> thresholds were available for 40 of the 83 chemicals in the data set with the distribution of differences between the minimum and the maximum LC<sub>50</sub> thresholds shown in Fig. S2.† When multiple experimental results for a single chemical were available, the geometric mean of the experimental LC<sub>50</sub> values was used because LC<sub>50</sub> values are typically log-normally distributed; under conditions of log-normality, geometric means are better estimators of centrality than arithmetic means.<sup>40</sup> Regardless of the geometric mean, the vast majority of experimental values resulted in classification of the chemical into the same EPA category of concern for aquatic toxicity with six substances having reported LC<sub>50</sub> values that spanned two categories. Experimental LC<sub>50</sub> values for an anticoagulant, Warfarin, span three regulatory categories, ranging from 0.037 mg L<sup>-1</sup> to >1000 mg L<sup>-1</sup>, and were independent of test duration. The complete data for these seven chemicals is given in Table S2.†





Table 1 Experimental and predicted toxicities for the 83 chemicals in the validation set

Chemical ID	Chemical class <sup>a</sup>	Experimental data		Predicted toxicity										
		LC <sub>50</sub> <sup>b</sup>	Cat	ADMET	CADRE-AT		ECOSAR		KATE		TEST			
Chemical name		LC <sub>50</sub> <sup>b</sup>	Cat	LC <sub>50</sub>	CP	Cat	CP	AD	CP	LC <sub>50</sub>	AD	CP	LC <sub>50</sub>	CP
(17beta)Estra-1,3,5(10)triene-3,17-diol	Phenols	1.55	2	0.797	No	2	Yes	Yes	Yes	1.58	Yes	Yes	8.35	No
5-Fluoro-2,4(1H,3H)-pyrionidione	Carbonyl Ureas	2420	4	1840	Yes	4	Yes	Yes	Yes	590	Yes	Yes	NA	NA
2-Bromo-2-nitro-1,3-propanediol	Halo Alcohols	27.6	2	660	No	2	Yes	Yes	No	778	Yes	No	3230	No
1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid	Pyrazoles/pyrroles-acid	81.9	2	0.761	No	2	Yes	Yes	No	0.878	Yes	No	2.32	No
(2S,5R,6R)-6-[[2R)-2-Amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]-heptane-2-carboxylic acid	Aliphatic amines-acid	1000	>4	17.9	No	3	No	Yes	Yes	1534	Yes	Yes	7920	No
Bromomethane	Neutral organics	1.82	2	699	No	2	Yes	Yes	No	429	Yes	No	220	No
Chloromethane	Neutral organics	550	4	639	Yes	4	Yes	Yes	No	274	Yes	No	194	No
Iodomethane	Neutral organics	1.26	2	391	No	2	Yes	Yes	No	272	Yes	No	185	No
Methanamine	Aliphatic amines	237	3	6349	Yes	3	Yes	Yes	Yes	323	Yes	Yes	NA	No
Ethanamine	Aliphatic amines	1000	4	1512	Yes	4	Yes	Yes	No	223	Yes	Yes	NA	Yes
Isopropyl amine	Aliphatic amines	1000	4	1056	Yes	4	Yes	Yes	No	155	Yes	No	NA	Yes
2-Methyloxirane	Epoxides, mono	215	3	2373	No	3	Yes	Yes	No	45.0	Yes	No	3.86	Yes
Acetone cyanohydrin	Nitrile alpha-OH	0.570	1	536	No	4	No	Yes	Yes	0.933	Yes	Yes	3450	No
Dimethyl sulfate	Esters	7.50	2	4119	No	2	Yes	Yes	No	200	Yes	No	1040	No
Triethyl phosphate	Esters (phosphate)	100	>3	582	Yes	4	Yes	Yes	No	9.83	Yes	No	1740	Yes
Propanoic acid	Neutral organics-acid	87.2	2	2739	No	2	Yes	No	No	11 521	Yes	No	1900	No
(2S)-2-Hydroxypropanoic acid	Neutral organics-acid	130	3	19 093	No	3	Yes	No	No	177 000	Yes	No	16.4	No
Camphene	Neutral organics	1.17	2	1.53	Yes	2	Yes	Yes	No	0.873	Yes	No	0.714	Yes
6,15-Dihydro-5,9,14,18-anthrazinetetrone	Neutral organics	46.0	2	0.026	No	2	Yes	No	No	0.003	No	No	NA	No
4-Hydroxy-3-(3-oxo-1-phenylbutyl)-2H-1-benzopyran-2-one	Vinyl/allyl alcohols	49.2	2	0.422	No	2	Yes	Yes	Yes	5.51	Yes	Yes	43.5	Yes
1,2-Benzenedicarboxylic acid	Neutral organics-acid	1000	>4	2079	Yes	3	No	No	Yes	9323	No	Yes	NA	No
o-Chlorobenzaldehyde	Aldehydes (mono)	2.62	2	5.62	Yes	1	No	Yes	Yes	5.24	Yes	Yes	5.75	Yes
2-Ethyl-1,3-hexanediol	Neutral organics	624	4	1187	Yes	4	Yes	Yes	No	275	Yes	Yes	580	Yes
2-Imidazolinedithione	Thioureas	502	>4	324	No	3	No	Yes	Yes	79 928	Yes	Yes	2790	No
Benzenesulfonyl chloride	Acid halides	3.00	2	3251	No	2	Yes	Yes	Yes	3.82	Yes	Yes	NA	Yes
alpha-Terpineol	Neutral organics	6.53	2	32.1	Yes	2	Yes	Yes	Yes	8.07	Yes	Yes	32.1	Yes
1-Chloro-4-nitrobenzene	Neutral organics	15.0	2	10.9	Yes	1	No	Yes	Yes	50.5	Yes	Yes	18.6	Yes
3-Phenyl-2-propenal	Vinyl/allyl aldehydes	4.64	2	1.07	Yes	2	Yes	Yes	No	0.201	Yes	Yes	8.74	Yes
1,2-Dibromoethane	Neutral organics	24.4	2	27.9	Yes	2	Yes	Yes	No	151	Yes	No	108	Yes
Butanoic acid	Neutral organics-acid	65.0	2	1405	No	2	Yes	No	No	4963	No	Yes	NA	No
2,5-Furandione	Neutral organics	138	3	3.18	No	3	Yes	No	Yes	177	No	Yes	NA	NA
N-(2-Aminoethyl)-1,2-ethanediamine	Aliphatic amines	1000	4	737	Yes	4	Yes	Yes	Yes	10 281	Yes	Yes	NA	Yes
1,2-Ethanediol, diacetate	Esters	90.0	2	40.4	Yes	2	Yes	Yes	No	167	Yes	No	297	No
1-Octene	Neutral organics	3.66	2	1.27	Yes	2	Yes	Yes	Yes	1.12	Yes	Yes	0.758	Yes
2-(2-Methoxyethoxy)ethanol	Neutral organics	2683	4	19 514	Yes	4	Yes	Yes	Yes	71 002	Yes	Yes	22 400	Yes
N,N,N-Trimethyl-1-hexadecanaminium, chloride	Neutral organics	0.158	1	0.924	Yes	1	Yes	Yes	No	22.7	Yes	No	NA	NA



Table 1 (Contd.)

Chemical ID	Chemical name	Chemical class <sup>a</sup>	Experimental data		Predicted toxicity						TEST						
			LC <sub>50</sub> <sup>b</sup>	Cat	ADMET	ECOSAR	CADRE-AT	KATE	AD	CP	AD	CP	LC <sub>50</sub>	CP			
1-Bromodecane		Neutral organics	18.7	2	0.295	No	2	Yes	0.107	No	No	Yes	No	0.087	No	0.420	No
2-(2-Butoxyethoxy)ethanol		Neutral organics	1300	4	1285	Yes	4	Yes	4555	Yes	Yes	Yes	Yes	2000	Yes	750	Yes
Benzoic acid, phenylmethyl ester		Esters	1.40	2	1.97	Yes	2	Yes	3.60	Yes	Yes	Yes	Yes	2.80	No	1.65	Yes
2-Octanol		Neutral organics	75.0	2	36.2	Yes	2	Yes	23.6	Yes	Yes	Yes	Yes	21.9	Yes	24.2	Yes
Decane		Neutral organics	530	>4	1.47	No	1	No	0.140	No	No	Yes	No	0.063	Yes	0.590	No
Methanesulfonyl chloride		Acid halides	11.0	2	38.841	No	2	Yes	16.1	Yes	Yes	Yes	No	11.8	No	98.3	Yes
<i>N,N</i> -Dimethyl acetamide		Amides	1000	4	2339	Yes	4	Yes	1558	Yes	Yes	Yes	Yes	NA	NA	1180	Yes
1-Aminonaphthalene		Amilines (unhindered)	7.00	2	6.96	Yes	2	Yes	13.6	Yes	Yes	Yes	Yes	NA	NA	13.9	Yes
Benzyl acetate		Esters	4.00	2	13.6	Yes	2	Yes	18.0	Yes	Yes	Yes	No	21.3	No	38.4	Yes
1 <i>H</i> -1,2,4-Triazole		Triazoles (non-fused)	498	3	331	Yes	3	Yes	3574	Yes	No	Yes	Yes	NA	NA	NA	NA
Cyanamide		Neutral organics	90.2	2	190	No	4	No	11.597	No	No	Yes	Yes	568	Yes	NA	NA
2-(1,3-Dihydro-3-oxo-2 <i>H</i> -indol-2-ylidene)-1,2-dihydro-3 <i>H</i> -indol-3-one		Vinyl/allyl ketones	42.0	2	2.54	Yes	1	No	37.7	Yes	Yes	Yes	Yes	NA	NA	0.200	No
<i>N</i> -[4-[Bis[4-(dimethylamino)phenyl]-methylene]-2,5-cyclohexadien-1-ylidene]- <i>N</i> -methylmethanaminium-chloride (1 : 1)		Neutral organics	0.100	1	0.107	Yes	1	Yes	2771	No	No	Yes	Yes	NA	NA	NA	NA
Nitroguanidine		Aliphatic amines	2268	>4	51.3	No	4	Yes	5563	Yes	Yes	Yes	Yes	55.900	No	NA	NA
2,5-Dichlorophenol		Phenols	3.30	2	12.3	Yes	2	Yes	6.96	Yes	Yes	Yes	Yes	5.42	Yes	5.93	Yes
Acetic acid, ammonium salt (1 : 1)		Neutral organics	72.0	2	NA	NA	2	Yes	1.27 × 10 <sup>6</sup>	No	No	Yes	Yes	NA	NA	NA	NA
2-Methoxy-2-methylbutane		Neutral organics	100	>3	315	Yes	4	Yes	99.0	Yes	No	Yes	Yes	167	Yes	403	Yes
Carbamic acid, monoammonium salt		Neutral organics	40.6	2	NA	NA	4	No	7.97 × 10 <sup>6</sup>	No	No	Yes	Yes	NA	NA	NA	NA
4-Chloro-2-methylphenol		Phenols	2.30	2	15.7	Yes	2	Yes	7.22	Yes	Yes	Yes	Yes	7.54	Yes	8.53	Yes
2-(1,1-Dimethylethyl)-1,4-benzenedio		Hydroquinones	0.274	1	10.8	No	1	Yes	0.099	Yes	Yes	Yes	Yes	NA	NA	21.0	No
2-(2,4-Dichlorophenoxy)acetic acid compd. with		Neutral organics	312	3	NA	NA	3	Yes	2427	No	No	Yes	Yes	NA	NA	NA	NA
<i>N</i> -methylmethanamine (1 : 1)		Neutral organics	0.154	1	0.510	Yes	NA	NA	0.180	No	Yes	Yes	Yes	0.218	No	1.10	No
2-[4-(1,1-Dimethylethyl)phenoxy]cyclohexyl-2-propynyl ester sulfurous acid		Aliphatic amines	1.01	2	0.078	No	2	Yes	1.39	Yes	Yes	Yes	Yes	0.586	Yes	NA	NA
<i>N</i> -(3-aminopropyl)- <i>N</i> 1-dodecyl-1,3-propanediamine		Aliphatic amines	0.012	1	107	No	NA	NA	627	Yes	No	Yes	Yes	NA	NA	NA	NA
Tanone 50		Neutral organics	12.0	2	7.45	Yes	2	Yes	16.2	Yes	Yes	Yes	Yes	8.10	Yes	7.82	Yes
1,2-Dichloro-3-nitrobenzene		Neutral organics	0.460	>1	NA	NA	1	Yes	NA	NA	NA	Yes	Yes	NA	NA	NA	NA
5-Chloro-2-(4-chlorophenoxy)-phenol		Neutral organics	2.85	2	1.96	Yes	2	Yes	8.95	Yes	Yes	Yes	Yes	3.13	No	2.37	Yes
2-(Octylthio)ethanol		Amides	40.2	2	0.002	No	NA	NA	0.027	No	No	Yes	Yes	NA	NA	NA	NA
CI pigment yellow 83		Carbonyl ureas	298	3	11.660	No	3	Yes	6331	Yes	No	Yes	Yes	2970	Yes	6890	No
1,3-Bis(hydroxymethyl)-5,5-dimethyl-2,4-imidazolidinedione		Neutral Organics	0.750	1	0.0004	Yes	1	Yes	1.22	Yes	No	Yes	Yes	4.57 × 10 <sup>-6</sup>	No	NA	NA
<i>N</i> -Decyl- <i>N,N</i> -dimethyl-1-decanaminium chloride (1 : 1)		Neutral organics	1.10	2	0.112	No	4	No	1.43 × 10 <sup>6</sup>	No	No	Yes	Yes	NA	NA	NA	NA
Dimethyldiallylammonium chloride		Neutral organics	1.10	2	0.112	No	4	No	1.43 × 10 <sup>6</sup>	No	No	Yes	Yes	NA	NA	NA	NA



Table 1 (Contd.)

Chemical ID	Chemical name	Chemical class <sup>a</sup>	Experimental data		Predicted toxicity												
			LC <sub>50</sub> <sup>b</sup>	Cat	ADMET	CP	Cat	CP	AD	CP	AD	CP	KATE	LC <sub>50</sub>	AD	CP	TEST
	7a-Ethyl(dihydro-1 <i>H</i> ,3 <i>H</i> ,5 <i>H</i> -oxazol-3,4- <i>c</i> )oxazole	Aliphatic amines	169	3	1555	No	3	Yes	308	Yes	Yes	Yes	73.2	Yes	No	2410	No
	1-(1-Butoxypropan-2-yloxy)propan-2-ol	Neutral organics	50.0	2	2905	No	2	Yes	45 354	Yes	No	7410	Yes	No	NA	NA	
	alpha-(Nonylphenyl)-omega-hydroxy(oxy-1,2-ethanediy) <i>N,N'</i> -1,2-Ethanediybis <i>N</i> -acetylacetamide	Neutral organics	5.54	2	2.63	Yes	2	Yes	2.15	No	Yes	0.181	Yes	No	NA	NA	
	<i>N,N'</i> -1,2-Ethanediybis <i>N</i> -acetylacetamide	Imides	140	>3	52.0	No	4	Yes	1911	Yes	Yes	1820	Yes	Yes	NA	NA	
	1,3-Dichloro-2-propanol phosphate (3 : 1)	Esters (phosphate)	3.60	2	2.57	Yes	2	Yes	2.47	Yes	Yes	902	No	No	0.650	No	
	<i>N,N</i> -Dimethyldecanamide	Amides	21.0	2	11.8	Yes	2	Yes	5.53	Yes	Yes	6.86	Yes	Yes	NA	NA	
	alpha-Methyl-4-(2-methylpropyl)-benzeneacetic acid	Neutral organics-acid	100	>3	28.6	No	3	Yes	41.6	No	No	19.4	Yes	No	2.55	No	
	(2 <i>R</i> )-2-(4-Chloro-2-methylphenoxy)propanoic acid	Neutral organics-acid	97.0	>2	38.5	Yes	3	Yes	254	No	Yes	10.4	No	Yes	21.0	Yes	
	Polypropylene glycol	Neutral organics	4123	4	9253	Yes	4	Yes	24 424	Yes	Yes	19 000	Yes	Yes	NA	NA	
	[2 <i>S</i> ]-[2alpha,5alpha,6beta(S*)]-6-[[Amino(4-hydroxyphenyl)acetyl]-amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid	Phenol amines -acid	1000	>4	38.5	No	3	No	370	Yes	No	20 400	No	Yes	2.27	No	
	Tetakis(hydroxymethyl) phosphonium, sulfate(2 : 1)(salt)	Neutral organics	95.5	2	1.03 × 10 <sup>6</sup>	No	4	No	4.26 × 10 <sup>22</sup>	No	No	NA	NA	NA	NA	NA	
	5-[2-Chloro-4-(trifluoromethyl)-phenoxy]-2-nitrobenzoic acid, 2-ethoxy-1-methyl-2-oxoethyl ester	Esters	0.528	1	0.061	Yes	2	Yes	1.22	No	No	0.923	No	Yes	0.028	Yes	
	<i>N</i> -[1,3-Bis(hydroxymethyl)-2,5-dioxo-4-imidazolidinyl]- <i>N,N'</i> -bis(hydroxymethyl)urea	Carbonyl ureas	150	>3	9006	Yes	4	Yes	6.70 × 10 <sup>6</sup>	Yes	Yes	9240	Yes	Yes	2760	Yes	
	2-[[5-Chloro-8-quinoliny]oxy]acetic acid, 1-methylhexyl ester	Esters	13.3	>2	0.809	No	2	Yes	0.544	No	No	0.502	No	No	0.630	No	
	2-Chloro-5-[3,6-dihydro-3-methyl-2,6-dioxo-4-(trifluoromethyl)-1(2 <i>H</i> )-pyrimidinyl]benzoic acid 1,1-dimethyl-2-oxo-2-(2-propenyloxy)ethyl ester	NA	6.02	2	NA	NA	2	Yes	NA	NA	NA	NA	NA	NA	0.095	No	
	1-(2,4-Dichlorophenyl)-4,5-dihydro-5-methyl-1 <i>H</i> -pyrazole-3,5-dicarboxylic acid 3,5-diethyl ester	Esters	4.20	2	0.196	No	1	No	1.13	Yes	Yes	1.97	No	Yes	0.370	No	

<sup>a</sup> Chemical class as identified by ECOSAR tool; Cat - regulatory category (1-4); AD - indicated whether the chemical is in the applicability domain; CP? - indicates correct and incorrect predictions based on regulatory categories; NA - not available. <sup>b</sup> Notes about the LC<sub>50</sub> measurements.

## Partition coefficients

All toxicity prediction tools evaluated in this study rely on octanol-water partition coefficient ( $\log P$ ) or octanol-water distribution coefficient ( $\log D$ ) at a particular pH to assess chemical toxicity to fish.  $\log P$  is commonly used to predict absorption and toxicity of narcotics to aquatic organisms.<sup>41–43</sup> In practice,  $\log P$  can be calculated with atom-based,<sup>44–46</sup> fragment-based,<sup>47</sup> whole molecule prediction methods<sup>48,49</sup> or more recently, from nuclear magnetic resonance spectra.<sup>50</sup> Most of these  $\log P$  estimation methods are based on neutral molecular species only. While both ionized and non-ionized forms are expected to contribute to absorption and toxicity,<sup>51–54</sup> the apparent partition coefficient of the ionic forms is expected to be at least three orders of magnitude smaller than that of the non-ionized forms<sup>55</sup> due to the differences in solute–solvent interactions and absorption kinetics.<sup>56,57</sup> Consequently,  $\log D$ , rather than  $\log P$ , reflects the contribution of all ionized and unionized species at a given pH, providing a more meaningful estimate of bioavailability.<sup>58</sup> In order to assess potential errors in partitioning estimates due to lack of ionization considerations, the  $\log P$  values estimated by each software tool were compared with corresponding  $\log D$  values, calculated at the biologically-relevant pH of 7.4, using Chem Axon's Marvin suite.<sup>59</sup> Compounds with differences between  $\log P$  and  $\log D_{7.4}$  estimates greater than one log unit were identified with  $\log P$  warnings.

## Predictive tools

The following five *in silico* tools were evaluated for predicting acute aquatic toxicity to fish: ADMET Predictor, CADRE-AT, ECOSAR, KATE and TEST. Brief description of each program is provided below while the pertinent details are summarized in Table 2.

**ADMET predictor™** estimates acute fish toxicity using two-dimensional (2D) Artificial Neural Network Ensemble (ANNE). Although only limited details for the ANNE are available due to proprietary nature of the algorithm, it is known that the

model relies on hundreds of structural, constitutional, topological, and electronic properties as descriptors. Two estimates of  $\log P$  are available – one based on the internal ANNE model, and another based on the atom fragment contribution (AFC) method outlined elsewhere.<sup>46</sup>  $\log D_{7.4}$  is calculated with the ANNE method trained on ionizable compounds.<sup>20</sup> ADMET is trained on fathead minnow data available from US EPA.<sup>60</sup> The program requires SMILES strings or 3D structure files as inputs to provide estimates of  $LC_{50}$  values and can process multiple substances in batch mode. ADMET generates predictions only for compounds that fall within its applicability domain, which is assessed automatically on the basis of descriptor space in the training set.

**CADRE-AT** uses a series of classification models to bin chemicals into categories of concern for acute and chronic aquatic toxicity. The models are based on mechanistically-relevant bioavailability and reactivity parameters that include distribution coefficient ( $\log D_{7.4}$ ), global quantum-mechanical reactivity indices and other physicochemical descriptors. Reactivity indices include frontier orbital energies, such as lowest unoccupied molecular orbital (LUMO), the highest occupied molecular orbital (HOMO) and the HOMO–LUMO energy gap ( $\Delta E$ ). These parameters are reflective of non-specific chemical reactivity with macromolecules.<sup>23,61</sup> Like ADMET, CADRE-AT was trained on the fathead minnow data available from US EPA.<sup>60</sup> Since descriptors are calculated at a high level of theory and require 3D chemical structures as inputs, CADRE-AT does not provide instantaneous predictions; typical processing times range from seconds to a few days per chemical, depending on the size and conformational flexibility of the structure (s) involved. CADRE-AT does not have an applicability domain and provides predictions for all organic chemicals that are amenable to the required computations.

**Ecological Structure Activity Relationships (ECOSAR) v1.11** estimates acute aquatic toxicity *via* the Mayer–Overton relationship for chemicals within a structurally similar class.<sup>13,62</sup> ECOSAR is trained on a large data set of Ecotoxicity studies from the ECOTOX database that follow OCSPP guidelines.<sup>15</sup> The database is divided into 111 structural classes, and linear

**Table 2** Predictive tool summary

	ADMET	CADRE-AT	ECOSAR	KATE	TEST
Free-ware?	No	No	Yes	Yes	Yes
Statistical method	2D ANNE	Classification system	Class-specific linear regression	Class-specific linear regression	Consensus model
AD definition	Molecular descriptor space	Molecular descriptor space	$\log P$ range and class categorization concerns	$\log P$ range and class categorization concerns	Molecular descriptor space
Training set size	490	565	1000s <sup>a</sup>	535	823
Training set species	Pimephales promelas	Pimephales promelas	All OCSPP approved species	Oryzias latipes, pimephales promelas	Pimephales promelas
Output	$LC_{50}$	Toxicity category ( $n = 4$ )	$LC_{50}$	$LC_{50}$	$LC_{50}$
# of chemicals in the AD <sup>b</sup>	78	80	61	35	57

<sup>a</sup> The exact number of compounds is not available. <sup>b</sup> Number of chemicals in the validation set ( $N = 83$ ) that are in the AD of each model. OCSPP – office of chemical safety and pollution prevention; 2D ANNE – two-dimensional artificial neural network ensemble. AD – applicability domain.



regression models between LC<sub>50</sub> toxicity estimates and log *P* were developed for substances in each class. When chemicals belong to multiple chemical classes the most conservative (most toxic) estimate is provided based on the principle of excess toxicity. Log *P* is calculated with the EPISUITE KOWWIN module v. 1.68 using the AFC method.<sup>63,64</sup> The KOWWIN module evaluates partitioning of neutral compounds only; thus, toxicity of organic acids and bases is estimated based on QSARs for non-ionized molecules of the same class. The program requires SMILES strings or CAS numbers as inputs to estimate LC<sub>50</sub> thresholds and can process multiple substances in batch mode. ECOSAR is designed to perform best on compounds with log *P* < 5 and molecular weight < 1000 amu.<sup>13,62</sup> Chemicals that do not meet the latter two criteria, or are structurally dissimilar from the domain of every QSAR model within ECOSAR, are considered outside the applicability domain.

**KAshinhou Tool for Ecotoxicity (KATE) on PAS 2011** estimates acute aquatic toxicity *via* Mayer–Overton relationship for chemicals within a structurally similar class, akin to ECOSAR. Forty structural chemical classes are used in KATE. Estimated LC<sub>50</sub> values are determined from linear regression models that use log *P*, which is obtained from an internal experimental database or is estimated with the AFC method.<sup>63,64</sup> KATE is trained on the US EPA fathead minnow (*Pimephales promelas*) and the Japanese Ministry of Environment *Oryzias latipes* datasets.<sup>65,66</sup> The program requires SMILES strings or CAS numbers as inputs to assess chemical toxicity and can process multiple substances in a single run with batch mode. The tool is available as a standalone application or as a web plug-in. The batch mode size is limited to 50 chemicals. KATE internally defines the applicability domains by comparing the log *P* of the test chemical to the range of log *P* values in each of the structural classes of the training set.<sup>18</sup>

**Toxicity Estimation Software Tool (TEST) v.4.1** consists of a number of models that estimate acute aquatic toxicity thresholds by read-across among structural analogs or *via* multivariate regression. The models are based on hundreds of structural, constitutional, connectivity, shape, topological, molecular distance, fragments, and electrotopological property descriptors. Several partition coefficient estimates are provided. Log *P* is calculated with two group contribution methods derived by Ghose<sup>45</sup> or Wang.<sup>67</sup> TEST is trained on Fathead minnow dataset from the EPA ECOTOX database.<sup>38,65</sup> The program requires only SMILES strings or CAS numbers as inputs to quickly assess chemical toxicity and can process multiple substances in a single run with batch mode. Each read-across or regression model has specific applicability domain. The program provides estimated LC<sub>50</sub> threshold based on each model's prediction, as well as a consensus average of the component models. Given that the consensus result was previously reported as the most accurate estimate provided by TEST,<sup>68</sup> it was used in this validation exercise.

### Structural alerts and modes of action

Model performance often varies between chemicals acting by different modes of action (MOAs). To evaluate model perform-

ance within common aquatic toxicity MOA, possible MOAs for chemicals in the test set were identified using the Verhaar scheme<sup>69–71</sup> coded in ToxTree v.2.6.13.<sup>72</sup> Briefly, the Verhaar classification scheme uses a series of structural alerts derived from chemicals with known toxic modes of action to sort organic chemicals into five categories according to the mechanism of toxicity. The five classes are Class 1 (inert chemicals or non-polar narcotics), Class 2 (less inert chemicals or polar narcotics), Class 3 (reactive chemicals), Class 4 (specifically acting chemicals), and Class 5 (not classifiable chemicals).<sup>70,71</sup>

### Statistical analysis

The R language and environment for statistical computing<sup>73</sup> (version 3.1.2) was used for data management, analysis, and performance metric valuation. A number of summary statistics were calculated to compare model performance and included both classification and regression model results whenever possible to provide the most comprehensive information for risk assessment.<sup>74</sup> Using logarithmically transformed measured and predicted LC<sub>50</sub> values, the root of mean squared error (RMSE), correlation coefficient (*R*<sup>2</sup>), total categorical accuracy, and predictive power that penalizes programs for predicting the wrong or for not predicting any toxicity category were determined. The percentage of substances with differences between predicted and measured LC<sub>50</sub> values within factors of 2, 5, 10, 100, and 1000, were also calculated for consistency with other studies. Failed predictions are defined as cases where no toxicity estimate was afforded by the model.

## Results

### Chemical diversity of external validation dataset

The 83 chemicals assessed represent a diverse array of commercial substances that includes aldehydes, halides, phenols, alcohols, ketones, amines, amides, esters, and carboxylic acids (Tables 1 and S1†). To test for structural similarity, structure clustering analysis was performed with the Marvin MSC software<sup>59</sup> and no large clusters were revealed. The three largest clusters were surfactant-like hydrocarbons (*n* = 6), chlorinated benzo ethers (*n* = 4), and benzo esters (*n* = 4). Additional cluster details can be found in Table S1.†

### Statistical distribution of experimental values

The experimental LC<sub>50</sub> thresholds of the 83 chemicals are approximately normally distributed and cover all regulatory categories (Fig. 1). Fig. 2A–C shows the relationships between experimental toxicity estimates and mechanistically relevant chemical parameters in the data set. The parameters include log *D*<sub>7,4</sub>, molecular volume, and HOMO–LUMO energy gap ( $\Delta E$ ). Consistent with prior work,<sup>21,29,41,75</sup> it is anticipated that log *D*<sub>7,4</sub> and molecular volume of the validation data set compounds show direct univariate relations to increased acute lethality (Fig. 2A and C), while  $\Delta E$  shows an inverse relationship (Fig. 2B).



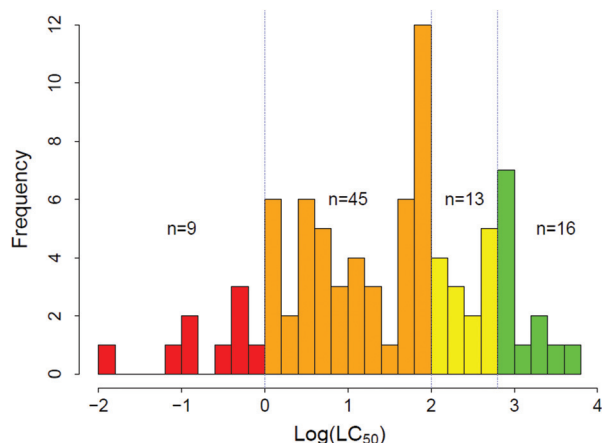


Fig. 1 The distribution of  $\log(LC_{50})$  thresholds ( $\text{mg L}^{-1}$ ).

### Model accuracy across the entire test set

The performance metrics for all programs tested in this evaluation are summarized in Table 3 and Fig. 2A–C. Total accuracy measures the fraction of chemicals correctly placed into regulatory categories, with missing prediction discounted in the analysis. Predictive power reflects an ability to assess the toxicity category of any given chemical, *i.e.* failed predictions are penalized in the computed score as previously discussed (Methods). Model performance is first evaluated on the entire data set regardless of the applicability domain (AD) to assess the tool utility for any new or existing chemical. Based on predictive power of classification into the four toxicity categories of the entire data set, the tested tools can be ranked in the following order from highest- to lowest-performers: CADRE-AT > ECOSAR = Admet > KATE > TEST. KATE and TEST failed to predict  $LC_{50}$  values for 26 and 23 of the 83 compounds, respectively. For the 67 chemicals for which KATE is able to provide an  $LC_{50}$  estimate, the total accuracy is relatively high (58%). However, owing to number of failed predictions, the

predictive power is much lower at 40%. Both total accuracy and predictive power of TEST are lower – 48% and 35%, respectively. ADMET, CADRE-AT, and ECOSAR, on the other hand, failed to yield predictions for only 5, 3, and 2 chemicals respectively. Among the toxicity prediction tools that estimate  $LC_{50}$  values (ADMET, ECOSAR, KATE, TEST), little difference is observed in the distribution of errors, as evidenced by percentage of predictions that fall within a factor 2, 5, 10, 100, and 1000 of the measured  $LC_{50}$  and RMSE (Table 3).

### Applicability domains

Robust and relevant applicability domain (AD) definition is essential for model performance.<sup>29</sup> ECOSAR and KATE readily generate toxicity estimates for chemicals outside their applicability domains. To gauge any changes in performance, chemicals solely within the applicability domain (AD) of these two tools were considered in a separate analysis (Table 4). For ECOSAR, the AD is defined in the user manual by structural domain alerts previously discussed in Methods and Table 2. The total accuracy of ECOSAR increases from 51 to 61% when the analysis is limited to chemicals within its AD; 59 of the 83 tested chemicals are within the ECOSAR's AD. The AD for KATE is defined entirely by the program alerts. KATE's total accuracy decreased from 58% to 46% when analysis is limited to the 35 (of the 83) compounds within its AD. As discussed above, little to no difference between the programs is observed in the distributed of errors (Table 3). However, ECOSAR AD definition identifies the large outliers in toxicity prediction. In addition, owing to the transparent nature of the ECOSAR algorithm, other large outliers in ECOSAR  $LC_{50}$  predictions can be attributed to substances for which the internal KOWWIN  $\log P$  estimates are substantially lower than the  $\log D_{7.4}$  estimates (Fig. 3 and 4). These compounds are flagged with  $\log P$  warnings. The large outliers explain the lowest coefficient of determination ( $R^2$ ) and the largest RMSE of  $LC_{50}$  thresholds estimated by ECOSAR (Table 3, Fig. 3). As parametric

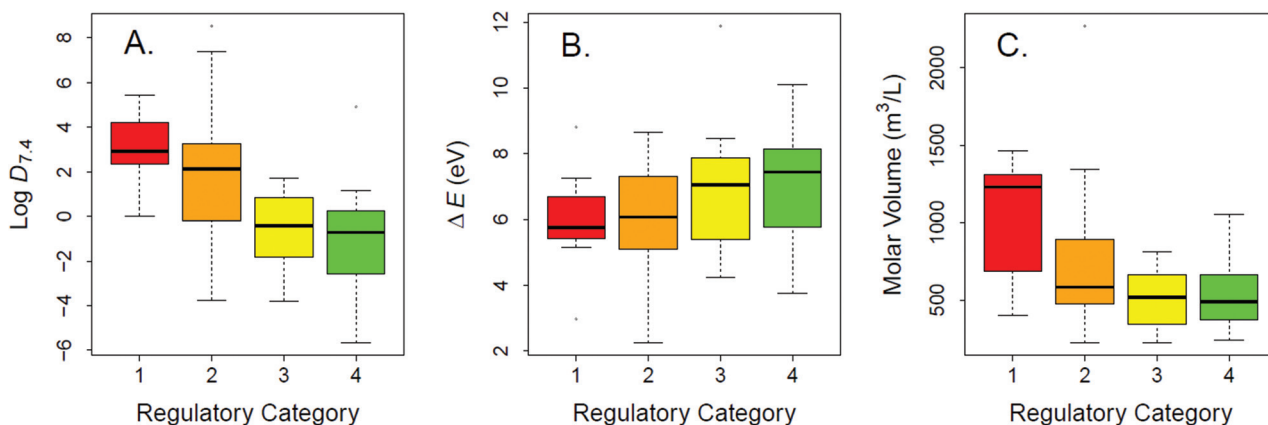


Fig. 2 Boxplot of properties mechanistically related to acute aquatic toxicity by acute aquatic toxicity category of concern: (A)  $\log D_{7.4}$ ; (B) energy gap between the highest occupied (HOMO) and the lowest unoccupied (LUMO) molecular orbitals, ( $\Delta E$ ); (C) molecular volume in the test data set. The four regulatory categories high ( $LC_{50} < 1 \text{ mg L}^{-1}$ ), medium ( $1 < LC_{50} < 100 \text{ mg L}^{-1}$ ), low ( $100 < LC_{50} < 500 \text{ mg L}^{-1}$ ), and no hazard ( $LC_{50} > 500 \text{ mg L}^{-1}$ ) are highlighted in red, orange, yellow, and green, respectively.





**Table 3** Tool performance and comparison summary statistics based on all 83 chemicals in the testing data set

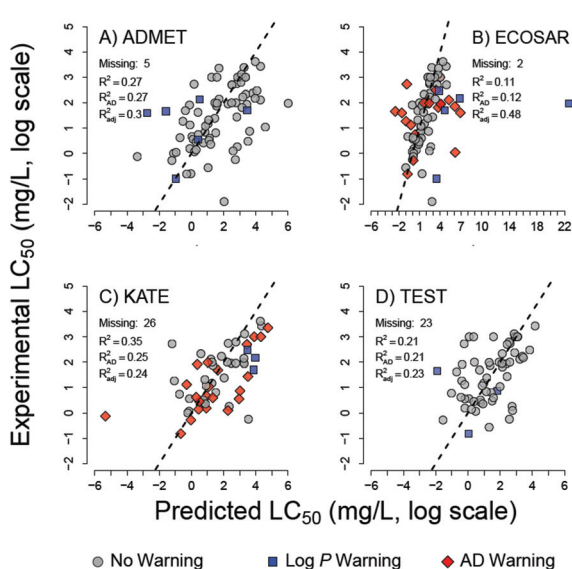
Measures of predictive accuracy	ADMET	CADRE-AT	ECOSAR	KATE	TEST
Total accuracy (%) <sup>a</sup>	53%	83%	51%	58%	48%
Predictive power (%) <sup>b</sup>	49%	80%	49%	40%	35%
Number of missing predictions	5	3	2	26	23
Coefficient of variance ( $R^2$ )	0.27	NA <sup>c</sup>	0.11 <sup>d</sup>	0.35	0.21
RMSE (log scale)	1.60	NA <sup>c</sup>	2.94 <sup>d</sup>	1.47	1.32
% within 1 regulatory category	80.8	92.5	85.2	85.5	88.3
% within a factor of 2 (%)	25.6	NA <sup>c</sup>	25.9	26.3	30.0
% within a factor of 5 (%)	48.7	NA <sup>c</sup>	54.3	47.4	50.0
% within a factor of 10 (%)	57.7	NA <sup>c</sup>	63.0	64.9	63.3
% within a factor of 100 (%)	80.8	NA <sup>c</sup>	76.5	82.5	85.0
% within a factor of 1000 (%)	91.0	NA <sup>c</sup>	86.4	94.7	98.3

<sup>a</sup>Total accuracy is the fraction of chemicals assessed by each tool for which the predicted LC<sub>50</sub> falls within the same regulatory category as the measured LC<sub>50</sub>. <sup>b</sup>Similar to total accuracy, predictive power measures the total number of correct category assignments. However, lack of prediction is treated as an incorrect assignment. <sup>c</sup>Cannot be calculated; software tool provides regulatory category designation only. <sup>d</sup>Parametric correlation might provide poor estimate of covariance due to extreme outliers. RMSE – root mean squared error.

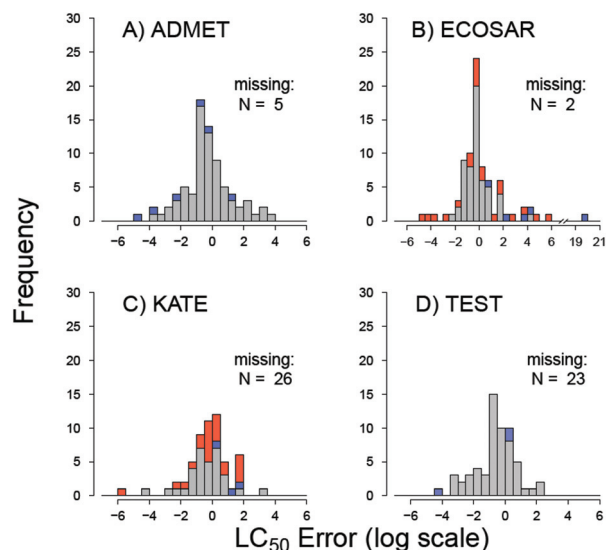
**Table 4** Model performance for chemicals within the tools' applicability domains

Measures of predictive accuracy	ADMET	CADRE-AT	ECOSAR <sup>a</sup>	KATE <sup>a</sup>	TEST
Total accuracy inside AD (%) <sup>b*</sup>	53%	83%	61%	46%	48%
Coefficient of variance ( $R^2$ )	0.27	NA <sup>c</sup>	0.13 <sup>c</sup>	0.25	0.21
RMSE (log scale)	1.60	NA <sup>c</sup>	1.29	1.35	1.32
Number of chemicals (out of 83) <sup>d</sup>	78	80	59	35	57

<sup>a</sup>This tool provides predictions when chemicals lie outside the applicability domain (AD). <sup>b</sup>Total accuracy within the AD is the fraction of chemicals assessed by each tool for which the predicted LC<sub>50</sub> falls within the same regulatory category as the measured LC<sub>50</sub>. The chemicals with AD warnings are excluded from the assessment. <sup>c</sup>Cannot be calculated; the tool provides regulatory category designation only. <sup>d</sup>Number chemicals for which the tool provided toxicity estimates that are also within the tool's AD. <sup>e</sup>Parametric correlation might provide poor estimate of covariance due to extreme outliers.



**Fig. 3** Correlations between predicted and experimental LC<sub>50</sub> values on log for (A) ADMET predictor, (B) ECOSAR, (C) KATE, and (D) TEST. Red: chemicals that lie outside the AD; blue: log *P* estimates used by the tool are >1 log unit below Marvin log *D*<sub>7.4</sub> estimates.  $R^2_{AD}$ : coefficient of determination for chemicals inside AD;  $R^2_{ADj}$  is the coefficient of determination for chemicals inside the AD and without log *P* warnings.



**Fig. 4** Distribution of prediction errors (predicted – experimental) in log LC<sub>50</sub> units for (A) ADMET predictor, (B) ECOSAR, (C) KATE, (D) TEST. Positive errors indicate predicted LC<sub>50</sub> above experimental LC<sub>50</sub> and toxicity underestimation. Red: “AD warning,” show the errors for chemicals that lie outside applicability domain (KATE and ECOSAR only); blue: “log *P* warning,” log *P* estimates are >1 log unit below Marvin log *D*<sub>7.4</sub> estimates.



**Table 5** Prediction accuracy by mode of action identified by Verhaar scheme (52 chemicals could not be classified)

Measures of predictive accuracy	ADMET	CADRE-AT	ECOSAR	KATE	TEST
Total accuracy – narcotics (total: 24)	68%	88%	52%	50%	71%
Missing prediction – narcotics	2/24	0/24	1/24	4/24	7/24
Total accuracy – neutral narcotics ( $\log P < 5$ )	87%	94%	53%	57%	77%
Missing prediction – neutral narcotics ( $\log P < 5$ )	1/17	0/17	1/17	2/17	3/17
Total accuracy – reactive chemicals (total: 6)	33%	67%	50%	40%	50%
Missing prediction – reactive chemicals	0/6	0/6	0/6	1/6	2/6

estimates,  $R^2$  and RMSE are poor measures of model fit when large outliers are present.

No explicit AD analyses for ADMET, CADRE-AT, and TEST were performed because the programs do not allow for predictions outside their respective ADs (ADMET, TEST) or lack applicability domain definitions (CADRE-AT). A narrow AD definition may decrease model performance due to large fraction of missed prediction and low predictive power. Furthermore, models trained on small data sets with narrow ADs may be overfitted, resulting in a poor accuracy during evaluation. Among evaluated tools that estimate  $LC_{50}$  values, ECOSAR showed the narrowest error distribution when analysis was limited to chemicals within its AD (Table 4).

### Structural alerts for modes of action

The majority of compounds ( $n = 52$ ) in the training set could not be classified into MOAs by the modified Verhaar scheme,<sup>70,71</sup> briefly discussed in Methods. Of the remaining 31 compounds, 24 were identified as narcotics, 6 as generally reactive, and 1 as specifically reactive. Chemicals designated as narcotics included ionized substances and compounds with  $\log P > 5$ . Since many of the charged and poorly soluble chemicals are outside the applicability domains of the tested tools (Table S1†), prediction accuracy was evaluated with and without these compounds (Table 5). Based on total accuracy for neutral chemicals with  $\log P < 5$  designated as narcotics, the tools performed in the following order: CADRE-AT > Admet > TEST > KATE > ECOSAR. All programs showed decreased performance on generally reactive chemicals ( $n = 6$ ).

## Discussion

Chemicals in the aquatic environment must pass biological membranes in order to interact with biological targets, propagating a cascade of biochemical events that leads to acute lethality.<sup>76,77</sup> Mechanistic insights into the bioavailability, distribution, and the nature of interactions between the chemical and its biological target that can be reflected in the predictive models are critical for improving model performance and interpretability.<sup>78–80</sup>

The majority of chemicals impact toxicity through non-specific, reversible interactions with biological membranes, known as narcosis.<sup>81–83</sup> Chemicals that primarily act *via* narcosis include aliphatic and aromatic hydrocarbons, chlorinated hydrocarbons, alcohols, ethers, ketones, aldehydes, weak acids

and bases, and some aliphatic nitro compounds.<sup>84–86</sup> Narcotics have been shown to exert toxic effects on fish at constant target tissue concentrations on the order of 220–470  $\text{mmol kg}^{-1}$  of lipids.<sup>87</sup> Thus, the toxicity of a narcotic is related to its ability to partition across the gill and target membranes and intercalate between the lipid bilayer. Conventionally, this process has been modeled using  $\log P$ , which provides an estimate of the degree of partitioning across the membranes and the affinity for the hydrophobic region of the lipid bilayer.<sup>81,88–94</sup>

KATE and ECOSAR predict aquatic toxicity based on single predictor ( $\log P$ ), which has been shown to be mechanistically relevant to compounds acting solely by narcosis.<sup>86</sup> Their respective algorithms assume that although toxicants with particular functional groups induce toxicity in excess of that estimated by  $\log P$ , the “excess” toxicity is constant factor for each category and can be adjusted with an appropriate class-specific correction factor.<sup>13,18</sup> Surprisingly, KATE and ECOSAR afford lower accuracy than the other three tools for assessing toxicity categories of chemicals identified as narcotics by the Verhaar scheme (Table 5). The results merit further investigation of the models and the Verhaar classification scheme. It is likely that errors in estimates of  $\log P$  by ECOSAR and KATE lead to errors in toxicity estimates. Indeed, underestimation of  $\log P$  by ECOSAR is directly related to its underestimation of toxicity (overestimating  $LC_{50}$ ) in the set of five chemicals (Fig. 5). The five chemicals are Crystal Violet dye (CAS# 548-62-9), DMDM Hydantoin (CAS# 6440-58-0), Dowanol 54B (CAS# 78491-02-8), and Butafenacil (CAS# 134605-64-4) (Table S1†). On the other hand, overestimated  $\log P$  values did not lead to proportionally overestimated  $LC_{50}$  results. These differences may arise from the differences in QSAR equations used to predict excess toxicity by ECOSAR; *i.e.* toxicity above prediction afforded by the baseline octanol-water partitioning equation for neutral organic narcotics.<sup>13</sup> Furthermore, these differences in ECOSAR and KATE may also be attributed to unstable regression models, as the QSARs for some chemical classes are based on as few as 2 data points (diazonium aromatics).<sup>13,31</sup> Previous study on an older ECOSAR version found that 22% of the QSAR equations in the tool were “reliable”.<sup>95</sup> In these cases, the tools’ performance would likely be improved if training set chemicals were partitioned into QSAR models by MOA rather than by chemical class.<sup>65,96</sup> Further work to test this hypothesis is ongoing. It should be noted that a significant portion of chemicals with large toxicity errors from predictions by KATE and



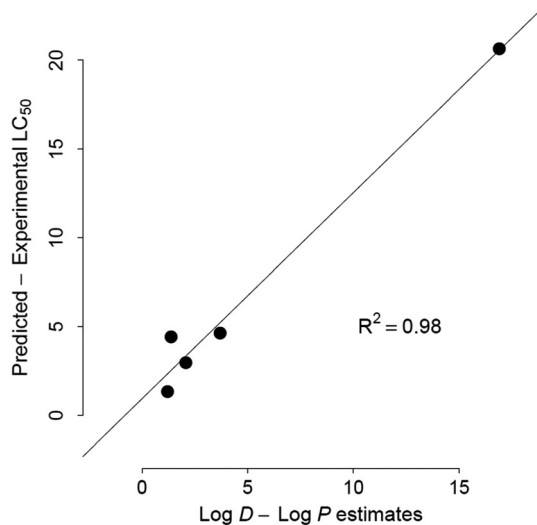


Fig. 5 Relationship between underestimated bioavailability, ( $\log P - \log D_{7.4}$ ) and acute toxicity thresholds for five chemicals with KOWWIN  $\log P$  estimates >one order of magnitude below Marvin  $\log D_{7.4}$ . ( $R^2 = 0.98$ ).

ECOSAR had  $\log P$  warnings, which should alert the user to discount the accuracy of prediction for those chemicals (Fig. 4).

Partitioning coefficients alone are poor predictors of acute aquatic toxicity for chemicals acting through specific interactions with biological macromolecules.<sup>97</sup> Such interactions include covalent reactivity with protein residues and nucleic acids, non-covalent binding to enzymes and receptors (e.g. acetylcholinesterase and estrogen receptors), oxidative phosphorylation uncoupling, and central nervous system stress.<sup>69,98,99</sup> Predictive toxicology models can be improved by considering chemical properties mechanistically relevant to these interactions, such as reactivity and steric parameters.<sup>100–103</sup> CADRE-AT uses global reactivity indices (such as frontier orbital energies) and physicochemical properties (such as  $\log D_{7.4}$ , molecular volume and accessible surface area) to bin chemicals into EPA's categories of concern. To this end, the higher performance and greater applicability domain (uniform predictive accuracy for soluble,

insoluble, charged and neutral compounds – Table 6) noted for CADRE-AT likely stem from the closer mechanistic relevance of its descriptors.<sup>23</sup>

TEST and ADMET rely on machine learning and consensus models with a diverse array of molecular predictors. Thus, it is not possible to ascertain the mechanistic relevance of the molecular parameters, other than partition coefficients, to MOAs. ADMET ANNE method outperforms the TEST consensus algorithm in accuracy (52% vs. 48%, respectively) and provides estimates for a wider range of compounds (5 vs. 23 missing predictions, respectively). However, due to high number of predictors and more complex statistical algorithms as it is nearly impossible to identify sources of misclassification.

### Structural alerts for modes of action

The two property-based chemical categories present a particular challenge to toxicity estimation are: (1) compounds with  $\log P$  values > 5 and (2) compounds that are ionizable at biological pH. All software tools, with the exception of CADRE-AT, suffer a decrease in accuracy when assessing chemicals with either of these attributes (Table 6). The reason is likely associated with the categorical model of CADRE-AT versus the linear QSAR models for estimating LC<sub>50</sub> of the other tools. While hydrophobic chemicals partition rapidly into organic layer, their diffusion in aqueous environment is limited. Thus, their toxicity may be controlled by diffusion limits,<sup>104</sup> which are not explicitly considered in the modeling approaches evaluated here and may lead to the observed high error rates for insoluble compounds. For example, all tools overestimated the 96 h LC<sub>50</sub> of decane due to its high hydrophobicity ( $\log P = 5.01$ ) and low solubility (0.052 mg L<sup>-1</sup> at 25 °C). However, decane has experimental LC<sub>50</sub> above 530 mg L<sup>-1</sup>, likely because it is unavailable in aquatic environment due to low solubility.

In contrast, ionized species diffuse rapidly through aqueous phase but have lower partitioning into tissues. However,  $\log P$  estimates do not consider the contribution of ionized molecular forms to chemical partitioning and toxicity. For this reason, using  $\log D_{7.4}$  is likely a better estimate of bioavailability than  $\log P$ , as the former takes into account the effect of ionization at biologically-relevant pH on the

Table 6 Prediction accuracy by chemical category: compounds ionized at biological pH and insoluble substances

Measures of predictive accuracy	ADMET	CADRE-AT	ECOSAR	KATE	TEST
Total accuracy – neutral ( $\log P \leq 5$ )	57%	83%	63%	62%	54%
No. of missing predictions – neutral ( $\log P \leq 5$ )	2/53	1/53	2/53	11/53	12/53
Total accuracy – neutral ( $\log P > 5$ )	38%	83%	25%*	33%	17%•
Missing prediction – neutral ( $\log P > 5$ )	0/8	2/8	0/8	2/8	2/8
Total accuracy – anionic	33%	83%	33%•	33%	57%
Missing prediction – anionic	3/12	0/12	0/12	3/12	3/12
Total accuracy – cationic	60%	80%	30%•	50%	75%
Missing prediction – cationic	0/10	0/10	0/10	8/10	6/10

The accuracy of the tool is significantly different from its accuracy for neutral molecules with  $\log P < 5$  at  $\alpha = 0.05$  (\*), and  $\alpha = 0.10$  (•). Two-sided Wilcoxon test was used.



hydrophobicity. Furthermore, ionized compounds may exhibit stronger interactions with biological membranes than other narcotics,<sup>105</sup> or cause toxicity by an entirely different mode of action than their neutral counterparts.<sup>106,107</sup> Consequently, estimations of the acute toxicity of ionizable compounds requires descriptors that reflect the properties of the compound in the predominant ionization state at biological pH. All tools that do not consider ionization exhibited decreased accuracy or failed predictions for a large fraction of ionizable compounds (Table 6). Notable exceptions to the decreased accuracy include ADMET's assessment of cationic compounds, and TEST's assessment of anionic compounds, which are on par with the programs' accuracy for neutral chemicals. CADRE-AT retained similar accuracy for ionizable compounds, likely because it uses  $\log D_{7.4}$  and calculates the reactivity parameters for predominant species at pH 7.4.

### Drug-like compounds

Aquatic toxicity of pharmaceuticals and hormone-like chemicals is of particular concern due to high biological activity of these compounds. Five pharmaceuticals compounds are included in the set of 83 chemicals considered here: estradiol hormone (CAS# 50-28-2), anticoagulant Warfarin (CAS# 81-81-2), and non-steroidal anti-inflammatory drug Indometacin (CAS# 53-86-1) and antibiotics Ampicillin (CAS# 69-53-4) and Amoxicillin (CAS# 26787-78-0) (Tables 1 and S1†). For this set of five pharmaceuticals, the log *P*-based tools give the most accurate predictions. KATE and ECOSAR correctly categorize 5/5 and 4/5 chemicals respectively. CADRE-AT predicts the toxicity categories of Estradiol, Indometacin and Warfarin correctly, but overestimates the toxicities of the two antibiotics by one category. TEST and ADMET performed worse, correctly categorizing 1/5, and 0/5 compounds, respectively. The tools that rely on large number of structural descriptors to predict toxicity could not accurately predict toxicity of the pharmaceutical compounds analysed here and consistently overestimated their toxicity by 1–2 orders of magnitude (Table 1). However, further research with larger data set of drug-like compounds is necessary to assess the applicability of aquatic toxicity prediction tool to pharmaceuticals more generally.

### Conclusions

This analysis assessed the performance of five acute aquatic toxicity prediction tools using an external validation dataset of 83 structurally diverse organic chemicals, which were distinct from the compounds used the training sets of all five tools. Overall, the toxicity estimation tools based on mechanistically-relevant chemical properties performed better than the purely statistical algorithms based on a hundreds of structural properties with unspecified relevance to toxicity. Based on total accuracy within each tool's applicability domains, the tools can be ranked in the following order (from best to worst): CADRE-AT (83%) > ECOSAR (61%) > ADMET (53%) > TEST

(48%) > KATE (46%). However, most programs showed a marked decrease in performance when considering insoluble and ionized chemicals. CADRE-AT showed uniform performance with accuracy above 80% in every chemical category. However, CADRE-AT does not provide an exact LC<sub>50</sub> estimate, but only a regulatory category assignment. Additionally, the latter is not currently available to the public as a standalone, user-operated tool. Among standalone, user-operated tools ECOSAR outperforms the other algorithms. When using any of these tools, users must be careful to consider the applicability domains and solubility warnings, which are not always available in batch mode. Faster processing times, easy compound entry, and clear AD warnings can help *in silico* tool integration in global regulatory environments. Model performance can be improved by developing models for specific MOAs, robust partitioning estimates such as distribution coefficients, and solubility considerations. Furthermore, while models based on mechanistically-relevant parameters perform best, chemical properties other than partition coefficients might be necessary to assess toxicity of diverse chemical space.

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