QSAR for baseline toxicity and classification of specific modes of action of ionizable organic chemicals in the zebrafish embryo toxicity test

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Highlights

- The applicability domain of the fish embryo baseline toxicity QSAR can be extended to ionizables organic compounds
- The use of speciation-corrected liposome-water distribution ratio log D_{lipw}(pH) as descriptor to predict FET baseline toxicity was successfully demonstrated
- Toxic ratio analysis resulted in a generally consistent MoA classification
- critical aspect for the effect analysis of IOCs is the pH

Abstract

The fish embryo toxicity (FET) test with the zebrafish *Danio rerio* is widely used to assess the acute toxicity of chemicals thereby serving as animal alternative to the acute fish toxicity test. The minimal toxicity of neutral chemicals in the FET can be predicted with a previously published Quantitative Structure-Activity Relationship (QSAR) based on the liposome-water partition coefficient K_{lipw}. Such a QSAR may serve to plan toxicity testing and to evaluate whether an observed effect is caused by a specific mode of action (MoA). The applicability domain of this QSAR was extended to ionizable organic chemicals (IOC) without any modification of slope and intercept simply by replacing the K_{lipw}

with the speciation-corrected liposome-water distribution ratio (D_{lipw}(pH)) as descriptor for the uptake into the embryo. FET LC_{50} values of IOCs were extracted from an existing FET database and published literature. IOCs were selected that are present concomitantly as neutral and charged, species, i.e., acids with an acidity constant pK_a <10 and bases with pK_a >5. IOCs were grouped according to their putative MoA of acute aquatic toxicity. The toxic ratios (TR) in the FET were derived by of the experimental FET-LC_{50} in comparison with the baseline toxicity QSAR. Baseline toxicants were confirmed to align well with the FET baseline toxicity QSAR (TR <10). Chemicals identified to act as specific or reactive chemicals with the toxic ratio analysis in the FET test (TR >10) were generally consistent with MoA classification for acute fish toxicity with a few exceptions that were suspected to have had issues with the stability of the pH during testing. One critical aspect for the effect analysis of ionizable chemicals is the pH, since the difference between pH and pK_a determines the speciation and thereby the D_{lipw}(pH).

Keywords: zebrafish, *Danio rerio,* fish embryo toxicity, ionizable chemicals, baseline toxicity QSAR

Introduction

Baseline toxicity, also called narcosis, is driven by a chemical's hydrophobicity and can be described as the minimal toxicity (van Wezel and Opperhuizen, 1995). The relationship between experimental toxicity and underlying baseline toxicity can serve to estimate the degree of specific effects (Russom et al., 1997; van Wezel and Opperhuizen, 1995; Verhaar et al., 1992). A specific or reactive mode of action would increase toxicity, resulting in lower lethal effect concentrations (LC_{50}) than expected for baseline toxicity. Quantitative structure-activity relationships (QSARs) for baseline toxicity have been developed for various aquatic species, mainly using the octanol-water partition constant (K_{ow}) as descriptor to predict the acute toxicity of many aquatic species (Barron et al., 1997; McKim et al., 1987; Vaes et al., 1998; Verhaar et al., 1996). A measure of the specificity of the effect of a chemical is the toxic ratio (TR), which is the quotient of the predicted LC_{50} with the baseline toxicity QSAR and the experimental LC_{50} (Verhaar et al., 1992). A TR ≥ 10 is typically considered as an indicator that a chemical exhibits a reactive or specific mode of toxic action. The higher the TR, the more potent is a chemical. This approach was used to generally categorize chemicals into baseline chemicals, less-inert chemicals, reactive chemicals and specifically acting chemicals (Verhaar et al., 1992). K_{ow} is a proxy for the lipophilicity of chemicals and serves as a surrogate for many environmental partitioning processes to condensed organic phases including the partitioning into biological membranes (Schwarzenbach et al., 2016). Thus K_{ow} is frequently used as descriptor for predicting baseline

toxicity, which occurs at a critical membrane concentration and results in disturbance of membrane integrity and functioning. Baseline toxicity QSARs often showed a distinct difference for non-polar and polar chemicals, which turned out to be an artifact that disappeared when the K_{ow} was replaced by the liposome-water partitioning coefficient K_{lipw} as descriptor (Vaes et al., 1998)

Liposomes are the simplest models for anisotropic biomembranes, the target site for baseline toxicants (van Wezel and Opperhuizen, 1995). The liposome-water partitioning coefficient K_{lipw} describes interaction with biological membranes much better than the K_{ow} especially for ionizable chemicals (Escher and Schwarzenbach, 1996; Ottiger and Wunderli-Allenspach, 1997). Baseline toxicity QSARs that use the speciation-corrected liposome-water distribution ratios D_{lipw} (pH) have much better predictability of baseline toxicity for ionizable and charged chemicals than speciation-corrected octanol-water distribution ratios $D_{ow,}$ which has already been successfully demonstrated for various bioassay, e.g. bacteria (Microtox), algae, daphnia and the fish acute toxicity test (Escher et al., 2017; Escher and Schwarzenbach, 2002; Neuwoehner and Escher, 2011).

We recently developed a log K_{lipw}-based baseline toxicity QSAR for the fish embryo toxicity (FET) with zebrafish (*Danio rerio*) and used it to identify specifically acting or reactive chemicals by determination of the TR (Klüver et al., 2016). This QSAR included only neutral chemicals. The goal of this study was to expand the applicability of the FET baseline toxicity QSAR to ionizable organic chemicals (IOCs) by using D_{lipw} (pH) as descriptor. In this study we included mainly monoprotic IOCs including acids with pK_a <10 and bases with pK_a >5 and considered the reported pH-value or pH-range of the exposure solution used in the FET tests. Furthermore we applied this QSAR to investigate degree of specificity in relation to the modes of action of chemicals classified as specifically acting or reactive chemicals.

2. Material and methods

2.1 Fish embryo toxicity LC_{50} *values*

Zebrafish embryo LC_{50} values of IOCs were extracted from a FET database (http://echa.europa.eu/documents/10162/13562/annex2_fet_en.xlsx) and an additional study was included (Bittner et al., 2018). We considered only data that started the exposures between 0 and 24 hours post fertilization (hpf) and for which effects were measured at 96-144 hpf after 96-120 hours total exposure time. LC_{50} data above water solubility were removed. IOCs that have a minimum of 1% charged species at the tested pH and a log D_{lipw}(pH) in the range of -1 to 6 were included. The pH is a critical parameter to calculate the speciation-dependent physicochemical parameter log D_{lipw}(pH);

however, the pH of the exposure solutions was provided for only 36% of LC_{50} data and in some cases a pH range was given, e.g., pH of 6.8-8. For studies that used a buffered exposure media, e.g. embryo medium or 10% of Hanks's balanced salt solution according to Westerfield (Westerfield, 2000), we assumed that the pH did not change after adding the chemical. Hence, studies, which did not report using a pH-buffered system, or did not provide any information regarding the pH of the exposure solution, were excluded from the analysis. For some chemicals more than one LC_{50} value was available because the LC_{50} values were from different studies with different exposure settings. Therefore, each LC_{50} value was considered separately rather than generating an average value.

2.2 Physicochemical properties

Experimentally determined acidity constants (pK_a) were extracted from literature and publicly available databases: Drugbank (https://www.drugbank.ca/), ChemIDplus (https://chem.nlm.nih.gov/chemidplus/), Physprop (http://esc.syrres.com/fatepointer/search.asp). If no experimental pK_a values were available, they were predicted with ACD/Percepta (Advanced Chemistry Development, Inc., Toronto, ON, Canada) based on the ACD/GALAS pK_a model. The fraction of neutral and charged species was calculated with the Henderson-Hasselbalch equation for monoprotic acids (1) and bases (2) as well as equation (3).

Acids: $$f_{neutral} = \frac{1}{1+10^{(pH-pK_a)}} \quad (1)$$

Bases: $$f_{neutral} = \frac{1}{1+10^{(pK_a-pH)}} \quad (2)$$

$$f_{ion} = 1 - f_{neutral} \quad (3)$$

The liposome-water distribution ratio at specific pH, D_{lipw} (pH), is defined as the sum of the fraction of each species *i* (f_i) times their respective K_{lipw}(i) of all species n ((Escher and Schwarzenbach, 1996), Eq. (4)).

$$D_{lipw}(pH) = \sum_{i=1}^{n} f_i \times K_{lipw}(i) \quad (4)$$

A poly-parameter linear free energy relationship (pp-LFER) was used to predict the K_{lipw} of the neutral species if no experimental data were available. Descriptors for the pp-LFER calculations were extracted from the UFZ-LSER database (Ulrich et al., 2017). Experimental descriptors were preferred over modeled descriptors and were used to predict K_{lipw} of the neutral species according to Eq. (5), (Endo et al., 2011). The following compound descriptors were used: *L*, the logarithm of hexadecane-

air partition; *S*, dipolarity/polarizability parameter; *A*, solute H-bond acidity; *B*, solute H-bond basicity; *V*, molar volume. Additionally, a one-parameter log K_{ow} model was used for the estimation of K_{lipw} (Endo et al., 2011) for chemicals, where pp-LFER descriptors were not available (Eq. (6)).

$$\log K_{lipw}(\text{neutral}) = 0.48 + 0.55L - 0.95S - 0.05A - 4.02B + 1.65V \quad (5)$$

$$\log K_{lipw}(\text{neutral}) = 1.01 \times \log K_{ow} + 0.12 \quad (6)$$

In the absence of experimental K_{lipw}(ion) data, K_{lipw}(ion) was predicted with COSMOmic. The COSMOmic model is a mechanistic model for the calculation of K_{lipw}(ion) based on quantum chemistry and fluid phase thermodynamics and has the widest applicability domain of any membrane partition model (Bittermann et al., 2014; Klamt et al., 2008). For two monoprotic acids we estimated K_{lipw}(ion) based on Δ*mw* (Eq. 7), which is the difference of the log K_{lipw} of the neutral and the log K_{lipw} of the charged species and in a rough approximation it is assumed to be a constant for different chemicals.

$$\Delta mw = \log K_{lipw}(\text{neutral}) - \log K_{lipw}(\text{ion}) \quad (7)$$

For most charged organic chemicals investigated so far Δmw was approximately one log unit, while for carboxylic acids two log units due to the stronger charge localization (Bittermann et al., 2016; Escher and Sigg, 2004).

2.3 MoA classification

The chemicals are identified in Table 1 by use and were grouped into their putative mode of action (MoA) class (baseline toxicity, specific MoA, uncouplers of oxidative phosphorylation, unspecific reactive MoA and unknown MoA). For the grouping, the detailed MoA information were extracted from PubChem (https://pubchem.ncbi.nlm.nih.gov/, (Kim et al., 2016)), T3DB (http://www.t3db.ca/, (Lim et al., 2010)) and from a recently established MoA and acute aquatic toxicity database for predictive model development (Barron et al., 2015). Additionally, the Verhaar scheme was used as a first screening for the MoA (Verhaar et al., 1992). For chemicals that are used as pesticides, especially herbicides and fungicides, the mechanism of toxicity for fish is unknown, thus we assigned unknown MoA. Chemicals known as endocrine active chemicals were considered to act as baseline toxicants in acute toxicity test because their endocrine mechanism would only manifest during chronic exposure. The detailed MoA information is included in the supplementary information (Table S1). A final MoA classification for the FET was assigned based on the toxic ratio (TR) analysis.

2.4 FET QSAR baseline toxicity

The common FET baseline toxicity QSAR for neutral chemicals was recently developed using the log K_{lipw} as hydrophobicity descriptor (Klüver et al., 2016). For IOCs, this QSAR was modified by replacing the log K_{lipw} with the ionization-corrected liposome water distribution ratio log D_{lipw}(pH) (Eq (8)). The rationale for the modification was that baseline toxicants have constant critical membrane burdens for neutral and charged chemicals (Escher et al., 2002); hence, the D_{lipw}(pH) would be an equivalent predictor of membrane concentration as K_{lipw} is for neutral chemicals.

$$\log\left(\frac{1}{LC_{50}(mM)}\right) = 0.99 \times \log D_{lipw}(pH) - 2.22 \qquad (8)$$

2.5 Toxic ratio analysis

A measure of the intrinsic potency of a chemical is the toxic ratio (TR) which is the quotient of the LC_{50} predicted with the baseline toxicity QSAR and the experimental LC_{50} (Eq. (9)). Any chemical with a TR>10 was considered specifically acting or reactive (Maeder et al., 2004; Verhaar et al., 1992).

$$TR = \frac{LC_{50}\ \text{(baseline toxicity QSAR)}}{LC_{50}\ \text{(experimental)}} \qquad (9)$$

3. Results and discussion

After quality control and considering the criteria listed in the material and methods section the FET dataset of ionizable chemicals comprised 66 different chemicals (46 acids and 20 bases, Table 1). The dataset included a total number of 83 LC_{50} values. All detailed information on the physicochemical properties and studies can be found in Table S1 of the supporting information (SI). The chemicals of each putative acute MoA class were evaluated together (Table 1). The log ($1/LC_{50}$) values of the different groups were plotted as function of log D_{lipw}(pH), which was calculated using Eq. 4 with the K_{lipw} of all species with respect to the specific pH or pH range of the individual studies.

The group of putative baseline toxicants fell into the baseline toxicity range ($0.1 < TR < 10$) and were confirmed as baseline toxicants (Figure 1A). No MoA for acetic acid in the FET could be assigned because the predicted log K_{lipw} of 0.57 for the anion was actually higher than the predicted log K_{lipw} of -0.16 of the neutral species. This unlikely discrepancy is presumably caused by high imprecision of

the prediction models at such low hydrophobicity and consequently high likelihood that acetic acid is outside the applicability domain of the QSAR model.

3,3',5,5'-tetraiodothyroacetic acid (TIA) just met the classification threshold with a TR of 10. TIA binds the thyroid hormone and thereby is an endocrine active substance. A compound with an endocrine MoA would be expected to act as baseline toxicant in acute toxicity tests (unless there is an additional acute specific MoA).

Consistent with this line of reasoning, genistein, butylparaben and propylparaben, all which act as endocrine disruptors in aquatic species (Boberg et al., 2010; Schiller et al., 2013; Serra et al., 2018), had TRs in the baseline toxicity range (0.1 < TR < 10) (Figure 1, Table 1).

The analysis with the D_{lipw}(pH) for putative baseline toxicants confirmed our hypothesis that the applicability domain of the general baseline toxicity QSAR can be extended to ionizable chemicals.

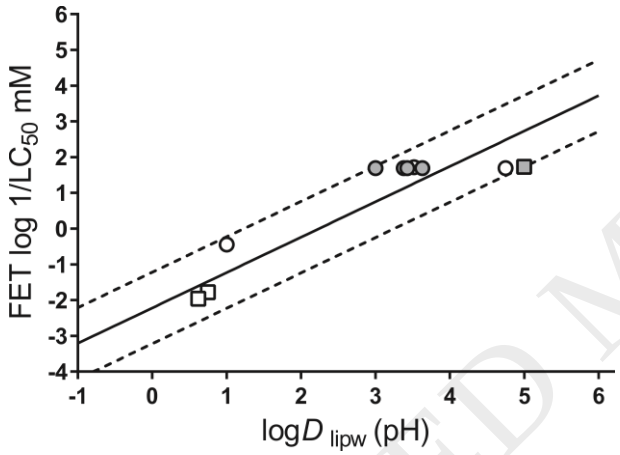

Figure 1: Experimental log(1/LC_{50}) of putative baseline toxicants plotted versus log D_{lipw}(pH). The solid line is not a regression line but constitutes the general baseline toxicity QSAR (Eq. 8) and the broken lines refer to lines with TR 0.1 and 10. Circles represent anionic chemicals and squares represent cationic chemicals. Grey shaded are chemicals that have been described to have an endocrine activity as chronic endpoint but were classified as baseline toxicants for the acute FET.

The group of potential reactive toxicants consisted of only three chemicals, namely menadione sodium bisulfite (MSB), perfluorooctane sulfonic acid (PFOS) and phenylhydrazine (PHZ). For MSB, a TR of about 19500 was observed which can be classified as a very potent reactive toxicant (Figure 2, Table 1). MSB is a synthetic naphthoquinone which is similar to vitamin K and has been reported to be very toxic to aquatic organisms (https://echa.europa.eu/substance-information/-/substanceinfo/100.004.535). Quinones are highly redox-active molecules which can result in

formation of reactive oxygen species (ROS). ROS are known to cause oxidative stress within cells by oxidizing cellular macromolecules, including lipids, proteins and DNA (Bolton et al., 2000).

PHZ exhibited an excess toxicity with a TR of 679-712 (Table 1). PHZ causes hemolytic anemia through the oxidization of hemoglobin, in zebrafish embryos, PHZ results in depletion of mature erythrocytes (Lenard et al., 2016). Furthermore, PHZ is a known lysyl oxidase (LOX) inhibitor and exposure of developing zebrafish embryos to LOX inhibitors is known to result in severe developmental effects, such as notochord malformations, head deformations and lack of blood flow (Strecker, 2013). Comparable phenotypes in zebrafish embryos are observed for dithiocarbamates, which, similar to PHZ, are LOX inhibitors (Tilton et al., 2006; van Boxtel et al., 2010). Thus, for PHZ a specific or reactive MoA could be confirmed.

PFOS is able to produce oxidative stress and induce apoptosis (Liu et al., 2007), however, five out of six PFOS studies in the FET resulted in a TR<10 but for one study a TR 28 was determined. This study had a 120h exposure. Two additional PFOS studies used the same exposure setup, the LC_{50} values deviated by a factor of 10. This study used a buffered system which was adjusted to pH 7.2 before solving the chemical, but no information on the pH value during the period of the test was provided. Since PFOS is a strong acid with a pK_a of -5.74, an additional toxic effect by acidification of the test medium cannot be excluded and the result would be questionable. Hence, we classified PFOS as a baseline toxicant in the FET.

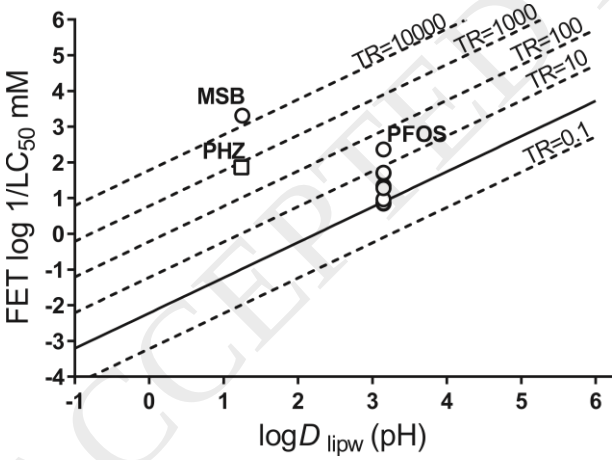

Figure 2: Experimental LC_{50} values of unspecific reactive toxicants plotted versus log D_{lipw} (pH). The solid line is the general baseline toxicity QSAR (Eq. 8) and the broken lines refer to lines with TR 0.1 to 10000. Circles represent anionic chemicals MSB and PFOS and the square represent cationic PHZ.

We split the chemicals with specific MoAs into two groups. The first group included substituted phenols that are known uncouplers of oxidative phosphorylation (Terada, 1990). The second group

included chemicals that interacted specifically with enzymes or receptors for which the exact mechanism was not further specified.

Uncouplers of oxidative- and photo-phosphorylation cause excess toxicity by interfering with the energy transduction common to both mitochondrial respiration and photosynthesis (Nicholls and Ferguson, 1992; Terada, 1990). Uncouplers destroy the proton gradient that is built up through the electron transport chain by shuttling the protons back across the membrane thereby short circuiting the ATP synthase (Nicholls and Ferguson, 1992). Uncoupling is accomplished by an interplay of the neutral and charged species. Both need to be present at the inner pH of mitochondria and the permeability of the anion is the rate-determining step of the overall processes, which can be enhanced by the formation of heterodimers between neutral and charged species, to form a more hydrophobic charged complex with enhanced permeability (Escher et al., 1996; Spycher et al., 2005). Hence the intrinsic uncoupling activity is pH dependent (Escher et al., 1999; Escher and Schwarzenbach, 1996). The internal pH in relation to the chemical specific pK_a determines if an intrinsic uncoupler (Escher and Schwarzenbach, 2002) is also a good uncoupler *in vivo* at the internal pH of the biological system. For the fish embryo, the internal pH is 7.55 (Mölich and Heisler, 2005); thus, we can expect that chemicals which were clearly identified as uncouplers at pH 7 to 8 in isolated energy-transducing membranes would also act as effective uncouplers in fish embryos.

LC_{50}-values of substituted phenols in the FET model were compared to measurements of intrinsic uncoupling performed by Escher and Schwarzenbach (2002) using isolated energy-transducing membranes. As expected the lower substituted chlorophenols were in the range of baseline toxicity. 2,4-Dichlorophenol had a TR of 1.5-1.9 in the FET (Figure 3), consistent with a TR of 3.4 for isolated energy-transducing membranes. The low TR of 1 for 2,4,5-trichlorophenol in the FET cannot be reconciled directly with the TR of 23 at pH 7 in energy-transducing membranes, while 2,4,6-trichlorophenol had a TR of 15 in FET and TR of 4 in isolated energy-transducing membranes. Another FET study of 2,4,6-trichlorophenol resulted in a TR range of 3-21, because only an pH range (pH 6.5-7.8) of the test has been provided rather than a specific pH. This pH range resulted in a difference of 31% and 2% of the neutral fraction for the lowest (6.5) and highest (7.8) pH, respectively. Distinct D_{lipw}(pH) values for this pH range were calculated which resulted in the TR range. Trichlorophenols have pK_a values between 6.15 and 7.73 depending on the substitution pattern of the chlorine substituents. Unless heterodimers are formed, the maximal activity of uncoupling occurs at pH values slightly higher than the pK_a, which is the case for 2,4,5- and 3,4,5-trichlorphenol but not for 2,4,6-trichlorophenol (Escher et al., 1999). Heterodimer formation lowers the maximum of intrinsic activity to pH values closer to the pK_a but increases the intrinsic potency (Escher and Schwarzenbach, 2002). The pH dependence of 2,3,5-trichlorophenol had not been measured in isolated energy-transducing

membranes but the analogy to 3,4,5-trichlorophenol and 2,3,4,5-tetrachlorophenol would suggest that it forms weak dimers and has the peak activity around its pK_a. 2,4,6-Trichlorophenol cannot form heterodimers so it is less intrinsically potent but it also has a lower pK_a and hence its maximum activity is expected at higher pH than the pK_a.

Pentachlorophenol (TR 13 in the FET test) (Figure 3) cannot form heterodimers due to steric constraints; therefore, its intrinsic maximum uncoupling activity is around pH 6.5 to 7.0, despite its low pK_a of 4.75 (Escher et al., 1999). Hence the TR of 100 observed in isolated energy-transducing membranes (Escher and Schwarzenbach, 2002) could be lower in the fish embryo which has an internal pH of 7.55 (Mölich and Heisler, 2005). Overall, TR of the chlorophenols increased with increasing number of chloro substituents as would be expected from kinetic uncoupling models.

In comparison to chlorophenols, the toxicity of the nitrophenols (except 4-nitrophenol) and phenols with bromo substituents was higher and these chemicals could therefore be unambiguously classified as uncouplers (Figure 3, Table 1). 4-Nitrophenol showed a TR range of 7 to 33 in the FET test, which was dependent on the pH range of the study and was consistent with an intrinsic TR of 9.9 in isolated energy-transducing membranes. The enhanced toxicity of 2-methyl-4,6-dinitrophenol (4,6-dinitro-o-cresol; TR 391 and 269), 2-s-butyl-4,6-dinitrophenol (dinoseb, TR 214), and 2,4-dinitrophenol (TR 191 and 132) are very much in agreement with the TR for intrinsic uncoupling in isolated energy-transducing membranes (Escher and Schwarzenbach, 2002).

While both chloro and nitro substituents are electron-withdrawing groups, the observed difference in uncoupling potency between nitrophenols and chlorophenols can be explained by the fact that the nitro-group additionally has a conjugating effect and especially the 4-nitro substituent in para position to the hydroxy substituent assures a smearing of the charge over the entire molecules. The effect of the nitro-substituent together with a shielding effect of ortho-alkyl groups increases the uncoupling activity. Bromoxynil (3,5-Dibromo-4-hydroxy-benzonitrile) is a pesticide that has also been reported to act as uncoupler of oxidative phosphorylation (Arena et al., 2017). The observed excess toxicity in the FET with TRs of 165 and 88 is consistent with the TR of 175 in isolated energy-transducing membranes (Escher and Schwarzenbach, 2002).

For two organochlorine compounds (hexachlorophene and triclosan) with an uncoupling MoA, a TR range with no deviation from baseline toxicity was determined (Table 1, Figure 3). Triclosan has intrinsic uncoupling potency (Newton et al., 2005; Shim et al., 2016) but with a pK_a of 7.90 acts as baseline toxicant in the FET test at pH 6.5-7.8 and pH 7.

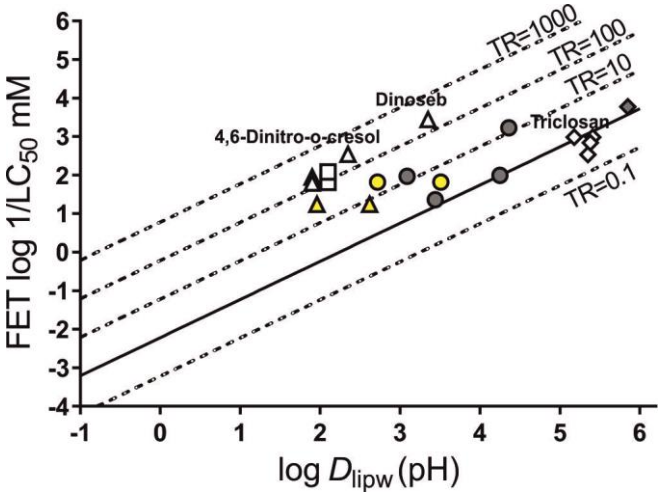

Figure 3: Experimental LC_{50} values of intrinsic uncouplers plotted versus log D_{lipw}(pH). The solid line is the general baseline toxicity QSAR (Eq. 8) and the broken lines refer to lines with TR 0.1 to 1000. Circles – chlorophenols, triangles – nitrophenols and squares – bromoxynil, white diamonds – triclosan, grey diamond – hexachlorophene. Yellow circles correspond to a 2,4,6-trichlorophenol study for which a pH range (6.5 – 7.8) was given. Yellow triangles correspond to a 4-nitrophenol study for which a pH range (6.5 – 7.8) was given.

The second group of specifically acting chemicals considered those which are either receptor-mediated or are known inhibitors of enzymes (Figure 4). The highest TR was found for nicotine (TR 2719), which is hydrophilic with a log D_{lipw}(pH 7) around -0.5. The applicability for the baseline toxicity QSAR might be limited since chemicals with a log D_{lipw}(pH 7) of 0 have no tendency to accumulate in the membrane, which is the target for baseline toxicity. Nicotine is a stimulant drug that acts as an agonist at nicotinic acetylcholine receptors and is a known developmental toxicant in animals including zebrafish embryos (Ali et al., 2014; DiFranza and Lew, 1995; Lantz-McPeak et al., 2015). In several *in vitro* assays nicotine was cytotoxic and it has been suggested that nicotine has the potential to induce ROS (Babich and Borenfreund, 1992; Nathiga Nambi et al., 2017). For neurotoxic chemicals it has been reported that the specific mechanism, such as inhibition of acetylcholinesterase and others, might not provoke mortality in fish embryos; rather mortality may be induced via unspecific effects, e.g., unspecific reactivity (Klüver et al., 2015; Klüver et al., 2016).

For all-trans retinoic acid (ATRA) two studies were available and resulted in TRs of 7 and 418 – 1348 (Figure 4). ATRA is a known teratogen that induces strong developmental effects in zebrafish embryos (Selderslaghs et al., 2009; Wang et al., 2014). The TR difference of more than a factor of one hundred in the two studies suggests that the lower TR might be an outlier. ATRA is highly labile and it is sensitive to light, oxygen and heat (Barua and Furr, 1998); therefore, it is possible that the outlier

study with a TR of 7 resulted from ATRA degradation in solution. ATRA concentrations of 8 nM showed an increase of apoptotic cells in the developing zebrafish embryo (Wang et al., 2014), a concentration which is close to the LC_{50} in the FET study that resulted in a high TR. Two dithiocarbamate fungicides metam (TR 320) and maneb (TR 81) were identified as specifically acting toxicants in zebrafish embryos. As discussed above the MoA of LOX inhibition and subsequent interference of extracellular matrix formation by dithiocarbamates has been demonstrated to result in strong axis deformations in zebrafish embryos (Tilton et al., 2006). Furthermore, dithiocarbamates showed high TRs in chronic fish toxicity of the early life stage (ELS) test (Scholz et al., 2018). It can be concluded that these teratogenic effects affect survival of the developing zebrafish.

Chemicals with the specific MoA of inhibition of cyclooxygenase (COX) also have TR>10 including salicylic acid (TR 51 – 110 and 53), acetyl salicylic acid (TR 31 – 37), and diclofenac (TR 35) (Figure 4). During zebrafish development it has been shown that an inhibition of COX in early stages result in gastrulation arrest or in later stages, in defective vascular tube formation (Cha et al., 2005; Chen et al., 2014). Thus, both literature and TR analysis clearly indicated a specific MoA in the FET for these chemicals.

Some chemicals are known to act via a specific pharmacological MoA for which the receptors are expressed in zebrafish yet do not exhibit high TRs. Drugs developed for human use might have a weaker affinity to their target in fish, which could result in a low TR in fish embryos. The β-blockers metoprolol and propranolol induced specific effects on the heart rate in fish embryos, which is a MoA-specific endpoint but only at concentrations that were as high as baseline toxicity (Bittner et al. 2018). Hence, both β-blockers were classified as baseline toxicants at pH 7 with TRs of 3.3 (Table 1).

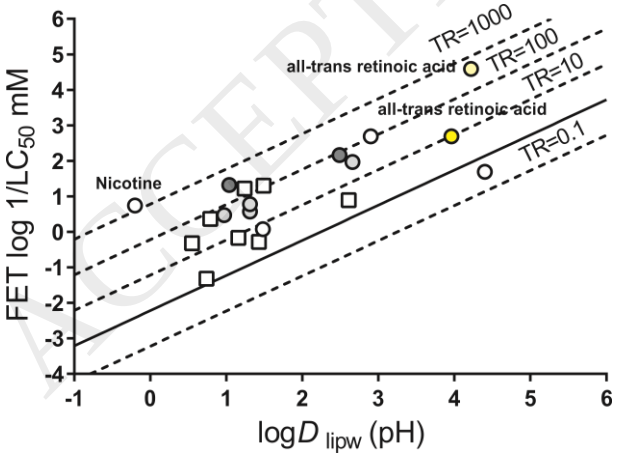

Figure 4: Experimental LC_{50} values of known specifically acting chemicals versus log D_{lipw} (pH). The solid line is the general baseline toxicity QSAR (Eq. 8) and the broken lines refer to lines with TR 0.1 to 1000. Circles represent anionic chemicals and squares represent cationic chemicals. COX inhibitors

are labeled in light grey; dark grey are dithiocarbamates. Yellow circles correspond to a study with ATRA, for which a pH range (6.8 – 8.0) was given.

The remaining chemicals that could not be classified on MoA information or any classification scheme are depicted in Figure 5. In this group of chemicals, there are several plant-protection products (PPPs) which are used as either herbicides or fungicides with a known MoA for plants and fungi but a specific target is not present or not known for vertebrate species including fish. However, based on their TR>10, imazamox, cyclanilide, propoxycarbazone, fenamidone, primisulfuron-methyl and butyrac can be classified as chemicals with a specific or reactive MoA in fish embryos (Figure 5, Table 1). The highest TR of 28120 was observed for dimethylaminoethanol (DMAE) which suggests a very high sensitivity in fish embryos and indicates a specific MoA. Adverse effects of DMAE were determined during embryonic development of sea urchin (Qiao et al., 2003). DMEA has been described as a competitive inhibitor of choline transport and co-treatment with acetylcholine or choline prevented the adverse effects (Qiao et al., 2003)

Interestingly, with increasing hydrophobicity the TR decreased and chemicals above a log D_{lipw}(pH 7) of 3 were mainly in the range of baseline toxicity (Figure 5). This has already been observed in our previous study for the neutral chemicals for those which were classified as specifically acting chemicals (Klüver et al., 2016).

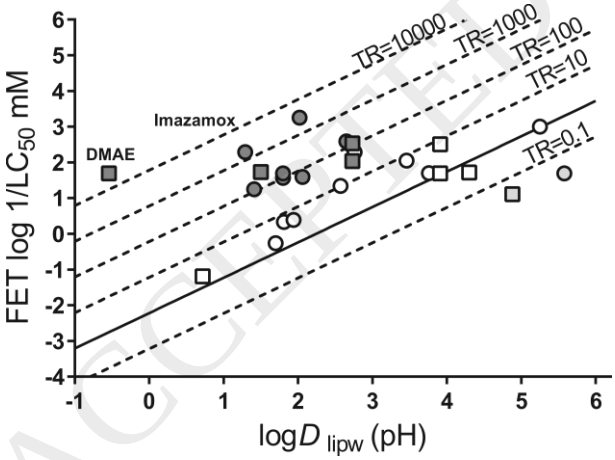

Figure 5: Experimental LC_{50} values of unclassified chemicals versus log D_{lipw}(pH). The solid line is the baseline toxicity QSAR (Eq. 8) and the broken lines refer to lines with TR 0.1 to 1000. Circles represent anionic chemicals and squares represent cationic chemicals. TR<0.1 are highlighted in light grey, which indicate that exposure concentrations may have been underestimated because of experimental limitations or a reduced internal bioavailability. Chemicals with TR>10 are highlighted in dark grey.

Conclusions

We demonstrated that the general baseline toxicity QSAR based on K_{lipw} as hydrophobicity descriptor can be extended to ionizable and charged chemicals by replacing K_{lipw} with the speciation-dependent log D_{lipw} (pH) without any further change of intercept and slope of the equation. The applicability of the baseline QSAR for ionizable chemicals was confirmed for 11 chemicals and 23 IOCs were newly identified as baseline toxicants in the FET. For 30 chemicals a specific or reactive MoA in the FET test was identified with the TR analysis, which was consistent with MoA information for most chemicals.

For 15 IOCs experimental K_{lipw} values (neutral and ion) were available and 51 IOCs had no experimental K_{lipw} values, so they had to be estimated. For most of the uncouplers used in this study experimental K_{lipw} values were available. The K_{lipw} (neutral) can be can be fairly well estimated and there is a good correlation of experimental K_{lipw} (neutral) and log K_{ow} which indicate that the two partition coefficient are generally in agreement (Endo et al., 2011). For charged chemicals the prediction of K_{lipw} (ion) a mechanistic model was necessary. The refined COSMOmic is the first mechanistic model that predicts K_{lipw} (ion) quite accurately (Bittermann et al., 2014). However, for 18 out of the 51 IOCs, the K_{lipw} of the charged species was higher than that of the neutral species, which is not only counterintuitive but also not supported by any experimental evidence. Hence, our analysis might have been somewhat limited by poor D_{lipw}(pH) estimates. Therefore, we recommend additional experimental studies to obtain more robust physicochemical descriptors for IOCs. The commonly used ionization-corrected octanol water distribution ratio D_{ow}(pH) (Huang et al., 2003; Vestel et al., 2016) would not be a suitable replacement because charged species partition into octanol only with a counterion or in the form of an ion pair (Escher and Schwarzenbach, 1996; Johnson and Westall, 1990), thereby underestimating the interaction with and partitioning into biological membranes.

Most of the standard regulatory guidelines for ecotoxicity testing do not address this issue of constant pH value, which is pertinent for IOCs. Even the OECD testing guideline 236 for the FET has a fairly wide acceptable pH range of 6.5 to 8.5 with a variation of less than 1.5 pH units during the exposure duration. Such a large pH range leads to high uncertainty of the speciation; in addition, the pH-dependence of bio-uptake needs to be accounted for, when there exists a difference in pH between the external medium and the inside of the fish embryo (Bittner et al., 2018). In the FET test for IOCs, it is crucial to keep the pH stable by the addition of a pH-buffer system rather than reporting a pH range. Depending on the pK_a of the IOC, such a permissible pH-shift might affect the speciation by more than a factor of 20. For baseline toxicants the LC_{50} may be so high that the IOC to

be tested may have an influence on the pH of the exposure medium. Hence, it is vital to include a measurement of the pH inside the exposure vessel at the beginning and end of testing to assure the constancy of pH during exposure, even if a pH-buffered system is used. Given that the market share of both pharmaceutical and pesticidal IOCs has been increasing over the last decades, we recommend that testing protocols be adapted for IOCs, e.g., by scrutinizing the standard pH range of the guidelines for toxicity testing.

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Table 1: Investigated IOCs, their physicochemical properties (more details and references are given in the SI, Table S1) and effects in the FET test grouped according to their putative MoA class in acute aquatic toxicity. The data within each group was sorted according to decreasing toxic ratios (TR , eq. 9). The TRs were used to classify the MOA of the chemicals in the FET (baseline = baseline toxicity; s = specific MOoA; sor = specific or reactive MoA; uc =uncoupler of oxidative phosphorylation; ur, unspecific reactive MoA). The star (*) indicates a buffered exposure medium but the pH test medium was not measured. a, (Ali et al., 2012; Ali et al., 2011); b, (Bachmann, 2002); c, (Bittner et al., 2018); d, (Busquet et al., 2014); e, (Carlsson et al., 2013); f, (Groth et al., 1993); g, (Hagenaars et al., 2011); h, (Huang et al., 2010); i, (Klüver et al., 2015); j, (Knöbel et al., 2012); k, (Oliveira et al., 2009); l, (Padilla et al., 2012); m, (Selderslaghs et al., 2009); n, (Strecker, 2013); o, (Truong et al., 2014); p, UFZ, unpublished data. A, acid; B, base.

Chemical	CAS	Use Group	acid or base	pK_a	pH or pH-range	$f_{neutral}$ (pH-range)	log D_{lipw} (pH) (range if a pH range was given)	log 1/LC_{50} (mM)	TR (range)	FET MoA classification
Baseline toxicity										
Acetic acid	64-19-7	Industrial chemical	A	4.76	7.46*	0.20%	0.57	-0.49[a]	15	-
3,3',5,5'-Tetraiodothyroacetic acid	67-30-1	Pharmaceutical	A	3.93	7.2*	0.05%	4.60	3.37[o]	10	baseline
Bisphenol AF	1478-61-1	Industrial chemical	A	8.75	7.2*	97.26%	3.00	1.69[o]	9	baseline
Sodium oxalate	62-76-0	Industrial chemical	A	4.40	7.46*	0.09%	1.00	-0.44[a]	6	baseline
Sodium dodecyl sulfate	151-21-3	Industrial chemical / surfactant	A	-0.09	7.46*	0.00%	3.52	2.00[a]	5	baseline

Chemical	CAS	Use Group	acid or base	pK_a	pH or pH-range	$f_{neutral}$ (pH-range)	log D_{lipw} (pH) (range if a pH range was given)	log 1/LC_{50} (mM)	TR (range)	FET MoA classification
Butylparaben	94-26-8	Food additive / preservative	A	8.47	7.2*	94.90%	3.38	1.69[o]	4	baseline
Propylparaben	94-13-3	Food additive / preservative	A	8.40	7.2*	94.06%	3.43	1.69[o]	3	baseline
Sodium dodecyl sulfate	151-21-3	Industrial chemical / surfactant	A	-3.00	6.5-7.8	0.00%	3.52	1.72[j]	3	baseline
Sodium tetradecyl sulfate	1191-50-0	Industrial chemical / surfactant	A	-3.00	6.5-7.8	0.00%	4.75	2.94[d]	3	baseline
Genistein	446-72-0	Pharmaceutical / Phytoestrogen	A	7.20	7.2*	50.00%	3.63	1.69[o]	2	baseline
2-Aminoethanol	141-43-5	Industrial chemical	B	9.50	8	3.07%	0.75	-1.78[f]	0.50	baseline
Isopropylamine	75-31-0	Industrial chemical	B	10.63	8	0.23%	0.62	-1.96[f]	0.44	baseline
Sodium tetradecyl sulfate	1191-50-0	Industrial chemical / surfactant	A	-3.00	7.2*	0.00%	4.75	1.69[o]	0.16	baseline

Chemical	CAS	Use Group	acid or base	pK_a	pH or pH-range	$f_{neutral}$ (pH-range)	log D_{lipw} (pH) (range if a pH range was given)	log 1/LC_{50} (mM)	TR (range)	FET MoA classification
Tamoxifen citrate	54965-24-1	Pharmaceutical	B	9.37	7.2*	0.67%	5.00	1.73[o]	0.10	baseline
Unspecific reactive										
Menadione sodium bisulfite	130-37-0	Pharmaceutical / food additive	A	-3.42	7.6-7.9	0.00%	1.25	3.31[n]	19539	ur
Phenylhydrazine	100-63-0	Industrial chemical	B	8.79	7.6-7.9	6-11%	1.24-1.26	1.86[n]	679-712	ur
Perfluorooctane sulfonic acid	1763-23-1	Industrial chemical	A	-5.74	7.2*	0.00%	3.15	2.36[h]	28	baseline
Perfluorooctane sulfonic acid	1763-23-1	Industrial chemical	A	-5.74	7.2*	0.00%	3.15	1.71[o]	6	baseline
Perfluorooctane sulfonic acid	1763-23-1	Industrial chemical	A	-5.74	7.2*	0.00%	3.15	1.69[o]	6	baseline
Perfluorooctane sulfonic acid	1763-23-1	Industrial chemical	A	-5.74	7.2*	0.00%	3.15	1.34[l]	3	baseline
Perfluorooctane sulfonic acid	1763-23-1	Industrial chemical	A	-5.74	7.2-7.5	0.00%	3.15	1.28[g]	2	baseline
Perfluorooctane sulfonic acid	1763-23-1	Industrial chemical	A	-5.74	7.2-7.5	0.00%	3.15	0.96[g]	1	baseline

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Uncouplers of oxidative phosphorylation										
4,6-Dinitro-o-cresol	534-52-1	Industrial chemical / Pesticide	A	4.31	6.5-7.8	0.64%	2.35	2.54[d]	267-270	uc
Dinoseb	88-85-7	Herbicide	A	4.60	7.2*	0.25%	3.35	3.44[o]	215	uc
2,4-Dinitrophenol	51-28-5	Industrial chemical	A	3.94	7.2*	0.05%	1.90	1.95[o]	191	uc
Bromoxynil	1689-84-5	Herbicide	A	4.09	7.2*	0.05%	2.10	2.08[o]	165	uc
2,4-Dinitrophenol	51-28-5	Industrial chemical	A	3.90	6.5-7.8	0.25%	1.90	1.79[d]	131-133	uc
Bromoxynil	1689-84-5	Herbicide	A	3.86	7.2*	0.05%	2.10	1.81[l]	89	uc
2,4,6-Trichlorophenol	88-06-2	Industrial chemical	A	6.15	7.2*	9.68%	3.09	1.97[o]	15	uc
Pentachlorophenol	87-86-5	Pesticide	A	4.75	7.2*	0.32%	4.36	3.23[o]	13	uc
4-Nitrophenol	100-02-7	Industrial chemical	A	7.08	6.5-7.8	18-82%	1.96-2.62	1.24[j]	7-33	uc

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2,4,6-Trichlorophenol	88-06-2	Industrial chemical	A	6.15	6.5-7.8	3-31%	2.72-3.51	1.82[j]	3.6-22	uc
2,4-Dichlorophenol	120-83-2	Industrial chemical	A	7.85	7.6-7.9	47-66%	3.32-3.42	1.36[n]	1.5-1.9	baseline
2,4,5-Trichlorophenol	95-95-4	Industrial chemical	A	6.94	7.2*	59.66%	4.25	1.99[o]	1.6	baseline
Hexachlorophene	70-30-4	Disinfectant	A	4.95	6.5-7.8	0-3%	5.85-6.32	3.77[j]	0.5-1.5	baseline
Triclosan	3380-34-5	Disinfectant	A	7.90	6.5-7.8	56-96%	5.18-5.41	2.98[d]	0.7-1.14	baseline
Triclosan	3380-34-5	Disinfectant	A	7.90	7	88.82%	5.38	2.84[k]	0.52	baseline
Triclosan	3380-34-5	Disinfectant	A	7.90	7.2*	83.37%	5.35	2.53[l]	0.27	baseline
Specific mode of action										
Nicotine	54-11-5	Pharmaceutical / Pesticide	B	8.50	7.46*	8.36%	-0.20	0.74[a]	1482	s
All-trans retinoic acid (ATRA)	302-79-4	Pharmaceutical	A	4.40	6.8-8.0	0.03%	3.70-4.22	4.59[m]	418-1348	s

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Metam-sodium hydrate	6734-80-1	PPP / various	A	2.99	7.2*	0.01%	1.04	1.32[l]	320	s
Strychnine hydrochloride	1421-86-9	Pharmaceutical	B	8.26	7.46*	13.68%	1.24	1.22[a]	162	s
Aconitine	302-27-2	Pharmaceutical	B	8.11	7.46*	18.29%	1.49	1.30[a]	110	s
cis-4-cyano-4-(1-cyclohexyl-3-ethyl-1H-indazol-6-yl)cyclohexanecarboxylic acid	199171-88-5	Pharmaceutical	A	4.66	7.2*	0.29%	2.90	2.69[o]	107	s
Maneb	12427-38-2	Herbicide	A	3.21	6.5-7.8	0.02%	2.49	2.16[p]	81	s
Coniine	3238-60-6	Pharmaceutical	B	11.10	7.46*	0.02%	0.79	0.37[a]	64	s
Salicylic acid	69-72-7	Pharmaceutical	A	2.97	7.46*	0.00%	0.97	0.47[a]	53	s
Salicylic acid	69-72-7	Pharmaceutical	A	2.97	4.5-7.4	0-3%	0.97-1.31	0.78[b]	51-110	s
Diclofenac sodium	15307-79-6	Pharmaceutical	A	3.99	7.2*	0.06%	2.66	1.97[o]	36	s
Acetyl salicylic acid	50-78-2	Pharmaceutical	A	3.49	4.5-7.4	0-9%	1.23-1.31	0.57[b]	31-37	s
Atropine	51-55-8	Pharmaceutical	B	9.43	7.46*	1.06%	0.55	-0.32[a]	23	s

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All-trans retinoic acid (ATRA)	302-79-4	Pharmaceutical	A	4.40	7.2*	0.16%	3.96	2.69[o]	10	-
Ropinirole hydrochloride	91374-20-8	Pharmaceutical	B	9.18	7.46*	1.87%	1.16	-0.17[a]	8	baseline
Valproate Sodium	1069-66-5	Pharmaceutical	A	4.60	6.8-8.0	0-1%	1.41-1.48	0.08[m]	6.7-7.9	baseline
Propranolol	318-98-9	Pharmaceutical	B	9.53	7	0.29%	2.61	0.89[c]	3	baseline
Metoprolol	56392-17-7	Pharmaceutical	B	9.68	7	0.21%	1.43	-0.28[c]	3	baseline
Morphine	57-27-2	Pharmaceutical	B	8.21	7.46*	15.10%	0.74	-1.32[a]	1	baseline
5HPP-33 (thalidomide derivate)	105624-86-0	Pharmaceutical / biochemistry	A	7.78	7.2*	79.17%	4.40	1.69[o]	0.35	baseline
unknown										
Dimethylaminoethanol	108-01-0	Industrial chemical	B	9.23	7.2*	0.92%	-0.54	1.69[o]	28120	sor
Phthalic acid, mono-2-ethylhexyl ester	4376-20-9	Industrial chemical	A	3.60	7.2*	0.03%	2.02	3.25[l]	2933	sor
Imazamox	114311-32-9	Herbicide	A	3.85	7.2*	0.04%	1.29	2.29[l]	1697	sor

Chemical	CAS	Use Group	acid or base	pK_a	pH or pH-range	$f_{neutral}$ (pH-range)	log D_{lipw} (pH) (range if a pH range was given)	log $1/LC_{50}$ (mM)	TR (range)	FET MoA classification
ethyl [(7-{[2-(3-chlorophenyl)-2-hydroxyethyl]amino}-5678-tetrahydronaphthalen-2-yl)oxy]acetate hydrochloride	929601-09-2	Industrial chemical	B	9.90	7.2*	0.20%	1.50	1.73[o]	291	sor
Toltrazuril	69004-03-1	Pharmaceutical	A	6.79	7.6	13.41%	2.65	2.59[e]	150	sor
Cyclanilide	113136-77-9	Herbicide	A	3.80	7.2*	0.04%	1.80	1.69[o]	134	sor
Propoxycarbazone-sodium	181274-15-7	Herbicide	A	2.10	7.2*	0.00%	1.41	1.25[l]	117	sor
Fenamidone	161326-34-7	Fungicide	B	7.77	7.2*	21.21%	2.73	2.53[l]	109	sor
Cyclanilide	113136-77-9	Herbicide	A	3.80	7.2*	0.04%	1.80	1.57[l]	102	sor
Primisulfuron-methyl	86209-51-0	Herbicide	A	3.47	7.2*	0.02%	2.76	2.29[l]	59	sor
2,4-DB (Butyrac)	94-82-6	Herbicide	A	4.95	7.2*	0.56%	2.06	1.59[l]	58	sor
Fenamidone	161326-34-7	Fungicide	B	7.77	7.2*	21.21%	2.73	2.04[o]	36	sor
Sulfentrazone	122836-35-5	Herbicide	A	6.56	7.2*	18.64%	2.57	1.34[l]	10	baseline

Chemical	CAS	Use Group	acid or base	pK_a	pH or pH-range	$f_{neutral}$ (pH-range)	log D_{lipw} (pH) (range if a pH range was given)	log $1/LC_{50}$ (mM)	TR (range)	FET MoA classification
Imazalil	35554-44-0	Fungicide	B	6.49	7.2*	83.68%	3.91	2.51[l]	7	baseline
Fenhexamid	126833-17-8	Fungicide	A	7.30	7.2*	55.73%	3.46	2.05[l]	7	baseline
Picloram	1918-02-1	Herbicide	A	2.30	6.8	0.00%	1.81	0.33[i]	6	baseline
Trichloroacetic acid	76-03-9	Industrial chemical	A	0.51	7.46*	0.00%	1.94	0.39[a]	5	baseline
Ethambutol dihydrochloride	1070-11-7	Pharmaceutical / antimycobacterial	B	9.30	7.46*	1.42%	0.72	-1.19[a]	2	baseline
2,4-Dichlorophenoxyacetic acid	94-75-7	Herbicide	A	2.58	7.5	0.00%	1.70	-0.26[p]	2	baseline
[2-({[2-(4-tert-Butyl-13-thiazol-2-yl)-1-benzofuran-5-yl]oxy}methyl)phenyl]acetic acid	149413-74-1	Industrial chemical	A	4.09	7.2*	0.08%	3.76	1.70[o]	2	baseline
Imazalil	35554-44-0	Fungicide	B	6.49	7.2*	83.68%	3.91	1.69[o]	1	baseline
Niclosamide	50-65-7	Pharmaceutical / antihelminthic	A	7.15	7.2*	47.12%	5.25	3.00[l]	1	baseline

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1'-{2-[2-(34-dichlorophenyl)-4-(phenylcarbonyl)morpholin-2-yl]ethyl}-NN-dimethyl-14'-bipiperidine-4'-carboxamide hydrochloride	NOCAS_47353	Industrial chemical	B	7.40	7.2*	38.69%	4.30	1.72[o]	0.47	baseline
Spiroxamine	118134-30-8	Fungicide	B	6.90	7.2*	66.61%	4.88	1.11[l]	0.03	-
Dodecylbenzenesulfonic acid	27176-87-0	Industrial chemical	A	0.70	7.2*	0.00%	5.58	1.69[o]	0.02	-