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Using membrane–water partition coefficients in a critical membrane burden approach to aid the identification of neutral and ionizable chemicals that induce acute toxicity below narcosis levels†

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The risk assessment of thousands of chemicals used in our society benefits from adequate grouping of chemicals based on the mode and mechanism of toxic action (MoA). We measure the phospholipid membrane–water distribution ratio (D_{MLW}) using a chromatographic assay (IAM-HPLC) for 121 neutral and ionized organic chemicals and screen other methods to derive D_{MLW} . We use IAM-HPLC based D_{MLW} as a chemical property to distinguish between baseline narcosis and specific MoA, for reported acute toxicity endpoints on two separate sets of chemicals. The first set comprised 94 chemicals of US EPA's acute fish toxicity database: 47 categorized as narcosis MoA, 27 with specific MoA, and 20 predominantly ionic chemicals with mostly unknown MoA. The narcosis MoA chemicals clustered around the median narcosis critical membrane burden (CMB_{narc}) of 140 mmol kg⁻¹ lipid, with a lower limit of 14 mmol kg⁻¹ lipid, including all chemicals labelled Narcosis_I and Narcosis_II. This maximum 'toxic ratio' (TR) between CMB_{narc} and the lower limit narcosis endpoint is thus 10. For 23/28 specific MoA chemicals a TR >10 was derived, indicative of a specific adverse effect pathway related to acute toxicity. For 10/12 cations categorized as "unsure amines", the TR <10 suggests that these affect fish via narcosis MoA. The second set comprised 29 herbicides, including 17 dissociated acids, and evaluated the TR for acute toxic effect concentrations to likely sensitive aquatic plant species (green algae and macrophytes *Lemna* and *Myriophyllum*), and non-target animal species (invertebrates and fish). For 21/29 herbicides, a TR >10 indicated a specific toxic mode of action other than narcosis for at least one of these aquatic primary producers. Fish and invertebrate TRs were mostly <10, particularly for neutral herbicides, but for acidic herbicides a TR >10 indicated specific adverse effects in non-target animals. The established critical membrane approach to derive the TR provides for useful contribution to the weight of evidence to bin a chemical as having a narcosis MoA or less likely to have acute toxicity caused by a more specific adverse effect pathway. After proper calibration, the chromatographic assay provides consistent and efficient experimental input for both neutral and ionizable chemicals to this approach.

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Environmental significance

To prioritize more detailed risk assessment for certain chemicals of concern, it is important for risk assessors to identify chemicals that induce toxicity by an adverse effect pathway other than narcosis. Our study shows that the membrane lipid–water distribution ratio (D_{MLW}) is a key descriptor for both neutral and ionizable organic chemicals. By measuring new D_{MLW} values for 121 chemicals we derive the critical membrane burden (CMB) threshold for fish acute toxicity below which a specific mode of action other than narcosis drives toxicity and use this CMB approach on the response of different (non-)target species to a variety of herbicides.

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1. Introduction

(i) Mode of action analysis to support priority setting and risk assessment

With increasing regulatory and ethical drive to accelerate and strengthen progress on chemical management and at the same time reduce reliance on *in vivo* data to fill data gaps, there is now an increased emphasis on the use of alternative approaches and data sources to support decision making.^{1,2} The approach taken to chemical management varies from region to region. However, widely applicable methods such as chemical grouping are employed under many regulatory schemes such as for read-across and data-gap filling under the EU REACH regulation where ECHA developed a Read-Across Assessment Framework (RAAF).³ One of the critical components for successful chemical grouping is an understanding of both the modes and mechanisms of action (MoA and MechoA) of the target and analogue structures in addition to comparable structural and bioavailability properties. If a chemical category includes members with disparate modes and mechanisms of action (including metabolites of parent chemicals in the category, if possible), the justification of the read-across for hazard information becomes less certain and may lead to false positives or false negatives.

The consideration of modes and mechanisms of action can also be very useful as one descriptor of hazards when prioritizing chemicals for further regulatory action. In Canada, for example, identifying organic chemicals with specific and non-specific modes and mechanisms of action was incorporated into version 1.0 of the Ecological Risk Classification (ERC1) approach^{2,4} used in 2016 by Environment and Climate Change Canada (ECCC) to reset priorities for 640 organic chemicals for phase three of the Chemicals Management Plan (CMP). ERC1 introduced the concept of data consensus weighting between critical body residue (CBR) derived toxicity ratios (TRs) and quantitative structure–activity relationship (QSAR) classification of MoA as one hazard descriptor for priority setting in ERC1. Specific modes of action were responsible for 40% of the high hazard classifications (*i.e.*, not final risk classification) identified in 2016 by ECCC using ERC1.

Building on ERC1, version 2.0 of the ERC⁵ (ERC2) was developed in 2018 and is currently in use by ECCC for identifying chemicals of concern among 12 200 organic chemicals for post 2020 work planning reasons. ERC2 is a weight of evidence logical model relying on data consensus to determine the risk classification, risk confidence and risk scale of organic chemicals for further regulatory consideration. ERC2 takes the MechoA concept further than ERC1 by expanding the number and type of tissue residue and QSAR approaches used for identifying specific and non-specific modes of action. ERC2 also integrates both molecular initiating event information (MIE) and modes and mechanisms of action using the adverse outcome pathway (AOP) concept.⁶

The degree of MoA consensus in ERC2 was also evaluated by comparing MoA classifications from the five MoA QSAR and five methods used to calculate tissue residue TRs associated with median lethality in fish² in ERC2. The confidence score

associated with the MoA classification in ERC2 is directly related to the degree of consensus between all methods above. The results, based on 929 organic chemicals with available averaged acute fish median lethality data gathered using the OECD QSAR Toolbox,^{7,8} revealed that 100% consensus between all ten methods was greater for non-specific (narcosis) chemicals (~53%) than those with specific modes of action (~29%). There was no large distinction in MoA classification among the five tissue residue methods and the authors suggest further curation of aquatic toxicity and water solubility data may improve the correlation among all methods.

A consensus MoA approach has also been incorporated into automated on-line tools for determining predicted no-effect concentrations (PNECs) for risk assessment using the Ecological Threshold of Toxicological Concern (Eco-TTC) and curated ENVIROTOX database tools.^{9–11} MechoA considerations have also been incorporated into the derivation of assessment (safety) factors for PNEC derivation used in risk assessments of new and existing substances by ECCC. The evaluation of modes and mechanisms of action is reviewed and documented to ensure that the selection of critical toxicity values used to derive a chronic PNEC are related to the mode and mechanisms of action.

A number of schemes exist to support the identification of the MoA of chemicals. Typically schemes such as those developed by Verhaar and subsequent updates,^{12–14} the EPA Mode of Action and Toxicity (MOAtox) database¹⁵ and the Acute Aquatic Toxicity MOA by OASIS (AAT OASIS) scheme,¹⁶ also incorporated into the US EPA inhouse expert system ASTER (ASsessment Tools for the Evaluation of Risk¹⁷) and the US EPA Toxicity Estimation Software Tool (TEST¹⁸), are easily accessible for such an application. The Verhaar and OASIS schemes also have been incorporated into the OECD QSAR toolbox as mechanistic profilers. More recently there has been a growing recognition that classifying chemicals using the mechanism of action can add more confidence in toxicity prediction.¹⁹ The MechoA scheme as one such example has recently been developed and available freely online is the KREATIS MechoA scheme²⁰ to predict the toxicity mechanism based on the chemical structure.^{21,22} The recent scheme of Sapounidou *et al.*^{23,24} is another such example which follows an analogous approach to the MechoA scheme. However, it remains that such schemes have potential for discrepancies in assigned MoA and also have limitations in being able to classify the full chemical space.⁹ With the benefits of having a reliable understanding of MoA for supporting chemical prioritization and risk assessment in addition to data gap filling, there is, therefore, a continued need to develop consensus on MoA.¹⁰ The development of new and complementary approaches to support MoA is thus needed.

In the current study, we investigate the expansion of an existing approach considering the application of the critical body residue (CBR) and critical membrane burden (CMB) to aid the determination of MoA. Specifically, we consider the role of the membrane lipid–water distribution ratio (D_{MLW} , as used for ionizable species, or K_{MLW} for neutral species) to distinguish between chemicals that operate in the range of baseline toxicants from chemicals which are likely to induce toxicity *via*



another MoA at a CMB lower than typical for narcosis. Since the cell membrane is not the target site for most specific adverse effect pathways (e.g., covalent interactions with DNA), the D_{MLW} approach is not intended to classify a chemical to a certain MoA. Our approach can be used to identify chemicals for which further information on MoA would be considered of high relevance for risk assessment, because they do not induce acute toxicity by baseline narcosis. The key aim is thus to define the lower limit CMB of polar and non-polar narcotic chemicals. D_{MLW} can be derived using a range of experimental methods (e.g. unilamellar liposome vesicles²⁵ or solid supported lipid membranes^{26,27}), or *in silico* approaches, such as COSMOmic^{28,29} or molecular dynamics.^{30–32} Here, we consider the use of a chromatographic column retention approach to derive D_{MLW} values for MoA determination. The overall aim was to generate two strategic D_{MLW} data sets that would allow for MoA assessment with high quality toxicity data, extending previous efforts in multiple aspects in terms of both chemical space, D_{MLW} data quality, and MoA domains. The first data set focuses on chemicals listed in the acute fish toxicity data from EPA's Fathead minnow (FHM) database. The second set involves herbicides for which toxicity data for several types of aquatic species are compared. Before discussing the experimental part and discuss the data interpretation, below we briefly introduce in Section (ii) the chromatographic approach and comparable studies that precede the current work, (iii) the link between D_{MLW} and toxicity, (iv) alternative ways to derive D_{MLW} , and (v) which toxicity data sets were used to evaluate the CMB-MoA analysis. The method section then starts with explaining which chemicals from those toxicity databases were selected to obtain the chromatographic retention data for.

(ii) Chromatographic retention measurements on phospholipid coated particles

Chromatographic retention time measurements allow for consistent experimental values of physico-chemical descriptors for a wide range of organic chemicals, because the retention capacity factors are proportional to the interaction energy with the column's coating material.^{33–42} The development of silica

particles coated with an immobilized artificial phospholipid membrane for use in high performance liquid chromatography (IAM-HPLC)^{43–46} has enabled measurements of retention capacity factors (k_{IAM}) that closely relate to descriptors that are valuable for drug development, toxicokinetic modeling and chemical risk assessment in general.⁴⁷

IAM-HPLC provides a cost-effective, high-throughput, consistent approach to experimentally derive indicative D_{MLW} values directly from these k_{IAM} measurements. Using k_{IAM} obtained in (or extrapolated to) fully aqueous eluent (i.e., k_{IAM}^0), multiplication with a pre-determined medium/phospholipid volume ratio of the IAM-column ($\varphi = 18.9$ (ref. 45)) gives the partition coefficient between the bulk aqueous eluent and IAM phospholipid monolayer (K_{IAM}), which should be analogous to D_{MLW} :

$$D_{MLW} - K_{IAM} = \varphi \times k_{IAM}^0 = 18.9 \times k_{IAM}^0 \quad (1)$$

A review on liposomal partition coefficients showed a linear relationship between D_{MLW} values and IAM-HPLC retention capacity factors (k_{IAM}) for 24 neutral organic chemicals, as shown in Fig. 1 by black squares (k_{IAM} already converted to K_{IAM} partition coefficients by using eqn (1)).²⁵ For most chemicals, the IAM-HPLC estimate was within a factor of ± 3 , but for some neutral chemicals with high H-bond donor capacities the K_{IAM} overestimated D_{MLW} by a factor of ~ 10 . Two studies, on anionic surfactants²⁷ (including perfluorinated anions) and cationic surfactants²⁶ (including quaternary ammonium chemicals), both determined K_{IAM} values and D_{MLW} (via solid supported lipid membranes), which extends the D_{MLW} - K_{IAM} comparison to also include ionic chemicals. The 19 cationic surfactants²⁶ are shown in Fig. 1 as green triangles, and the 10 (fluorinated and non-fluorinated) anionic surfactants as red dots. Since the aim is to derive quantitative and precise D_{MLW} values from k_{IAM} for the CMB approach, and not work with relative chromatographic indices, we need to take into account that the silica particles used in the IAM-columns cause confounding electrostatic effects on the retention time of ionic chemicals. Only intrinsic IAM phospholipid-partition coefficients ($K_{IAM, intr}$) are to be used for predominantly/fully ionized chemicals, which are corrected

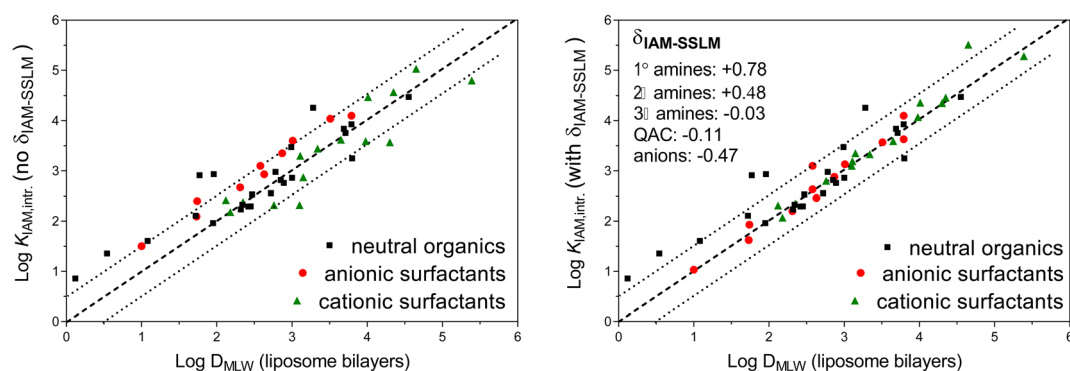


Fig. 1 Liposome membrane–water distribution ratios (D_{MLW}) for neutral organics and surfactants plotted against (left) intrinsic (i.e. corrected for confounding electrostatic surface attraction/repulsion for ionic chemicals): IAM coating partition ratios ($K_{IAM, intr}$); (right) $K_{IAM, intr}$ values corrected for empirical increments ($\delta_{IAM-SSLM}$) reported for specific types of ionic chemicals. The broken line indicates unity between $K_{IAM, intr}$ and D_{MLW} (starting in 0,0), and the dotted lines represent a 0.5 log unit (factor of 3.3) difference.



for this confounding attraction using empirically based or theoretically derived Boltzmann corrective factors.⁴⁸ This Boltzmann correction is in more detail reviewed for dissociated acids and protonated bases in ESI Section S1.† While the neutral data in Fig. 1 were obtained from a wide variety of studies and different experimental approaches to derive D_{MLW} and K_{IAM} , the scatter between the series of K_{IAM} and D_{MLW} values obtained for ionic surfactants was obtained in a single research institute. Consistent empirical differences for K_{IAM} and D_{MLW} for specific types of ionic moieties on the surfactant data indicated that it may be necessary to adjust the IAM-HPLC results by several empirical increments ($\delta_{IAM-SSLM}$) to improve the K_{IAM} – D_{MLW} consistency, as shown in the plot on the right in Fig. 1: -0.5 log units for all anionic chemicals, $+0.8$ log units for primary (1°) amine cations, $+0.5$ log units for secondary (2°) amine cations, -0.03 log units for tertiary (3°) amine cations, and -0.1 log units for quaternary ammonium cations (see also ESI-Table S3†). The reasons for these ionic-type specific increments to align the phospholipid-coating based K_{IAM} data and phospholipid bilayers based D_{MLW} data remain to be elucidated.

The primary goal of the current study was to use a measured D_{MLW} value to predict the baseline toxicity to aquatic organisms for a large and diverse set of organic chemicals, and identify whether a chemical is not a baseline toxicant and very likely induces acute toxicity by a reactive or more specific MoA. Since IAM-HPLC allows for high-throughput and experimental consistency, the K_{IAM} values obtained by this method are considered to be the best D_{MLW} proxies to do so. Several studies have already demonstrated that k_{IAM} values (*i.e.*, IAM-HPLC retention capacity factors) strongly correlate with the acute effect concentrations of non-specific toxicants to aquatic organisms ($LC_{50,Narcosis}$),^{49–54} for example tadpoles (*Rana temporaria*, from the data set on narcotics by Overton and Meyer) and the fathead minnow fish (*Pimephales promelas*). The set-up of these valuable k_{IAM} – $LC_{50,Narcosis}$ comparative studies, however, are limited in several aspects, and we aimed to extend these aspects in this study. First, while the reported studies used k_{IAM} retention capacity factors that were based on measurements with eluent that contained 40% acetonitrile, the current study aims to derive new IAM-retention data for a large number of strategic chemicals (regarding available toxicity data, ionization, and data scarcity) obtained at, or adequately extrapolated to, fully aqueous eluent. As a result, our IAM-HPLC data set allows for simple conversion of k_{IAM}^0 (0% solvent, 100% water) to K_{IAM} values that are direct proxies for D_{MLW} , rather than using k_{IAM} as a relative scaling index. Second, whereas the reported studies included only neutral chemicals, the current study aimed to include largely ionized organic bases (or permanently charged cations) and strong organic acids in toxicity evaluations. The K_{IAM} – D_{MLW} data set on ionic surfactants allows for a validation set in deriving the intrinsic K_{IAM} values to relate to D_{MLW} values for ionic species (Fig. 1). Third, while the reported studies included only chemicals with narcosis MoA, we aim to include chemicals with proven specific strongly toxic MoA alongside those with only narcosis MoA. This should exemplify the extent to which IAM-based D_{MLW} values

accurately distinguish specific MoA and narcosis MoA chemicals.

(iii) D_{MLW} values predict baseline toxicity

Like all D_{MLW} proxies, IAM-HPLC based retention capacity factors can provide a reasonable estimate of the dissolved concentration of a chemical that is toxic, because organic chemicals are toxic at a certain accumulated cellular concentration. For a wide variety of organic chemicals considered to only act through a non-specific “narcosis” MoA, the total critical body residue (CBR_{wet} , in $mmol\ kg^{-1}$ wet weight) is found to be in the range of 2–8 $mmol\ kg^{-1}$ for a range of different aquatic organisms.^{55–57} Narcosis MoA is most often related to a critical accumulation level of molecules in cell membranes, which relates to the “baseline toxicity” for all chemicals. At the critical membrane burden (CMB, in $mmol\ kg^{-1}$ phospholipid) basic cellular membrane functions become impaired.⁵⁶ The relationship between CBR_{wet} and CMB can be expressed as the ratio between the two driving partition coefficients.

$$\frac{CMB}{CBR_{wet}} = \frac{D_{MLW}}{K_{organism/water}} \quad (2)$$

According to partition coefficients to different tissue phases, membranes being one specific component besides for example storage lipids, carbohydrates, (structural) proteins, and water,^{58–62} it was derived from a reviewed set of CBR data for narcosis chemicals that CMB_{narc} ranges between 80 and 250 $mmol\ kg^{-1}$ membrane, with a geometric mean of 140 $mmol\ kg^{-1}$.⁶³

Using this narrow range of CMB_{narc} , dissolved concentrations that are acutely lethal to 50% of aquatic organisms due to narcosis ($LC_{50,narc}$) can be back-calculated for any chemical using D_{MLW} , as defined by the target lipid model by Di Toro *et al.* (2000):⁶⁴

$$LC_{50,narc} \text{ (in } mmol\ L^{-1} \text{ water)} = CMB_{narc} \text{ (} \sim 140\ mmol\ kg^{-1} \text{ membrane)} / D_{MLW} \text{ (in L water per kg membrane)} \quad (3)$$

Chemicals that are considered to distinctively exert acute toxicity by a MoA other than narcosis are expected to have a critical membrane burden significantly below 140 $mmol\ kg^{-1}$, and hence have an acute (lethal) effect concentration well below the D_{MLW} -calculated $LC_{50,narc}$. It is important for risk assessors to identify this level of endpoint specificity, as near baseline cytotoxic levels many specific cellular pathways will also be affected, as a so-called “cytotoxic burst”.^{65,66} The primary aim of the current study was to derive a minimum CMB_{narc} for as large a set of chemicals as possible classified as narcotics, with the cell membrane as the target site of action for narcosis, and a measured membrane–water partition coefficient. Any organic chemical is likely to induce acute toxicity due to baseline narcosis above this range, whereas chemicals that act *via* a specific mechanism of action are expected to display a CMB below this minimum CMB_{narc} and thus indicative of a specific MoA driving the observed acute toxicity. Note that the chronic MoA to aquatic organisms is not taken into account in this



CMB-MoA approach. For most baseline toxicants the acute to chronic ratio (ACR) is small ($\sim 10\times$), and the chronic CMB_{narc} is thus not much lower than the acute CMB_{narc} . This suggests that below the threshold of basic membrane disturbance the exposed organism may withstand this toxic pressure for a prolonged period, with a certain loss of energy used for maintenance. However, a small fraction of chemicals identified as baseline toxicants were found to have an acute to chronic ratio of more than 30, indicating that upon chronic exposure these chemicals may act *via* a specific MoA.⁶⁷ It is therefore not possible to rely on the current selection of chemicals classified by acute effect studies.

A prediction of the baseline toxic concentration ($LC_{50,narc}$) of any organic chemical is relevant for risk assessment. First, it easily translates to an initial approach to set maximal allowable concentrations for chemicals for which no toxicity data are available. Second, it could be a check whether the adverse effect concentration reported for a certain chemical is due to a specific MoA, or whether the adverse effect occurred at a level where baseline toxicity is expected (apparently specific effects may have occurred only as part of the cytotoxic burst).⁶⁸ Third, most environmental pollution occurs as complex chemical mixtures, and in most cases chemicals with a specific MoA are dissolved at concentrations well below the level that induces a specific effect. However, any chemical in a mixture contributes to the accumulation of chemicals in membranes, and each chemical therefore contributes to the narcosis CMB of the total mixture in a (molar) concentration-additive way.^{69–71}

The CMB approach is generally evaluated using the octanol–water partition coefficient (K_{OW}) as a proxy for D_{MLW} , using toxicity data sets for algae, daphnids, and fish.^{57,64} Baseline toxicity has also been expressed as a function of chemical activity, using the maximum water solubility (S_w) in relation to the LC_{50} as a metric to assess the MoA.⁷² There are relevant uncertainties related to using both the K_{OW} and S_w that could lead to false classification of chemicals having or not having a specific MoA.⁷³ This involves uncertainties surrounding the K_{OW} and S_w values, but also the relevance of these values in relation to the D_{MLW} values driving the actual CMB, as discussed below. The approach of the current study aims to obtain D_{MLW} experimentally, which would by-pass several uncertainties relating to K_{OW} and S_w and thereby assess MoA specificity with a higher level of confidence.

(iv) Different ways to derive K_{MLW}/D_{MLW}

There are multiple approaches of deriving the D_{MLW} in eqn (2) and (3), either by experiment or calculations. Below, we first describe commonly used and more recent ways to calculate sorption affinity to phospholipid membranes ('phospholipophilicity'). For many neutral organic chemicals, K_{MLW} values are closely related to octanol–water partition coefficients,²⁵ with a nearly 1 : 1 relationship based on a structurally diverse K_{MLW} data set:

$$\log K_{MLW} = 1.01(\pm 0.02) \times \log K_{OW} + 0.12(\pm 0.07) \quad (n = 156, SD = 0.426, R^2 = 0.948) \quad (4)$$

Although accurate K_{OW} values can be obtained according to standardized protocols, there can be wide margins in reported values for many chemicals. And although large experimental K_{OW} data sets have been used to create a variety of commonly used predictive algorithms, predicted K_{OW} values further contribute to uncertainty in derived K_{MLW} values according to eqn (4). Octanol also does not necessarily reflect the specific interactive properties of neutral chemicals with phospholipids in a cell membrane. Therefore, a phospholipid–water specific poly-parameter linear free energy relationship (ppLFER) has been constructed (eqn (5)).²⁵ This ppLFER uses five chemical descriptors that should adequately cover the contribution of different solute/system interactions: molecular volume (V_x), hydrogen-bond acidity (A) and basicity (B), hexadecane–air partition coefficients (L), and a parameter to account for excess polarizability (S).

$$\log K_{MLW} (25^\circ C) = 1.65(\pm 0.17) \times V_x + 0.55(\pm 0.03) \times L - 0.95(\pm 0.09) \times S - 0.05(\pm 0.09) \times A - 4.02(\pm 0.10) \times B + 0.48(\pm 0.09) \quad (n = 131, SD = 0.294, R^2 = 0.977) \quad (5)$$

While V_x is calculated, the descriptors S , A , and B are recommended to be derived experimentally from consistent column retention studies, as collected in the UFZ LSER database (<https://www.ufz.de/lserd>), as to avoid stacking of estimation uncertainties for each descriptor. So instead of the more generic hydrophobicity descriptor K_{OW} , more accurate values can be calculated *via* ppLFER if descriptors are available. This indeed requires the experimental ppLFER descriptors S , A , B and L to be available or to be newly derived, *e.g.* from multiple chromatographic retention data.

For ionizable organic chemicals (IOCs), however, neither K_{OW} nor the ppLFER approach adequately takes into account the ionic interactions between charged sites in the phospholipid headgroup domain with charged groups in ionized organic chemicals. The octanol–water distribution ratio ($\log D$) of the largely ionized form of a strong acid ($pK_a \ll$ testing pH, *e.g.* <3 units) or strong base ($pK_a \gg$ testing pH, *e.g.* >3 units) is mostly several orders of magnitude lower than the octanol–water partition coefficient ($\log P$) of the fully neutral acid or base, because the ionized form strongly prefers the readily polarizable aqueous phase.^{74,75} Moreover, the affinity of the ionic form for octanol strongly depends on the presence and concentration of counterions. However, the majority of cell membrane phospholipids contain a zwitterionic phosphatidylcholine headgroup. In anisotropically organized phospholipid layers of liposomes and cell membranes, the zwitterion moieties together form a partially hydrated headgroup 'region', which shields off the highly hydrophobic 'region' formed by the densely packed fatty acid tails from the bulk water phase. Due to strongly favorable ionic interactions with the zwitterionic headgroups, combined with partial embedding in the hydrophobic bilayer core, ionized forms of many organic bases and organic acids have a phospholipid membrane–water distribution ratio ($D_{MLW,ion}$) only marginally lower than, or even equal to, the $K_{MLW,N}$ of their corresponding neutral forms.^{29,76–81}



The affinity of ionic organic chemicals for phospholipid membranes can be estimated rather crudely for both predominantly charged acids and bases as a first approach.⁸²

$$\log D_{\text{MLW,ion}} = \log K_{\text{MLW,N}} - 1 \quad (6)$$

However, a single increment between the D_{MLW} values of the ionic and neutral forms is overly simplistic, and can be further refined for different ion types. Based on rather small sample sizes, ion-type specific scaling factors (Δ_{MW}) have been derived according to eqn 7

$$\log D_{\text{MLW,ion}} = \log K_{\text{MLW,N}} - \Delta_{\text{MW}} \quad (7)$$

The Δ_{MW} describes the average difference between $\log D_{\text{MLW,ion}}$ and $\log K_{\text{MLW,N}}$: -0.75 for phenolates, -2 for all other anionic chemicals, -0.3 for primary amines, -0.5 for secondary amines, and -1.25 for tertiary amines and other cationic chemicals.^{80,81,83–85}

After defining the D_{MLW} of both ionic and neutral forms, the pH-dependent fractions of neutral forms (f_{N}) and ionic forms ($1 - f_{\text{N}}$), the overall distribution ratio at a certain pH ($D_{\text{MLW(pH)}}$) can be calculated according to eqn (8):

$$D_{\text{MLW(pH)}} = f_{\text{N}} \times K_{\text{MLW,N}} + (1 - f_{\text{N}}) \times D_{\text{MLW,ion}} \quad (8)$$

where the pH and dissociation constant ($\text{p}K_{\text{a}}$) define f_{N} according to the Henderson–Hasselbalch eqn (9):

$$f_{\text{N}} = \frac{1}{1 + 10^{\alpha(-\text{pH} + \text{p}K_{\text{a}})}} \quad (9)$$

in which $\alpha = 1$ for bases and -1 for acids.

While the Δ_{MW} -approach takes the specific ion-type differences into account, the $K_{\text{MLW,N}}$ is often still based on K_{OW} values and eqn (4). For certain chemicals, particularly for IOCs, it becomes relevant that octanol is a bulk solvent, while phospholipid membranes are anisotropically structured. The charged moiety will favorably position in the headgroup region while the most hydrophobic molecular portion will extend into the core, and this position may strongly differ for the neutral form of the same IOC. Computational methods such as the COSMOmic and COSMOconf modules of the commercial software package COSMOlogic (3ds Dassault systèmes/BIOVIA) and molecular dynamics simulations can be of use in spatially oriented prediction of D_{MLW} . COSMOmic combines quantum chemistry and thermodynamics and uses the three-dimensional (3D) structure of both the solute and the hydrated phospholipid membrane. The internal membrane potential and surface charge density distributions of ionogenic chemicals as well as the phospholipid structure can also be considered.^{26,29,86,87} Molecular dynamics simulations consider the conformation of, and interactions between, all compounds in the membrane, water and solute, and can also be used in the study of the impact of conformation on D_{MLW} .

Experimental measurements on phospholipophilicity may be preferred over descriptor calculated values for chemicals of environmental concern that require higher tier level risk assessment, particularly for ionizable chemicals. Many

pharmaceuticals and illicit drugs are ionizable chemicals,⁸⁸ as well as a considerable fraction of high production volume chemicals for which chemical fate and hazard assessment needs to be more detailed.⁸⁹ Using the neutral form of an acid or base to calculate the phospholipophilicity of the anionic or cationic species, *e.g.* via eqn (4) + (6), or (5) + (6), entails considerable uncertainty. Although various batch tests with pure artificial phospholipids are possible,^{79,90–92} no widely recognized testing protocols, such as OECD or ASTM, are available. Chromatographic methods, however, are already standardized in OECD guideline 117 to determine octanol–water distribution ratios⁹³ and OECD 121 to screen for soil organic carbon sorption affinities. Retention on a commercially available HPLC-column with an immobilized artificial membrane (IAM) facilitates consistent measurements of large numbers of chemicals, with simple HPLC systems pumping aqueous eluent into various detectors (RI, ELSD, UV, FLU, and MS/MS).^{43–46} The experimentally feasible range covers $\log D_{\text{MLW}}$ 0–6, depending on detection limits for the strongest sorbing chemicals.^{94–96} Although the silica IAM packing can have confounding coulombic electrostatic effects on the retention of anions and cations, as discussed in Section S1,[†] this can be corrected for by empirical or modeled Boltzmann factors.^{27,48,97} Consistent IAM-based D_{MLW} data sets have been derived for both cationic (>150 chemicals^{26,77,78,98,99}) and anionic chemicals (>20 (ref. 27 and 100)).

(v) Rationale for selecting toxicity data and chemicals to derive K_{IAM}

In order to evaluate whether the CMB-approach can adequately distinguish chemicals with different MoA based on K_{IAM} values, the collection of toxicity data should be of high quality. Ideally, this experimental toxicity data are obtained using a standardized test protocol, measured exposure concentrations, and consistent handling of test procedures, and have minimum uncertainty margins due to differences between species of the same type, *e.g.* fish.

The first MoA-evaluation approach we selected was to use a widely recognized consistent database of acute toxicity values on a single fish species, performed by a single institute with measured exposure concentrations. The United States Environmental Protection Agencies Fathead Minnow (FHM) database¹⁶ provides such MoA data for 616 organic chemicals. We selected 74 neutral chemicals for IAM-measurement: 47 narcotics, 27 with a specific MoA. One of the other criteria for selecting neutral chemicals was that we wanted to address the scarcity of physicochemical information for many chemicals in the FHM set, and selected only chemicals for which no other estimate of K_{MLW} was available other than (estimates of) K_{OW} , as discussed in the Methods section in more detail.

The second MoA-evaluation approach selected was to use chemicals that are designed to exert specific MoA on a certain type of species, while they are expected to have only baseline effects (non-specific MoA) for non-target species. For pesticides, standard toxicity tests on different representative species under strict testing protocols are mandatory in the regulatory process.



Also, from the interest of (eco)toxicological assessment, typically pesticides have high quality toxicity data sets on diverse test organisms that allow for adequate comparisons. In this case, we performed IAM-measurements for 12 neutral herbicides as well as for a set of 17 strongly acidic herbicides, which under physiological pH exist for >99.9% as organic anions. For these herbicides, we used the CMB_{narc} approach to determine to what extent toxicity to 'likely target' organisms was more specific than toxicity to 'non-target' organisms. For example, algae are likely affected specifically by herbicides, whereas fish are hopefully only reacting non-specifically to herbicides at levels predicted by the CMB_{narc} approach.

2. Materials and methods

(i) Chemical selection

The chemical selection was primarily limited by the toxicity data set used for CMB evaluation, but furthermore based on the following criteria and goals: ease of purchase in adequate purity, ease of detection based on expected retention time and detector sensitivity, the goal of extending the k_{IAM} database with new chemicals,⁹⁴ the goal of including a substantial number of ionizable acids and bases for which also liposome based $D_{MLW,ion}$ data exist for comparison with k_{IAM} , and the goal of covering a sufficient number of chemicals to compare narcosis I and II (set 1), to compare narcosis with several specific MoA (set 1), and herbicides (set 2) with a specific MoA expected for algae/plants but nonspecific for other aquatic organisms, and to extend the K_{IAM} data for anionic chemicals (set 3). In total, this study determined new K_{IAM} values for 121 chemicals.

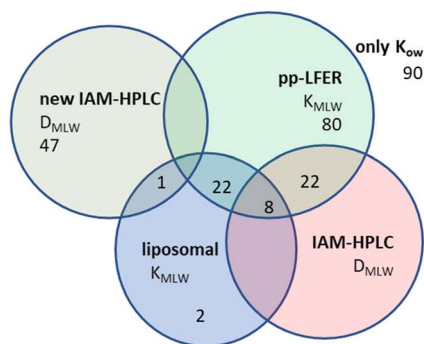
Chemical set 1: chemicals with different MoA in EPA's fathead minnow (FHM) database. The FHM database classifies the toxic MoA to each of the 616 chemicals according to results from behavioral toxicity assessments, studies on physiological responses, and assessment of additivity in tests with chemical mixtures, using a weight of evidence approach.¹⁶ As shown in Fig. 2A, from the 273 classified neutral narcosis chemicals out of this total of 616 chemicals, experimental K_{MLW} was available for 55 chemicals based on either liposomal partition coefficients or IAM-HPLC retention values. For 80 additional

chemicals, established ppLFER descriptors were available to predict K_{MLW} with eqn (5). We selected a set of 47 neutral narcosis chemicals outside the chemicals covered by the existing IAM-HPLC database and pp-LFER calculations of K_{MLW} , which only relate to FHM narcosis chemicals with level 1 or 2 confidence (see further details in ESI Section S2, and further details in the file Table S1A†). These narcosis MoA FHM-chemicals are listed in Table 1, sorted by decreasing reported FHM LC₅₀ in mmol L⁻¹.

As shown in Fig. 2B, we also selected a set of 27 neutral FHM chemicals that were categorized by MoA, 17 with electrophile/pro-electrophile reactivity ('REACTIVE'), 7 acetylcholinesterase inhibitors ('ACHE'), 1 uncoupler of oxidative phosphorylation, and 1 that acts as a respiratory blocker/inhibitor ('BLOCK'). The selection was aimed at minimizing overlap with the existing IAM-HPLC/liposomal database (see further details in ESI section S2, Table S1B†). These specific MoA FHM-chemicals are listed in Table 2, sorted per MoA by decreasing reported FHM LC₅₀ in mmol L⁻¹.

A substantial number of chemicals in the FHM database are ionizable chemicals: forty nine are bases that are >95% ionized at tested pH ($pK_a > 8.5$) and nineteen are acids with $pK_a < 6$ (>95% ionized at physiological pH 7.4). MoA classification of most of the ionizable chemicals is equivocal. For chemical set 1, we selected fifteen strong bases/cations listed in the FHM database for which K_{IAM} values were already available, *i.e.* three neurotoxic bases (amphetamine, strychnine, and nicotine)⁷⁸ and twelve bases/cations of the type "UNSURE AMINES".⁷⁷ The five strongly dissociated acids selected from the FHM database for chemical set 1 had different MoA labels, including three phosphorylation uncouplers. The fifteen selected cations were assumed to be a relevant set of chemicals to evaluate the MoA approach based on the CMB for cations, with expectations that the "UNSURE AMINES" would be considered non-specific (narcosis) chemicals, as they lack specific functional moieties other than the charged amine group, while the three neurotoxic cations are expected to have a CMB significantly below the narcosis level. The set of anions was considered too small to evaluate the MoA approach based on the CMB, which is another reason we included the strongly acidic herbicides for chemical

A. 273 FHM chemicals with MoA Narcosis I or II



B. FHM chemicals with specific MoA

97 REACTIVE 17 new IAM-HPLC 1 IAM-HPLC 0 liposomal 24 ppLFER 42 only K_{OW}	17 ACHE 7 new IAM-HPLC 0 IAM-HPLC 0 liposomal 8 ppLFER 2 only K_{OW}	12 UNCOUPLER 2 new IAM-HPLC 0 IAM-HPLC 6 liposomal 5 ppLFER 3 only K_{OW}
9 NEUROTOX 3 new IAM-HPLC 1 IAM-HPLC 0 liposomal 3 ppLFER 3 only K_{OW}	4 BLOCK 1 new IAM-HPLC 1 IAM-HPLC 0 liposomal 0 ppLFER 3 only K_{OW}	6 NEURODEP 0 new IAM-HPLC 2 IAM-HPLC 0 liposomal 1 ppLFER 4 only K_{OW}

Fig. 2 Selection of test chemicals from the fathead minnow databases for which new membrane–water partition ratios are derived using IAM-HPLC (A) narcosis chemicals; (B) chemicals with a specific mode of action.



Table 1 Chemical set 1 – selected FHM chemicals labeled narcosis^a

Code	47 narcosis FHM chemicals	CAS	FHM LC ₅₀ (mmol L ⁻¹)	FHM LC ₅₀ (mg L ⁻¹)	Log K _{ow} (EpiSuite)	New log K _{IAM}	Nr injections	Solvent range (%)	CMB _{IAM} (mmol kg ⁻¹)
1N_I-01	2-Hydroxyethyl ether	111-46-6	709	75 200	-1.30	-0.46	2	0	246
1N_I-02	Triethylene glycol	112-27-6	399	59 900	-1.24	0.01	1	0	408
1N_I-03	2-Methyl-2,4-pentanediol	107-41-5	90.5	10 700	-0.67	0.75	1	0	509
1N_I-04	Urethane	51-79-6	58.8	5240	-0.15	0.48	3	0	178
1N_I-05	3-Methyl-1-pentyn-3-ol	77-75-8	12.4	1220	0.86	1.19	1	0	192
1N_I-06	<i>n</i> -Phenyldiethanolamine	120-07-0	4.06	735	0.44	1.84	2	0	281
1N_I-07	3-Methyl-3-pentanol	77-74-7	6.58	672	1.53	1.43	5	0	177
1N_I-08	2,4,5-Trimethylloxazole	20 662-84-4	4.04	449	1.79	1.78	3	0	243
1N_I-09	<i>cis</i> -3-Hexen-1-ol	928-96-1	3.80	381	1.34	1.58	2	0	144
1N_I-10	2-Methyl-3,3,4,4-tetrafluoro-2-butanol	29 553-26-2	3.64	582	1.03	1.53	1	0	123
1N_I-11	<i>trans</i> -3-Hexen-1-ol	928-97-2	2.71	271	1.34	1.62	2	0	113
1N_I-12	2-Phenoxyethanol	122-99-6	2.49	344	1.16	1.70	5	0	125
1N_I-13	2,6-Dichlorobenzamide	2008-58-4	2.47	469	1.25	1.51	3	0	80
1N_I-14	1-Ethynyl-cyclohexanol	78-27-3	2.06	256	1.73	1.80	2	0	130
1N_I-15	3,4-Dimethyl-1-pentyn-3-ol	1482-15-1	1.84	205	1.26	1.59	2	0	72
1N_I-16	Diethyl benzylphosphonate	1080-32-6	1.47	336	1.59	2.41	3	0	378
1N_I-17	2-(Bromomethyl)-tetrahydro-2H-pyran	34 723-82-5	1.14	205	1.61	1.77	4	0	67
1N_I-18	2',3',4'-Trimethoxy-acetophenone	13 909-73-4	1.09	229	1.12	2.39	3	0	268
1N_I-19	2-Dimethylaminopyridine	5683-33-0	1.04	127	1.43	1.96	3	0	95
1N_I-20	2-Amino-4-chloro-6-methylpyrimidine	5600-21-5	1.02	147	1.13	1.76	3	0	59
1N_I-21	2-Phenyl-3-buten-2-ol	127-66-2	0.773	113	1.68	2.15	2	0	109
1N_I-22	2,5-Dimethylfuran	625-86-5	0.740	71.1	2.62	2.05	4	0	83
1N_I-23	2,3-Dihydrobenzofuran	496-16-2	0.680	81.7	2.14	2.18	4	0	113
1N_I-24	5-Ethyl-2-methylpyridine	104-90-5	0.669	81.1	2.49	2.51	3	0	216
1N_I-25	Benzyl- <i>tert</i> -butanol	103-05-9	0.404	66.4	2.57	2.59	3	0	157
1N_I-26	Benzyl sulfide	621-08-9	0.348	80.1	1.96	2.73	3	0	187
1N_I-27	2-(<i>n</i> -Ethyl- <i>m</i> -toluidino)ethanol	91-88-3	0.295	52.9	2.49	2.74	3	0	163
1N_I-28	α,α,α -Trifluoro- <i>o</i> -tolunitrile	447-60-9	0.247	42.2	2.46	2.54	3	0	86
1N_I-29	α,α,α -4-Tetrafluoro- <i>m</i> -toluidine	2357-47-3	0.168	30.1	2.62	2.64	4	0	73
1N_I-30	α,α,α -4-Tetrafluoro- <i>o</i> -toluidine	393-39-5	0.165	29.6	2.62	2.54	7	0	57
1N_I-31	4-Ethoxy-2-nitroaniline	616-86-4	0.143	26.0	2.47	2.87	3	0	106
1N_I-32	4-(Diethylamino)-benzaldehyde	120-21-8	0.135	23.9	2.94	3.10	6	0	170
1N_I-33	4-Phenylpyridine	939-23-1	0.104	16.1	2.59	3.05	3	0	117
1N_I-34	1,1-Diphenyl-2-propyn-1-ol	3923-52-2	0.053	11.1	2.71	3.34	3	0	116
1N_I-35	Butyl phenyl ether	1126-79-0	0.0384	5.8	3.65	3.64	7	15–30%	168
1N_I-36	4-(Diethylamino)-salicylaldehyde	17 754-90-4	0.0277	5.4	3.34	3.35	2	0	62
1N_I-37	Flavone	525-82-6	0.0157	3.5	3.56	3.90	6	15–30%	125
1N_I-38	2-Amino-4'-chloro-benzophenone	2894-51-1	0.0092	2.1	3.95	4.21	6	15–30%	149
1N_I-39	Di- <i>n</i> -butylisophthalate	3126-90-7	0.0032	0.9	5.53	4.67	9	20–30%	150
1N_I-40	3-(4- <i>tert</i> -Butylphenoxy)-benzaldehyde	79 124-76-8	0.0015	0.4	5.93	5.41	4	20–30%	386
1N_I-41	3-(3,4-Dichlorophenoxy)-benzaldehyde	69 770-23-6	0.0011	0.3	5.49	4.98	4	20–30%	105
					Average				160
					St. dev.				105

Table 1 (Contd.)

Code	47 narcosis FHM chemicals	CAS	FHM LC ₅₀ (mmol L ⁻¹)	FHM LC ₅₀ (mg L ⁻¹)	Log K _{ow} (EpiSuite)	New log K _{IAM}	Nr injections	Solvent range (%)	CMB _{IAM} (mmol kg ⁻¹)
1N_II-01	6-Chloro-2-pyridinol	16 879-02-0	1.65	214.0	1.78	1.34	3	0	36
1N_II-02	2-Amino-5-bromopyridine	1072-97-5	1.02	177.0	1.39	2.12	3	0	134
1N_II-03	2-Chloro-4-methylaniline	615-65-6	0.253	35.9	2.58	2.55	5	0	90
1N_II-04	4-Amino-2-nitrophenol	119-34-6	0.235	36.2	0.96	1.74	6	0	13
1N_II-05	2-Chloro-4-nitroaniline	121-87-9	0.109	18.9	2.17	2.84	4	0	75
1N_II-06	3-Trifluoromethyl-4-nitrophenol	88-30-2	0.0441	9.1	3.00	3.28	3	0	84

^a Coding of MoA of chemicals in the FHM database (set 1): N_I = Narcosis_I; N_II = Narcosis_II (polar narcosis); log P as reported in the FHM database.

set 2 as part of this study. ESI Section S2† provides more details on the chemical selection procedure, and ESI Table S2† presents a list of the purity and suppliers of the chemicals purchased to create Chemical set 1.

Chemical set 2: acidic and neutral herbicides. We selected twenty nine herbicides to evaluate the CMB_{narc} approach using D_{MLW} values based on IAM-HPLC, as listed in Table 3, sorted by herbicide MoA and increasing K_{IAM} in mg L⁻¹. This set contained seventeen acidic herbicides with $pK_a < 5$ (one also listed in set 1), and twelve neutral herbicides (of which two are listed in FHM, but not part of set 1). For these herbicides, acute toxicity data (2–10 d exposure) were collected mostly from EU-dossiers (Table 2), often focused on one standard fish species (rainbow trout, *Oncorhynchus mykiss*), one crustacean species (water flea, *Daphnia magna*), fresh water green algal species (mostly *Raphidocelis subcapitata*, formerly known as *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata*), and aquatic plants (*Lemna gibba*, *Lemna minor*, and *Myriophyllum spicatum*). In the case of data gaps, endpoint data were retrieved from EPA's ECOTOX database (<https://cfpub.epa.gov/ecotox/search.cfm>) or via Pubchem ([https://pubchem.ncbi.nlm.nih.gov/chemical/\[name\]](https://pubchem.ncbi.nlm.nih.gov/chemical/[name])). In case no data were retrieved for one of the standard species, the Pesticide Property Database (PPDB) from the University of Hertfordshire was checked (<http://sitem.herts.ac.uk/aeru/ppdb/en/index.htm>). Endpoint data for herbicides are collected in Table 6. While the selected FHM chemicals contained 16 cationic chemicals for which D_{MLW} was derived by IAM-HPLC, it only contained 5 predominantly dissociated acids for which this data existed. The acidic herbicides were therefore specifically selected to determine whether the IAM-HPLC analysis of D_{MLW} could be used to support and facilitate the MoA evaluation based on the CMB_{narc} approach.

Chemical set 3: additional acidic chemicals with reported K_{MLW} . Liposomal D_{MLW} values were available for only a few of the seventeen herbicides in chemical set 2 to verify the accuracy of IAM-HPLC based values. We therefore selected an additional set of eighteen acidic chemicals with a $pK_a < 5.6$, based on recent liposomal D_{MLW} reviews on ionogenic chemicals,^{29,76} listed as set 3 in Table 4 (sorted per anion type and by increasing $D_{MLW,anion}$). Five of these selected strong acids were also in the FHM database (toxic concentration and MoA listed), among which three were 'uncouplers' of oxidative phosphorylation. Dinoseb, pentachlorophenol and bromoxynil are used as pesticides. The reviewed D_{MLW} data set for ionizable chemicals lists thirty acids with a $pK_a < 6$, where D_{MLW} is reported for the anionic form. Except for phenolic acids, for most acids the $K_{MLW,N}$ is ~ 2 log units higher than $D_{MLW,ion}$ (e.g., $D_{MLW,ion}$ and $K_{OW,N}$ differ by 1.86 log units for the carboxylic acid ibuprofen, "3Ca03" in Table 4), and therefore the retention on the IAM column may still be influenced by a neutral fraction of $>1\%$ for acids with a $pK_a > 6$ when testing at the maximum recommended IAM eluent pH of 8. Electrostatic repulsion of anions from the IAM particles at pH > 6 results in even lower apparent K_{IAM} for the dissociated anion form, so in order to determine the IAM retention for the anionic form only, the pK_a needs to be < 5.5 . The selection includes carboxylates (code Ca#), sulfonates





Table 2 Chemical set 1 – selected FHM chemicals with specific MoA or ionic properties

Code ^a	27 specific MoA FHM chemicals	CAS	FHM LC ₅₀ (mmol L ⁻¹)	LC ₅₀ (mg L ⁻¹)	Log K _{OW} (Epi-Suite)	New log K _{IAM}	Nr inj.	Solvent range (%)	CMB _{IAM} (mmol kg ⁻¹)
1Ach-01	Carbaryl	63-25-2	0.0444	8.93	2.46	2.98	3	0	42.40
1Ach-02	Propoxur	114-26-1	0.0421	8.80	1.45	2.27	3	0	7.84
1Ach-03	Diazinon	333-41-5	0.0307	9.35	4.19	4.05	7	15–30%	344.46
1Ach-04	Aminocarb	2032-59-9	0.0094	1.95	2.16	2.48	4	0	2.84
1Ach-05	Aldicarb	116-06-3	0.0045	0.86	1.12	1.91	4	0	0.37
1Ach-06	Carbofuran	1563-66-2	0.0038	0.84	2.32	2.46	4	0	1.10
1Ach-07	EPN	2104-64-5	0.0002	0.08	3.85	5.10	7	20–30%	25.18
1Ach-08	Azinphos-methyl	86-50-0	0.0002	0.06	2.75	3.57	7	15–30%	0.74
1Rea-01	Butanal	123-72-8	0.2219	16.00	0.88	1.02	1	0	2.32
1Rea-02	4-Fluoroaniline	371-40-4	0.1521	16.90	1.15	1.63	3	0	6.49
1Rea-03	2,4-Dinitrotoluene	121-14-2	0.1334	24.30	2.00	2.42	3	0	35.09
1Rea-04	4-Nitrobenzaldehyde	555-16-8	0.0668	10.10	1.50	1.90	3	0	5.31
1Rea-06	Benzaldehyde	100-52-7	0.0717	7.61	1.48	1.80	3	0	4.52
1Rea-05	2,4,5-Tribromoisimidazole	2034-22-2	0.0261	7.96	1.96	2.27	3	0	4.86
1Rea-07	4-Dimethylamino-cinnamaldehyde	6203-18-5	0.0337	5.90	NA	3.11	3	0	43.41
1Rea-08	Salicylaldehyde	90-02-8	0.0188	2.30	1.81	1.91	3	0	1.53
1Rea-09	4-Chlorocatechol	2138-22-9	0.0109	1.58	1.97	1.60	1	0	0.43
1Rea-11	1-Benzoylacetone	93-91-4	0.0068	1.10	1.05	2.57	3	0	2.53
1Rea-10	Pentafluorobenzaldehyde	653-37-2	0.0056	1.10	2.45	1.83	3	0	0.38
1Rea-12	α,α -Trifluoro- <i>m</i> -tolualdehyde	454-89-7	0.0053	0.92	2.47	2.46	3	0	1.53
1Rea-13	1,3,5-Trichloro-2,4-dinitrobenzene	6284-83-9	0.0008	0.22	2.65	4.04	9	20–30%	8.77
1Rea-14	2-Methyl-1,4-naphthoquinone	58-27-5	0.0006	0.11	2.20	2.72	3	0	0.31
1Rea-15	α -Bromo-2',5'-dimethoxyacetophenone	1204-21-3	0.0003	0.09	2.39	3.12	3	0	0.40
1Rea-16	1,3-Dichloro-4,6-dinitrobenzene	3698-83-7	0.0002	0.05	2.49	2.64	4	0	0.09
1Rea-17	N-Vinylcarbazole	1484-13-5	0.0000166	0.0032	NA	4.85	7	20–30%	1.18
1Blo-01	Rotenone	83-79-4	0.0000114	0.0045	4.10	4.58	6	20–30%	0.43

5 Mostly anionic FHM chemicals									
		FHM LC ₅₀ (mmol L ⁻¹)	LC ₅₀ (mg L ⁻¹)	Log K _{OW}	New log K _{IAM}	Log K _{IAM} ^b	CMB _{IAM} (mmol kg ⁻²)		
1AUns-01	Salicylic acid	54-21-7	13.5	2160	2.26	1.49	0.99	1	0
1AUnc-01	2,4-Dinitrophenol	51-28-5	0.072	13.3	1.67	2.32	1.82	2	0
1AUnc-02	Dinoseb	88-85-7	0.0029	0.70	4.62	3.76	3.26	2	0
1AUnc-03	Pentachlorophenol	87-86-5	0.00083	0.22	5.12	4.45	3.95	7	15–30%
1AN-01	2,3,4,6-Tetrachlorophenol	58-90-2	0.0044	1.03	4.12	3.74	3.29	8	15–30%

15 Mostly cationic FHM chemicals									
		FHM LC ₅₀ (mmol L ⁻¹)	LC ₅₀ (mg L ⁻¹)	Log K _{OW}	New log K _{IAM}	Log K _{IAM} ^b (ref. 77)	CMB _{IAM} (mmol kg ⁻²)		
1CNeu-01	Amphetamine sulfate	60-13-9	0.078	28.80	1.76	2.31	1.76	2.31	15.9
1CNeu-02	Nicotine sulfate	65-30-5	0.029	12.20	1.17	0.96	1.17	0.96	0.3
1CNeu-03	Strychnine hemisulphate	60-41-3	0.0029	1.11	1.93	2.28	1.93	2.28	0.6
1CUnsA-01	Phenyltrimethylammonium methosulfate	98-04-4	0.924	243	NA	0.64	NA	0.64	4.0
1CUnsA-02	Benzyltriethylammonium chloride	56-37-1	0.952	161	NA	1.37	NA	1.37	22.3
1CUnsA-03	N,N-Dimethylbenzylamine	103-83-3	0.707	37.8	1.95	1.08	1.95	1.08	8.5
1CUnsA-04	Hexylamine	111-26-2	0.559	56.6	2.06	2.12	2.06	2.12	73.7

Table 2 (Contd.)

15 Mostly cationic FHM chemicals		FHM LC ₅₀ (mmol L ⁻¹)	LC ₅₀ (mg L ⁻¹)	Log K _{OW}	Log K _{IAM} ^b (ref. 77)	CMB _{IAM} (mmol kg ⁻²)
1CUnsA-05	N-Ethylbenzylamine	14321-27-8	0.422	2.04	1.64	18.4
1CUnsA-06	Benzylamine	100-46-9	0.280	1.09	1.73	15.0
1CUnsA-07	N-Heptylamine	111-68-2	0.190	2.57	2.54	65.9
1CUnsA-08	tert-Octylamine	107-45-9	0.189	2.43	2.29	36.9
1CUnsA-09	1-Adamantanamine	768-94-5	0.165	2.44	2.44	45.4
1CUnsA-10	Octylamine	111-86-4	0.040	3.04	3.08	48.1
1CUnsA-11	Di-n-hexylamine	143-16-8	0.0065	4.77	3.28	12.4
1CUnsA-12	N-Decylamine	2016-57-1	0.0042	4.10	4.40	105.5

^a Coding of MoA of chemicals in the FHM database (set 1): Ach = acetylcholinesterase inhibitors; Rea = electrophile/proelectrophile reactivity; Unc = uncouplers of oxidative phosphorylation (AUnc = acidic uncoupler); Blo = respiratory blocker/inhibitor; 1CNeu = cationic neurotoxin; CunsA = cationic unsure amine mode of action; AN = acidic narcosis chemical. ^b $\delta_{IAM-SSLM}$ corrected. NA = not available. $\delta_{IAM-SSLM}$ corrections for 1°, 2°, and 3° amines: +0.78, +0.47, and -0.03, respectively, as in ref. 77.

(code Su#), and phenolates (code Ph#). This allows for a structurally more diverse comparison between IAM-HPLC retention data and liposomal D_{MLW} than the alkylsulfonate and alkylsulfate surfactants²⁷ in Fig. 1.

(ii) Chromatographic conditions

A 100 × 4.6 mm IAM.PC.DD2 column (Regis Technologies, Inc., Morton Grove, IL USA), with an IAM.PC.DD2 10/300 guard cartridge, was operated with typical phosphate buffer saline (PBS, pH 7.4) as the eluent, at a flow rate of 1.0 mL min⁻¹ (23 ± 2 °C). Eluting chemicals were detected by using a UV-diode array (Agilent 1100 system) at various wavelengths (207, 220, 254, and 278 nm) relative to the signal at 360 nm. This way, in all cases there was at least one wavelength that clearly detected the eluting peak of all of the chemicals tested on the IAM-HPLC system with a single detector setting. The PBS was composed of 10 mM buffer with 8.0 g L⁻¹ NaCl (137 mM) and 0.2 g L⁻¹ KCl (2.7 mM). Triplicate IAM-injections (20 µL) were run on the same day for most chemicals if fully aqueous eluent was used. 3-Nitroaniline was used as the neutral reference chemical to check the IAM column performance throughout the testing period. The negative peak apex signal of injected pure water (MilliQ, Millipore Merck) was used as a neutral non-retained tracer (t_0) in UV-diode array detection. The peak apex of the eluted peak (t_r) on both detectors was used to calculate retention capacity factors (k_{IAM}) based on the ratio $t_r/(t_r - t_0)$. The intrinsic phospholipid sorption coefficient ($K_{IAM,intr}$) was obtained by (i) multiplying k_{IAM} by the solvent/sorbent phase ratio of 18.9 for the IAM.PC.DD2 column, and (ii) accounting for electrostatic repulsion by IAM surfaces at a certain pH with Boltzmann factors.²⁷

A series of at least 3 different eluent mixtures with acetonitrile (≤30%) were applied to chemicals with a log K_{OW} >3 as a first indication (Fig. S2†). At least 3 measurements were performed on 3 different solvent mixtures for these chemicals. Linear trends between log K_{IAM} and fraction solvent in the eluent mixtures were extrapolated to estimate log K_{IAM} values with a fully aqueous eluent composition in MS Excel.

3. Results and discussion

The key purpose of this study was to evaluate the MoA-classification based on critical membrane burdens for chemical set 1 (fish toxicity with different MoA, using the FHM database) and chemical set 2 (chemicals with a herbicidal MoA, but evaluated for different species). The chromatographic method IAM-HPLC is used to obtain membrane-lipid/water distribution coefficients for the more than 100 chemicals selected (details on all IAM measurements in Table S4†).

For 28 chemicals in set 1, 2, or 3, where IAM-HPLC retention capacity was measured for a series of (water/acetonitrile) compositions, ESI-fig. S2† shows the extrapolated linear trendlines to derive the $K_{IAM,intr}$ at 100% water. For seven chemicals (2A-16, 2A-17, 2N-01, 2N-02, 2N-11, 3Ca-05, and 3Ca-06), a solvent range was determined as well as measurements at 0% solvent, confirming the linearity of the trendline in this



Table 3 Chemical set 2 – acidic and neutral herbicides for CMB-evaluation^a

Code	Name (FHM set 1 code, LC ₅₀ in mg L ⁻¹ , MoA)	CAS	Herbicide MoA	pK _a	log K _{OW} , neutral ACD labs	New log K _{IAM} (anion K _{IAM,intr})	New log K _{IAM} δ _{IAM-SSLM} corrected	Nr inj.	Solvent range (%)	Toxicity endpoint source
Anionic herbicides										
2A-01	Clopyralid	1702-17-6	Synth. auxin	2.02	1.31	0.64	0.14	1	0	EFSA scientific report, 2005, 50, 1–65
2A-02	Fluroxypyr	69377-81-7	Synth. auxin	2.22	3.16	1.90	1.40	3	0	EFSA J., 2011, 9(3), 2091
2A-03	Triclopyr	55335-06-3	Synth. auxin	2.26	2.98	2.16	1.66	3	0	EFSA scientific report, 2005, 56, 1–103
2A-04	MCPA	94-74-6	Synth. auxin	3.14	2.49	2.14	1.64	6	0	EC (2008), Review report for the active substance MCPA (SANCO/4062/2001-final; pp. 1–62)
2A-05	2,4-D	94-75-7	Synth. auxin	2.6	2.59	2.13	1.63	3	0	EFSA J. 2014; 12(9), 3812
2A-06	MCPP	93-65-2	Synth. auxin	3.19	2.84	2.28	1.78	6	0	EFSA J. 2017; 15(5), 4832
2A-07	2,4-DP	120-36-5	Synth. auxin	3.1	3.43	2.29	1.79	3	0	EFSA J. 2018; 16(6), 5288
2A-08	MCPB	94-81-5	Synth. auxin	4.84	3.42	2.74	2.24	3	0	EC (2005). Review report for the active substance MCPB (No. SANCO/4063/2001-final; pp. 1–42)
2A-09	2,4-DB	94-82-6	Synth. auxin	4.95	3.53	2.78	2.28	3	0	EFSA J. 2016; 14(5), 4500
2A-10	2,4,5-T	93-76-5	Synth. auxin	2.88	3.3	2.59	2.09	3	0	US EPA ECOTOX/PPDB
2A-11	DNOC	534-52-1	Uncoupler	4.31	2.13	2.65	2.15	3	0	US EPA ECOTOX (EC (1998). Review report for the active substance DNOC (no. 7777/VI/98-rev. 3; pp. 1–3))
2A-12	Dinoseb	88-85-7	Uncoupler	4.62	3.60	3.76	3.26	2	0	REACH registration dossier https://echa.europa.eu/nl/registration-dossier/-/registered-dossier/12446
2A-13	Bromoxynil	1689-84-5	PS-II inh.	4.09	2.95	2.70	2.20	2	0	EFSA J., 2017; 15(6), 4790
2A-14	Ioxynil	1689-83-4	PS-II inh.	3.96	3.43	3.31	2.81	2	0	EC (2004). Review report for the active substance ioxynil (no. SANCO/4349/2000 final; pp. 1–98)
2A-15	Triasulfuron	82097-50-5	AHAS inh.	4.34	2.36	2.05	1.55	3	0	EFSA J., 2015; 13(1), 3958
2A-16	Bensulfuron-methyl	83055-99-6	AHAS inh.	3.50	2.38	2.82	2.32	6	10–20%	EFSA scientific report, 2008, 178, 1–102
2A-17	Fomesafen	72178-02-0	PROTOX	2.42	2.94	3.48	2.98	6	0–20%	Australian pesticides and veterinary medicines authority (APVMA): public release summary on fomesafen in the product reflex herbicide, ISSN 1443–1335



Table 3 (Contd.)

Code	Name (FHM set 1 code, LC ₅₀ in mg L ⁻¹ , MoA)	CAS	Herbicide MoA	log K _{OW} neutral ACD labs	New log K _{IAM} (anion K _{IAM,INT})	New log K _{IAM} δ _{IAM-SSLM} corrected	Nr inj.	Solvent range (%)	Toxicity endpoint source
Neutral herbicides									
2N-01	Alachlor	15972-60-8	Cell div. inh. (VLCFA)	3.59	3.31		10	0–30%	EC (2007). Review report for the active substance alachlor (SANCO/4331/2000-final)/US EPA ECOTOX
2N-02	Metolachlor	1418095-19-8	Cell div. inh. (VLCFA)	3.45	3.39		8	0–30%	Public consultation_S-Metolachlor_RAR_23_LoEP_2018-09-06.pdf
2N-03	Simazine	122-34-9	PS-II inh.	1.78	2.49		3	0	US EPA ECOTOX (EC (2003)), Review report for the active substance simazine SANCO/10495/2003-rev. Final)
2N-04	Ametryn	834-12-8	PS-II inh.	2.60	3.14		3	0	US EPA ECOTOX/REACH registration dossier https://echa.europa.eu/nl/registration-dossier/-/registered-dossier/2171/6/2/1
2N-05	Terbuthylazin	5915-41-3	PS-II inh.	2.48	3.36		3	0	EFSA J. 2011; 9(1):1969/US EPA ECOTOX
2N-06	Diuron	330-54-1	PS-II inh.	2.53	3.38		3	0	EFSA scientific report, 2005, 25, 1–58
2N-07	Linuron	330-55-2	PS-II inh.	2.30	3.51		3	0	EFSA J., 2016; 14(7), 4518
2N-08	Chloroxuron	1982-47-4	PS-II inh.	3.43	4.26		6	15–30%	US EPA ECOTOX
2N-09	Ethofumesate	26225-79-6	Lipid synth inh.	2.34	3.25		3	0	EFSA J., 2016, 14(1), 4374
2N-10	Prosulfocarb	52888-80-9	Lipid synth inh.	4.17	4.62		7	15–30%	EFSA scientific report, 2007, 111, 1–81
2N-11	Glomazone	81777-89-1	Lycopene cyclase inh.	2.93	2.93		14	0–30%	EFSA scientific report, 2007, 109, 1–73
2N-12	Trifluralin	1582-09-8	Mitosis inh.	4.60	5.48		4	20–30%	EFSA scientific report, 2009, 327, 1–111

^a Coding of MoA of chemicals in Set 2: A = acid; N = neutral; ¹ Lemna endpoint (or *Myriophyllum* endpoint between brackets) as listed in Table 5; ² alg = EC₅₀ *Raphidocelis subcapitata* if no data on aquatic plants were retrieved; ³ *Oncorhynchus mykiss* (rainbow trout) as listed in Table 5. ⁴ 7d ErC₅₀ at pH 7.8, reported as 0.000202 mg L⁻¹ (nom) for the Frond number of *Lemna gibba* in EFSA J. 2015; 13(1):3958. ⁵ 14d ErC₅₀, reported as 0.0008 mg L⁻¹ for the Frond number of *Lemna gibba* in EFSA Scientific Report 2008; 178, 1.



Table 4 Chemical set 3 – acids for $K_{IAM, intr}$ comparison with reviewed $D_{MLW, ion}$ ^a

Code	Name (code in Set 1; FHIM LC ₅₀ in mg L ⁻¹ , MoA)	CAS	pK _a	Log K _{OW}	New log K _{IAM}			Solvent range (%)	Log D _{MLW, ion}		New log K _{IAM}
					Anion	K _{IAM, intr}	Nr inj.		Liposome	Log units	δ _{IAM-SSLM} corrected
3Ca-01	Salicylic acid (1AUns-01; 2160, UNSURE)	54-21-7	2.75	2.26	1.49		1	0	1.03	0.46	0.99
3Ca-02	5-Phenylvaleric acid	2270-20-4	4.88	2.70	1.97		1	0	1.66	0.31	1.47
3Ca-03	Ibuprofen	15 687-27-1	4.45	3.50	2.78		1	0	1.81	0.97	2.28
3Ca-04	Fenamic acid	91-40-7	3.99	4.36	3.43		3	0	2.28	1.15	2.93
3Ca-05	Diclofenac	15 307-86-5	3.99	4.5	3.76		6	0	2.64	1.12	3.26
3Ca-06	Diflunisal	22 494-42-4	3.00	4.44	3.73		6	0	2.73	1.00	3.23
3Su-01	4-Octylbenzene-1-sulfonate	6149-03-7	<0	4.65 ACD	4.81		2	0	3.63	1.11	4.31
3Su-02	C ₁₀ -2-LAS	NA	<0	5.53 ACD	5.58		8	15–30%	4.79	0.79	5.11
3Ph-01	Warfarin	81-81-2	4.9	2.7	3.06		5	20–30%	1.40	1.66 ^b	2.56
3Ph-02	Pentafluorophenol	771-61-9	5.53	3.05 ACD	2.20		1	0	1.74	0.46	1.70
3Ph-03	2,4-Dinitrophenol (not in set 1; 13.3, UNCOUPLER_1)	51-28-5	3.94	1.67	2.36		1	0	1.90	0.46	1.86
3Ph-04	Bromoxnil	1689-84-5	4.09	2.8	2.70		2	0	2.10	0.78	2.20
3Ph-05	2-Methyl-4,6-dinitrophenol	534-52-1	4.31	2.20 ACD	2.77		2	0	2.35	0.46	2.27
3Ph-06	4-tert-Butyl-2,6-dinitrophenol	4097-49-8	4.11	3.56 ACD	3.38		1	0	3.23	0.18	2.88
3Ph-07	Dinoseb (1AUnc-01; 0.7, UNCOUPLER_3)	88-85-7	4.62	3.56	3.76		1	0	3.35	0.43	3.26
3Ph-08	2,3,4,6-Tetrachlorophenol (1AN-01; 1.03, NARCOSIS_I_3)	58-90-2	5.40	4.12	3.79		2	0	3.46	0.28	3.29
3Ph-09	2,3,5,6-Tetrachlorophenol	935-95-5	5.14	3.88	3.78		8	15–30%	3.49	0.28	3.28
3Ph-10	Pentachlorophenol (1AUnc-02; 0.22, UNCOUPLER_1)	87-86-5	4.75	5.12	4.45		8	15–30%	4.35	0.10	3.95
									Average	0.60	
									St. dev.	0.36	

^a Coding of MoA of chemicals in set 3: Ca = carboxylic acid; Su = sulfonate acid; Ph = phenolic acid. ^b Considered outlier (>2 time st. dev.), not included in the calculation of average δ_{IAM-liposome}.

solvent range. For most chemicals with a solvent range extrapolation, the 95% confidence limit for $K_{IAM, intr}$ at 0% solvent was <0.2 log units (details in Fig. S2†), particularly if multiple measurements were made per solvent composition. In some cases, with single measurements per solvent composition (3Ca-04 fenamic acid), or one deviating point (3Ca-05 diclofenac), and with a limited range of 20–30% solvent (1Ach-07 EPN), the 95% confidence limits around the extrapolated 100% aqueous medium $K_{IAM, intr}$ are actually too high to derive a reliable D_{MLW} value for further interpretation. For all other chemicals measured in 100% aqueous medium, replicate IAM measurements demonstrate high consistency (<0.1 log unit deviations for $K_{IAM, intr}$), and as such also single K_{IAM} measurements for 15 chemicals in 100% aqueous buffer are considered sufficiently reliable to derive the D_{MLW} value.

Since both set 1 and set 2 contain largely dissociated acids, for which the chromatographic method is used to determine the D_{MLW} , the intrinsic K_{IAM} (accounting for electrostatic repulsion from the IAM surface at neutral pH) obtained for the acids of chemical set 3 will be presented and discussed first. Whilst the alignment between liposomal D_{MLW} and $K_{IAM, intr}$ values has been presented in other studies for neutral organics²⁵ and organic cations,^{26,77,78} the current study provides data for a substantial set of organic anions in addition to the anionic surfactants²⁷ that are already presented in Fig. 1. This collection should demonstrate the uncertainty margins with which IAM-HPLC can be used to derive D_{MLW} for a wide chemical domain that includes both neutral and ionizable organic chemicals.

(i) Assessment of the alignment between IAM-retention factors and liposomal D_{MLW} (chemical set 3)

Table 4 shows the IAM-HPLC results for the strong acids, with the number of injections included to derive $K_{IAM, intr}$, and the solvent (acetonitrile) range applied. Nearly all acids were at least 99% dissociated at the tested pH 7 because of the very low pK_a

values (>3 units lower than the tested pH). The observed retention time is thus due to the interaction of the anionic form with the IAM surface. In Table 4, the IAM retention capacity factor (k_{IAM}) is already converted to the IAM phospholipid–water partition coefficient (K_{IAM}) using eqn (1). The logarithmic Boltzmann factor ($\log B$) for pH 7.0 and eluent salinity of 0.1 M ($\log B_{pH\ 7.0/0.1\ M}$) is 0.5 (see ESI-Fig. S1†). The left plot in Fig. 3 shows the same surfactant data as shown in the left plot of Fig. 1, but now also includes the newly derived $K_{IAM, intr}$ data for organic anions (open red circles). In addition, data for organic cations other than the cationic surfactants in Fig. 1 are now shown (open green triangles), using the consistent $K_{IAM, intr}$ data set for organic cations⁷⁸ and liposomal K_{MLW} data collected elsewhere (see details in ESI-Table S3†).^{29,76} These data sets present the widest chemical space of the K_{IAM} – K_{MLW} comparison that is currently available.

The data for non-surfactant organic ions demonstrate more scatter than the surfactants. Obviously, surfactants have very simple hydrocarbon or fluorocarbon structures, and don't account for the influence of polar groups on the interaction difference between the IAM monolayer and bilayer liposomes. The empirical incremental $\delta_{IAM-MLW}$ correction of -0.47 log units derived from the different anionic surfactants was also applied to all corresponding types of organic anions. As shown in the right plot of Fig. 3, for all anions this indeed brings nearly all $K_{IAM, intr}$ values for anions closer to the 1:1 line with liposomal $K_{MLW, anion}$ values. Warfarin (3Ph-01, indicated by the red arrow in Fig. 4) is the organic anion with the highest deviating $K_{IAM, intr}$ ($\delta_{IAM-MLW}$ adjusted $K_{IAM, intr}$ still 1.2 log units above liposomal $K_{MLW, anion}$). Warfarin also showed a higher K_{IAM} compared to liposomal K_{MLW} (pH 7.4) in another study, although the slightly different IAM.PC.DD column was used.¹⁰¹ It is not clear what features of warfarin are responsible for this deviation, although it has a very delocalised charge in comparison to the other carboxylic acids and phenolic acids in the selection. Leaving out warfarin as an atypical outlier, the average difference between $K_{IAM, intr}$ and $K_{MLW, anion}$ for 27

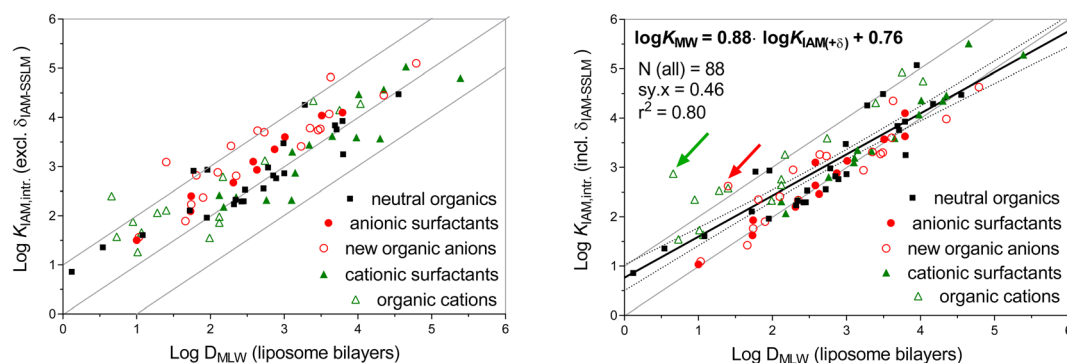


Fig. 3 Liposome membrane–water distribution ratios ($\log D_{MLW}$) plotted against: (left plot) experimental intrinsic phospholipid–water partition ratios obtained with the chromatographic IAM-HPLC method ($K_{IAM, intr}$) for all neutral and ionic chemicals (reported and new from this study); (right plot) the same $K_{IAM, intr}$ data set as in the left graph, but now corrected by empirical ion-type specific corrective increments ($\delta_{IAM-SSLM}$ -0.5 for all anions, $+0.8$ for primary amines, $+0.5$ for secondary amines, 0 for tertiary amines and -0.1 for quaternary ammonium chemicals, not defined for neutral chemicals). The fitted linear black trendline used data for all types of chemicals, dotted lines are 95% confidence intervals, grey solid lines represent unity (starting in 0,0) and factor 10 differences between $K_{IAM, intr}$ and D_{MLW} . The red arrow points to warfarin, green to acebutolol.



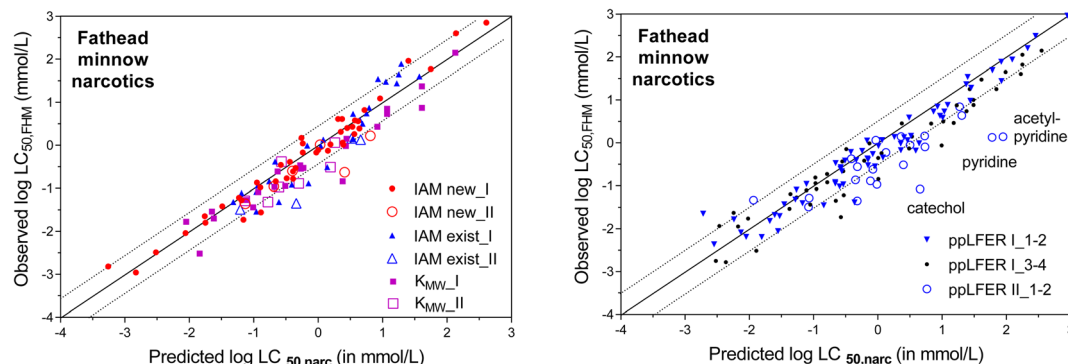


Fig. 4 Predicted acutely narcosis toxic concentrations ($LC_{50,narc}$) for chemicals in the EPA fathead minnow data set using the CMB_{narc} approach with several sets of D_{MLW} estimates, where $\hat{a} \in \hat{a}^{TM}$ denotes chemicals classified in the EPA's database as Narcosis_I, and $\hat{a} \in \hat{a}^{TM}$ chemicals classified as Narcosis_II. (left plot) IAM-HPLC partition ratios (new \hat{a} chemicals used in the current study and existing \hat{a} chemical measured in other studies) and liposomal partition coefficients (K_{MLW} , measured in other studies); (right plot) ppLFER calculated D_{MLW} values, 1 and 2 are the highest confidence groups for this classification, and 3 and 4 lower confidence groups. The solid line represents 1 : 1 correspondence, and dotted line deviations by $\hat{A} \pm 0.5$ or ± 1 log units.

anionic compounds is 0.54 log units, so the final $\delta_{IAM-MLW}$ remains at -0.5 . The root mean square error (RMSE) for all 27 $\delta_{IAM-MLW}$ adjusted anion $\log K_{IAM,intr}$ values compared to $\log K_{MLW,anion}$ is 0.38. This indicates that there is about a factor of ± 3 uncertainty when extrapolating IAM-HPLC measurements for anions (incl. $\delta_{IAM-MLW}$) to liposomal D_{MLW} .

For cations, $\delta_{IAM-MLW}$ was defined for various types of simple amine structures in another study.²⁶ Surprisingly, the diverse set of organic cations does not seem to converge to the 1 : 1 line with the $\delta_{IAM-MLW}$ increments set by the cationic surfactants (Fig. 3B). Several adjusted $K_{IAM,intr}$ values even deviate by more than a log unit from D_{MLW} data, and not one cation has an adjusted K_{IAM} partition coefficient lower than the K_{MLW} values. The most outlying cation is acebutolol (indicated by the green arrow in Fig. 4, $\log K_{MLW,ion}$ 0.66, $\log K_{IAM,intr}$ 2.4, and $\log K_{IAM,intr} + \delta_{IAM-MLW}$ 2.9). For the majority of chemicals in the right plot of Fig. 4, the $\delta_{IAM-MLW}$ corrected $K_{IAM,intr}$ values are within a factor of 0.7–10 of the liposomal D_{MLW} data, with a tendency to particularly overestimate D_{MLW} for lower affinity chemicals. Using all data on neutral, anionic and cationic chemicals, the overall double-log linear trendline shows a standard deviation of the residuals ($sy \cdot x$, the square root of the average squared deviation) of 0.46 log units:

$$\log D_{MLW} = 0.88 \times (K_{IAM,intr} + \delta_{IAM-MLW}) + 0.76 \quad (10)$$

Eqn (10) may be used to further minimize the error margins between $(K_{IAM,intr} + \delta_{IAM-MLW})$ and D_{MLW} for the wide variety of neutral and ionizable chemicals. However, for the current evaluation we only used $\delta_{IAM-MLW}$ corrective increments for anionic and cationic surfactant D_{MLW} determination, no further corrections were applied for the K_{IAM} of neutral chemicals.

(ii) Evaluation of the CMB_{narc} -approach based on chemicals with a narcosis MoA from the FHM database (chemical set 1)

Neutral chemicals with a narcosis MoA are the easiest data set to start the evaluation of the CMB_{narc} -approach. Table 3 lists the

measured IAM partition coefficients for FHM chemicals classified as having a narcosis MoA. The acutely toxic concentrations observed for narcotic chemicals to fathead minnow ($\log LC_{50,FHM}$), as classified in EPA's FHM database as Narcosis_I and Narcosis_II, are plotted against CMB predicted narcosis concentrations ($\log LC_{50,narc}$), using eqn (1) with a CMB_{narc} of 140 mmol kg^{-1} as defined by Endo (2016)⁶³ (Fig. 4). To calculate $\log LC_{50,narc}$, different membrane–water partition coefficients were available as input values, we used:

(i) 29 liposomal sorption coefficients (K_{MLW} in the left plot of Fig. 4)

(ii) 30 existing IAM-HPLC partition coefficients ($K_{IAM-exist}$ in the left plot of Fig. 4)

(iii) 47 new IAM-HPLC based K_{IAM} values obtained in this study ($K_{IAM-new}$ in the left plot of Fig. 4)

(iv) 138 ppLFER predicted K_{MLW} values (right plot of Fig. 4).

As shown in the two plots of Fig. 4, most of the predicted toxic concentrations are within a factor of ± 3 of the observed acutely toxic concentrations for fathead minnows. When using experimental membrane lipid–water distribution coefficients (Fig. 4 left), this is the case for 79% of the $LC_{50,narc}$ values based on liposomal K_{MLW} values (97% within a factor of 10), and 84% of the $LC_{50,narc}$ values based on (existing and new) IAM-HPLC values (96% within a factor of 10). When using K_{MLW} calculated with ppLFER descriptors (Fig. 4 right), this is the case for 71% of the $LC_{50,narc}$ values based on ppLFER calculated K_{MLW} (93% within a factor of 10).

Fig. 4 demonstrates that the prediction of baseline toxic MoA based on IAM-HPLC derived partition coefficients is accurate for 96% of the tested neutral narcosis chemicals; only for 4% of the tested narcosis chemicals in the FHM set, the LC_{50} deviates by more than a factor of 10 from the $LC_{50,narc}$. Fig. 4 also indicates that the previously derived CMB of 140 mmol kg^{-1} seems to apply equally to Narcosis_I and Narcosis_II classified chemicals, as discussed further below in section (iii). The ppLFER predictions show comparably successful predictions of acutely toxic concentrations, but this typically is only possible when



experimental ppLFER descriptors are available.¹⁰² The three Narcosis_II classified chemicals that have a ppLFER calculated CMB <14 mmol kg⁻¹ (catechol, pyridine and acetylpyridine) may even be re-classified as having a more specific mode of toxic action.

(iii) What is the lower CMB range for narcosis chemicals, and do Narcosis_II chemicals have a lower CMB than Narcosis_I chemicals (chemical set 1)?

The distinction between Narcosis_I and Narcosis_II chemicals (or non-polar narcosis and polar narcosis) stems from observed differences in respiratory–cardiovascular responses in fish acute toxicity syndromes (FATS), and the fact that lethal body burdens are generally lower for polar narcosis. When LC₅₀ values are plotted against K_{OW} values as the chemical descriptor of hydrophobicity, Narcosis_I and Narcosis_II chemicals typically are somewhat separated clusters.¹⁰³ We examined this using only chemicals labeled in the FHM data set as Narcotics with confidence classes 1 and 2 used (highest confidence groups in EPA classification) and only for those chemicals for which experimentally derived K_{OW} values were available (listed in EpiSUITE) to avoid co-influence of K_{OW}-algorithm prediction biases. As listed in Table 5, multiplying K_{OW} with LC₅₀ indeed results in a higher CMB_{narc} for Narcosis_I chemicals (CMB = 169 mmol kg⁻¹) compared to Narcosis_II chemicals (CMB = 29.1 mmol kg⁻¹), on average a factor 5.8 difference. A comparable distinction based on K_{OW} was observed in the study by Vaes *et al.*¹⁰³ for guppy toxicity data; the recalculated average CMB for 8 Narcosis_I chemicals (342 mmol kg⁻¹) and 10

Narcosis_II chemicals (75.6 mmol kg⁻¹) according to eqn (2) indicates a difference of a factor of 4.5.

The current study involves a much larger data set on Narcosis_I and Narcosis_II chemicals than the study by Vaes *et al.*¹⁰³ Instead of using IAM-HPLC partition coefficients to calculate LC_{50,narc} for comparison with reported LC₅₀ values, as done in the previous section (ii), the K_{IAM} for narcosis FHM chemicals can also be used to re-calculate the CMB_{narc}. We can now do this for 82 Narcosis_I chemicals (64 classes 1, 2 and 18 class 3) from the FHM database for which K_{IAM} is available. For one Narcosis_I chemical, 2-methyl-2-propanol, a very high CMB_{narc} of 3832 mmol kg⁻¹ was calculated based on an IAM-HPLC based log D_{MLW} of 1.65 (k_{IAM} = 0.37 from ref. 104, and as such included in a review¹⁰⁵ on IAM-HPLC capacity factors). This CMB_{narc} is more than 6 times higher than the second highest CMB value of 570 mmol kg⁻¹ for Narcosis_I chemicals. The reported log D_{MLW} may be a considerable overestimation, because the pp-LFER-based log D_{MLW} estimate is 0.05, which would result in a 40 times lower CMB. The same IAM-HPLC study also reported an almost 3 times lower retention capacity factor for the more hydrophobic homolog 2-methyl-2-butanol, which would have a log D_{MLW} of only 1.20. Without this outlier, a CMB_{narc} range of 19–570 mmol kg⁻¹ (average 151 ± 114 s. d.), which closely compares to the average value of 140 mmol kg⁻¹ derived recently.⁶³ For Narcosis_II chemicals still only a limited set of 17 K_{IAM} are available (15 classes 1, 2). This Narcosis_II set shows an average CMB_{narc} range of 13–247 mmol kg⁻¹ (average 71 ± 56 s. d.). Based on this set of 98 chemicals (the one outlier excluded) with a defined narcosis MoA and measured D_{MLW}, there is a significant distinction between the CMB_{narc} for Narcosis_I and Narcosis_II chemicals

Table 5 Groups of MoA chemicals from the FHM database (classes 1 and 2) with average critical membrane burdens (CMB) based on experimental K_{MLW} (IAM + liposomes), ppLFER predicted K_{MLW}, experimental K_{OW} (+eqn (5)), and COSMOmic predicted K_{MLW}

MoA class		Using experimental D _{MLW} neutral/ions	Using ppLFER (neutral only)	Using experimental K _{OW} neutral only	Using COSMOmic neutral/ion
Narcosis_I (EPA)	CMB mmol kg ⁻¹	155	132	169	397
Classes 1 and 2 only	St. dev.	119	202	138	1039
(130 out of 225 classes 1–4)	Min–max	30–568 ^a	13–1645	2–825	2–10599
	N used	63	65	87	130
Narcosis_II (EPA)	CMB mmol kg ⁻¹	72	46	29	35
Classes 1 and 2 only	St. dev.	59	45	36	51
(26 out of 36 classes 1–4)	Min–max	13–247	2–152	0.9–97.4	3–275
	N used	15	23	24	29
Specific toxic	CMB mmol kg ⁻¹	21	6	20	n.a.
Mode of action	St. dev.	64	11	66	
	Min–max	0.09–341	0.24–32	0.08–285	
	N used	26	8	18	
Unsure amines	Average CMB mmol kg ⁻¹	45			179
	St. dev.	45			244
	Min–max	3–164			2–734
	N used	12			12
Neurotox amphetamine	CMB mmol kg ⁻¹	16			23
Neurotox nicotine	CMB mmol kg ⁻¹	0.27			0.06
Neurotox strychnine	CMB mmol kg ⁻¹	0.55			0.06

^a The highest calculated CMB of 3832 was considered an outlier, see the text.



(using Graphpad Prism V9, unpaired t -test, $p = 0.006$, $t = 2.813$, and $df = 96$). This is consistent with a data compilation of several studies where internal whole body residues were measured and it was found that the range of polar narcosis overlaps with non-polar but goes lower in all data sets used. Still, a valuable distinction seems to be the lowest observed CMB of 13 mmol kg^{-1} for both types of narcosis chemicals.¹⁰³ For simplicity, we set this limit to 14 mmol kg^{-1} from here on, as 10% of the average CMB_{narc} of 140 mmol kg^{-1} .⁶³ Chemicals with CMB_{narc} calculated using LC_{50}/D_{MLW} above 14 mmol kg^{-1} most likely do not exert lethal toxicity *via* a specific MoA, while chemicals with CMB_{narc} below 14 mmol kg^{-1} may be considered to have lethal adverse effects *via* some kind of specific or reactive MoA.

Experimental DMPC membrane–water partition coefficients have been used as the chemical descriptor to plot LC_{50} values against.¹⁰³ The difference between Narcosis_I and Narcosis_II chemicals in their analysis was still a factor 1.8 lower average CMB_{narc} for Narcosis_II chemicals, comparable to our evaluation. For the 8 Narcosis_I chemicals, the CMB_{narc} ranged between 33 and 513 mmol kg^{-1} (average 173 mmol kg^{-1}), and for the 10 Narcosis_II chemicals, 11– 174 mmol kg^{-1} (average 94 mmol kg^{-1}). This set did not show a significant difference ($p = 0.13$, $t = 1.555$, and $df = 17$). As discussed in that study, the sorption affinities to the DMPC phospholipids (K_{DMPC}) of Narcosis_II chemicals are relatively higher than to octanol, compared to Narcosis_I chemicals. The fact that Lethal Body Burden (LBB) values differ for Narcosis_I and Narcosis_II chemicals in this study may be related to the fact that these values relate to the whole body of the fish, including storage lipid, and the more polar Narcosis_II chemicals have a distinctly lower affinity for neutral storage lipids compared to polar phospholipids. The rationale behind a similar CMB_{narc} for all chemicals with a narcosis MoA is that the target site is the cell membrane, and that when normalized to this specific lipid pool all organic chemicals exert baseline toxicity at a comparable molar cell membrane loading.

In conclusion, the CMB_{narc} is on average about a factor 2 lower for more polar chemicals with a narcosis MoA, and based

on experimental D_{MLW} values, there is a significant difference in CMB_{narc} for Narcosis_I and Narcosis_II classified chemicals. The lower CMB_{narc} limit of 98 narcosis chemicals (classes 1–3) of the current study and the 18 narcosis chemicals from Vaes *et al.*¹⁰³ is 11 mmol kg^{-1} , or about 10% of the average CMB_{narc} of 140 mmol kg^{-1} phospholipid. If for a chemical the CMB is lower than 14 mmol kg^{-1} , the toxic effect is thus very likely associated with an adverse effect pathway other than narcosis.

(iv) Using the IAM-based CMB to evaluate the classification of neutral FHM chemicals with a MoA other than narcosis (chemical set 1)

Table 2 lists the IAM partition coefficients for 28 chemicals with a specific MoA, and the 20 largely ionized chemicals, all selected from the FHM database. Fig. 5 plots all neutral FHM chemicals for which IAM-HPLC data are available, including the narcosis chemicals similar to those in Fig. 4 (black symbols), but now plotted together with the specific MoA chemicals selected from the FHM database (blue symbols in the left plot of Fig. 5). Each reported LC_{50} for Fathead minnow ($LC_{50,FHM}$) is plotted against the calculated $LC_{50,narc}$ which is derived by dividing the CMB_{narc} of 140 mmol kg^{-1} by the chemical's D_{MLW} obtained with IAM. The first dotted line next to the solid line in Fig. 5 marks a tenfold higher toxicity than expected based on the CMB_{narc} level of 140 mmol kg^{-1} , *i.e.* a CMB of 14 mmol kg^{-1} , which represents the lower limit of any evaluated narcosis chemical's CMB (13 mmol kg^{-1} derived in the discussion section above).

Most of the tested FHM chemicals (23 out of 28) that were originally classified to have a specific MoA are on the right of this dividing line, *i.e.* these chemicals had lethally toxic effects occurring at membrane burdens below 14 mmol kg^{-1} (according to the IAM-based D_{MLW} values). This confirms that these chemicals act *via* a MoA other than narcosis. It is interesting to see that several acetylcholinesterase inhibitors (AChE, indicated by + signs: diazinon, carbaryl, and EPN) are not as specifically (acutely) toxic to fish *via* the AChE mechanism as compared to other AChE chemicals, but have lethal membrane concentrations associated with a nonspecific narcosis effect. Although these chemicals may still adversely affect fish *via* the AChE

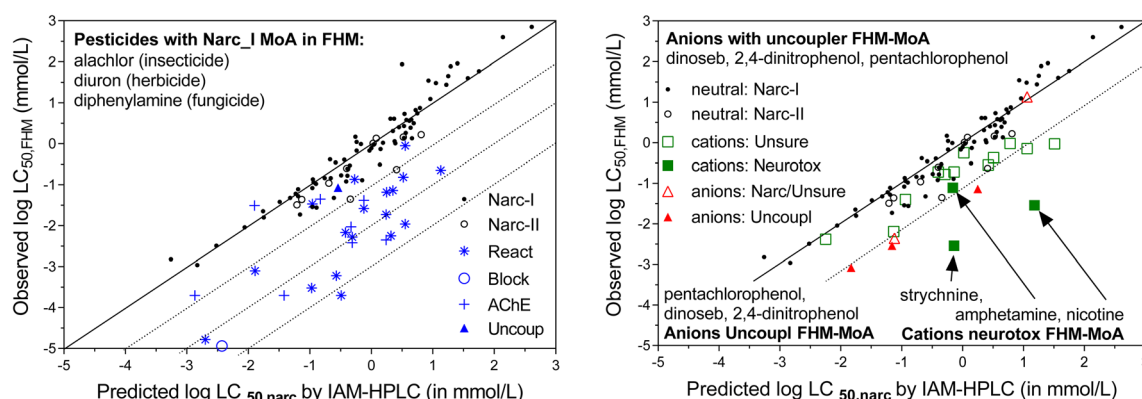


Fig. 5 Predicted acutely narcosis toxic concentrations ($LC_{50,narc}$) for chemicals in the EPA FHM data set using the CMB_{narc} approach with K_{IAM} estimates for: (left) neutral narcosis chemicals and chemicals with a specific MoA; (right) cationic and anionic chemicals for which the MoA was often not classified (‘‘unsure’’). The solid line represents 1 : 1 correspondence, and dotted line deviations in steps of +1 log units.



mechanism, the potencies with regard to binding to the enzyme is rather low for these chemicals.

The AChE pesticide most toxic to fathead minnow fish is aldicarb (2.6 log units more toxic than $LC_{50,narc}$). The maximum difference between the observed fish $LC_{50,FHM}$ and $LC_{50,narc}$ is 3.2 log units (*i.e.* an “excessive toxicity factor” of 1600) for the reactive chemical 1,3-dichloro-4,6-dinitro-benzene. The most toxic chemical tested in terms of dissolved concentrations was the respiratory blocker/inhibitor rotenone ($0.01 \mu\text{mol L}^{-1}$), although the reactive chemical 1,3-dichloro-4,6-dinitro-benzene is toxic at the lowest calculated cell membrane concentration (0.1 mmol kg^{-1}). Whether any organic chemical is likely to exert a specific MoA at levels below or within the CMB_{narc} range is not readily derived by the current CMB approach. This is part of more detailed risk profile assessments, which may be done using the various MoA and MechoA tools available mentioned in the Introduction, or even based on the likelihood of interactions with the key initiating receptor using the chemical properties of the solute using a polyparameter approach.¹⁰⁶

(v) Using the IAM-based CMB to evaluate whether largely ionized FHM chemicals act by a specific MoA (chemical set 1)

The right graph of Fig. 5 plots the largely cationic (green symbols) and anionic chemicals (red symbols) in the FHM data set for which IAM-HPLC data are available, alongside the neutral (black symbols) narcosis chemicals. The tested cationic amines classified as ‘unsure amines’ all have a molecular formula alike the cationic surfactants: $C_xH_yN^+$.⁷⁷ It is thus expected that the $K_{IAM,intr}$ values adjusted with $\delta_{IAM-MLW}$ increments (set by $C_xH_yN^+$ cationic surfactants) give realistic K_{MLW} values to apply to the CMB_{narc} approach. For most of these “Unsure MoA amines”, the observed $LC_{50,FHM}$ deviates less than a factor of 10 from the predicted $LC_{50,narc}$ – ranging on average a factor of 8.4 with a range between factors 1.3 and 35. Only the quaternary ammonium chemical phenyltrimethylammonium methosulfate (factor 35) and the tertiary amine *N,N*-dimethylbenzylamine (factor 16) have a more than tenfold lower $LC_{50,FHM}$ than $LC_{50,narc}$. The average CMB for all 12 unsure amines is 38 mmol kg^{-1} phospholipid, suggesting that on average these cationic amines with a simple molecular formula of $C_xH_yN^+$ are not toxic to fathead minnow fish by a MoA other than narcosis. Three FHM neurotoxic amines that are mostly cationic at neutral pH are strychnine (rat poison), amphetamine, and nicotine. Based on the adjusted $K_{IAM,intr}$ values, both strychnine and nicotine are calculated to have an $LC_{50,FHM}$ that is 250 and 520 times lower, respectively, than the predicted $LC_{50,narc}$, clearly indicative of a specific MoA operating at lower levels than narcosis. Amphetamine, however, is calculated to be only 9 times more toxic than the predicted $LC_{50,narc}$, positioning more in the range of the other ‘unsure amines’ and appears to have an acute lethal effect concentration based on narcosis for this fish species.

The organic anion salicylic acid is classified in the FHM database as “unsure” MoA, but according to the CMB-approach this organic anion acts as a narcosis organic acid to fish. The acid 2,3,4,6-tetrachlorophenol (pK_a 5.4) is classified as

a Narcosis_I-3 chemical, but falls in line with the other uncoupler acids, acting at the same level as dinoseb (17 and 24 times more toxic than $LC_{50,narc}$, respectively). This was already established for guppy fish data based on liposomal distribution coefficients for these same two acids.¹⁰⁷ The four tested acidic uncouplers are lethally toxic to FHM at a level 17–24 times below the predicted $LC_{50,narc}$, and thus also appear to act as toxicants by a specific MoA based on the IAM-HPLC derived K_{MLW} values. Unfortunately, the number of largely dissociated acids in the FHM database with a narcosis MoA is limited, so we focused on herbicides to further evaluate the use of IAM-HPLC values for acidic chemicals to distinguish between specific MoA and baseline toxicity.

(vi) Chemical set 2: predicting baseline toxicity for herbicides

For 29 herbicides, divided in a set of 17 predominantly anionic and 12 nonionic chemicals, IAM-HPLC based D_{MLW} values were measured, as listed in Table 2. We use the herbicide toxicity data as a second case study to (i) demonstrate how the CMB_{narc} approach can perform risk assessment on the specificity of adverse effects of chemicals to suspected sensitive organisms (*i.e.*, herbicides affecting aquatic primary producers *via* a specific MoA), and to non-sensitive non-target organisms (*i.e.* only *via* baseline narcosis if the key molecular initiating event is not present), and (ii) whether IAM-HPLC is a useful tool in deriving the $LC_{50,narc}$ also for organic anions. The goal of this CMB evaluation with herbicides was not to prove whether herbicides are specifically toxic to various non-target organisms, as this would require inclusion of all available toxicity data (including chronic endpoints) and more detailed analysis of the possible adverse outcome pathways.

As discussed above, in order to translate the IAM-HPLC based $K_{IAM,intr}$ values to D_{MLW} , an empirical corrective increment $\delta_{IAM-MLW}$ of -0.47 for anions was applied to the values listed in Table 2. Using the critical membrane burden of 140 mmol kg^{-1} as derived for neutral chemicals, the baseline $LC_{50,narc}$ was calculated, in Table 6 shown in mg L^{-1} for comparison to the toxicity data.

Dividing CMB_{narc} by D_{MLW} , the $LC_{50,narc}$ was calculated as the aqueous concentration at which non-specific MoA was expected to result in a 50% (sub-)lethal effect (Table 6). Just as we did for the evaluation above for 1 fish species (fathead minnow), the $LC_{50,narc}$ serves as the benchmark value to compare the observed toxic concentrations for the four different aquatic organisms evaluated here.

Using the baseline CMB_{narc} approach, the specificity of an observed toxic effect is more easily expressed by the toxic ratio (TR) in eqn (11):

$$TR = \frac{LC_{50,narc}}{\text{observed } EC_{50} \text{ or } LC_{50}} \quad (11)$$

When including the margins of CMB_{narc} between 80 and 250 mmol kg^{-1} , and residual uncertainty in the $K_{IAM}-D_{MLW}$ relationship, a $TR > 10$ is a strong indicator of a chemical





Table 6 Predicted and observe acute toxicity concentrations, and Toxicity Ratios (TR) for the 29 herbicides

	LC _{50,narc} (mg L ⁻¹) (CMB _{narc} of 140 mmol kg ⁻¹)	Algal EC ₅₀ ^b (mg L ⁻¹)	Algal TR	Macrophyte EC ₅₀ (mg L ⁻¹) <i>Lemna</i> sp. ^a (<i>Myrio-phyllum</i>)	Macrophyte TR	Invertebrate EC ₅₀ ^c (mg L ⁻¹)	Invertebrate TR	Fish LC ₅₀ ^d (mg L ⁻¹)	Fish TR
Anionic herbicides^e (MoA, as in Table 3)									
Clopyralid (SA)	19 297	30	643	89	217	130	148	53	364
Fluroxypyr (SA)	1417	35.3	40	12.3	115	100	14	14.3	99
Triclopyr (SA)	779	75.8	10	✗	✗	131	6	117	7
MCPA (SA)	647	32.9	20	0.152	4258	190	3	50	13
2,4-D (SA)	717	0.68	1054	10.66(0.011)	67(65 169)	134.2	5	100	7
MCPP (SA)	503	23.9	21	1.6(0.027)	315(18 708)	91	6	93	5
2,4-DP (SA)	532	100	5	50(0.156)	11(3411)	46.6	11	46.6	11
MCPB (SA)	184	1.5	123	37	5	55	3	4.3	43
2,4-DB (SA)	181	16.4	11	23.47(0.51)	8(355)	21.2	9	3	60
2,4,5-T (SA)	286	100.8 ^g	3	✗	✗	5.0	57	38.2	7
DNOC (Unc)	267	3.4	79	0.81	330	3.67	73	0.066	4050
Dinoseb (Unc)	19	0.74	25	✗	✗	0.24	77	0.058	319
Bromoxynil (PS-II)	242	0.12	2018	0.118	2052	12.5	19	23	11
Ioxynil (PS-II)	80	0.15	533	0.027	2959	3.14	25	0.64	125
Triasulfuron (AHAS)	1586	0.39	4067	0.000202	7 852 263	100	16	100	16
Bensulfuron-methyl (AHAS)	274	0.0077	35 558	0.0008	342 249	130	2	66	4
Fomesafen (PROTOX)	64	0.42	151	0.48	133	25	3	>99	1
Neutral herbicides (MoA)									
Alachlor (VLCFA)	18	0.0016	11 279	0.0023	8043	18.4	1	2.24	8
Metolachlor (VLCFA)	16	0.056	289	0.0367	441	11.24	1.4	1.23	13
Simazine (PS-II)	91	0.10	914	0.14	653	1.10	83	60.2	2
Ametryn (PS-II)	23	0.0032	7205	0.009	2506	15	2	3.6	6
Terbutylazine (PS-II)	14	0.028	501	0.0128	1097	36	0.4	2.2	6
Diuron (PS-II)	14	0.019	716	0.0183	743	1.1	12	6.7	2
Linuron (PS-II)	11	50.7	0.2	0.017(0.0317)	634(340)	5.81	2	6.7	2
Chloroxuron (PS-II)	2.2	0.0160	140	✗	✗	2.95	0.8	0.43	5
Ethofumesate (LSI)	23	4.7	5	42(0.38)	0.5(59)	13.5	2	11.9	2
Prosulfocarb (LSI)	0.8	0.049	17	0.69	1.2	0.51	2	11.9	1
Clomazone (LCI)	39	0.14	290	34	1.2	12.7	3	15.5	3
Trifluralin (MIT)	0.2	0.0122	13	0.0435	4	0.245	0.6	0.088	2

^a 7 d 50% biomass reduction for duckweed species. ^b 3 d 50% growth reduction of fresh water green algae *Raphidocelis subcapitata* (formerly *Pseudokirchneriella subcapitata*). ^c 2 d 50% immobilization of *Daphnia magna*. ^d 4 d 50% lethal effects on rainbow trout (*O. mykiss*). ^e SA: synthetic auxin; Unc: uncoupler of phosphorylation; AHAS inh.: inhibits plant amino acid synthesis - acetoxyhydroxyacid synthase AHAS; PROTOX: inhibits protoporphyrinogen oxidase (PROTOX), leading to irreversible cell membrane damage. PS-II: inhibitor for photosystem II. (VLCFA: very-long-chain fatty acid (inhibition of cell division); LSH: lipid synthesis inhibitor; LCI: lycopene cyclase inhibitor; MIT: mitosis inhibitor. ^f ✗ = no reliable ecotoxicity data retrieved. ^g Italic values: not from regulatory dossiers but through the US EPA ECOTOX database.

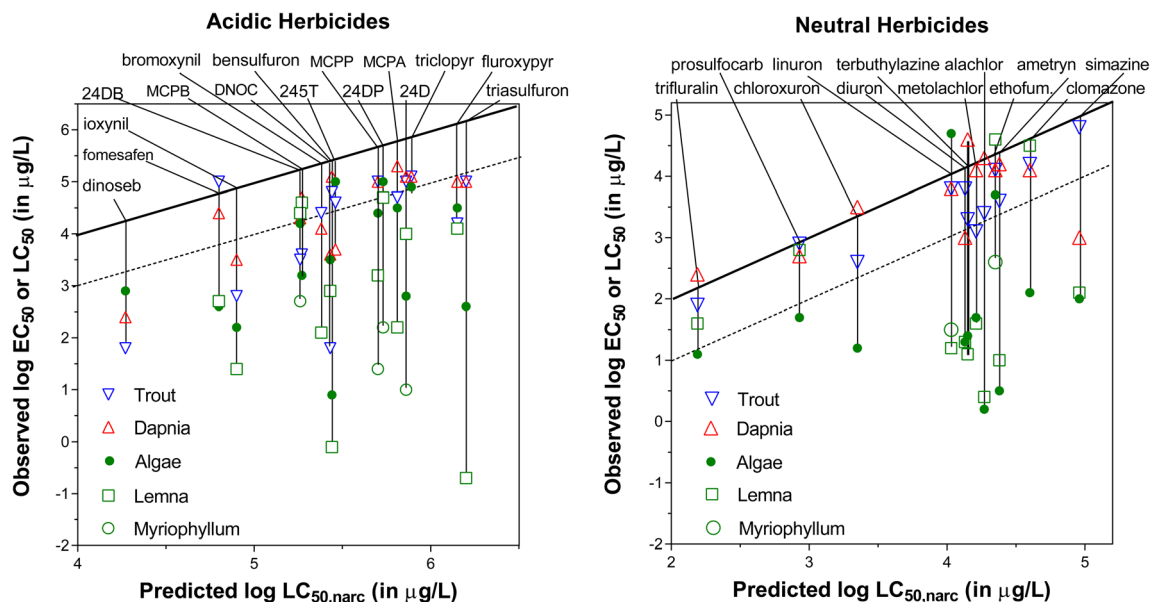


Fig. 6 Predicted acutely narcosis toxic concentrations ($LC_{50,narc}$) for largely deprotonated herbicides (left) and neutral herbicides (right) using the CMB_{narc} approach with k_{IAM} estimates, compared to observed acute toxicity for 4 non-target species: the aquatic plant duckweed (green squares, either *Lemna gibba* or *Lemna minor*, 7 day exposure), dicotyl macrophyte *Myriophyllum spicatum*, green algae (green dots), planktonic crustaceans (red upward triangle, mostly *daphnia magna*), and fish (blue downward triangle, mostly rainbow trout *Oncorhynchus mykiss*). Full diagonal line represents the 1 : 1 line, and the broken line a $10\times$ higher observed toxicity. Data for each herbicide are connected via a vertical line.

exerting adverse effects other than *via* a non-specific MoA. Since $LC_{50,narc}$ is based on the CMB_{narc} of 140 mmol kg^{-1} , a $TR > 10$ is set as the cut-off concentration of the critical membrane burden of 14 mmol kg^{-1} below which chemicals induce toxicity through a specific MoA. This corresponds to the observation for narcosis chemicals in chemical set 1 with the US-EPA fathead minnow database, where out of 78 Narcosis_I and Narcosis_II chemicals the lowest CMB was 13. The CMB_{narc} is a fairly constant value across all kinds of organisms^{56,57,107} and the FHM evaluation above already showed adequate predictions for a broad range of narcosis chemicals in fish. Table 6 lists the TR values for the herbicide endpoints for different aquatic organisms relative to $LC_{50,narc}$.

We assumed that all herbicides are toxic to aquatic plants *via* a specific MoA, but mostly act by narcosis (non-specific MoA) on non-target invertebrates and fish, except for uncouplers of oxidative phosphorylation (DNOC and dinoseb). Fig. 6 shows the range of toxicity endpoints for various aquatic species for each herbicide along the Y-axis, in relation to the expected baseline $LC_{50,narc}$.

Chemical set 2: do all herbicides induce a specific effect on aquatic plants? The tested herbicides were expected to act by a specific toxicity to aquatic primary producers with at $TR > 10$. This seems obvious when the inhibition of the photosystem is the main herbicidal MoA. Algae and aquatic macrophytes likely also share other features with target terrestrial weeds that many herbicides specifically act upon, such as cell division structures, and inhibition of necessary lipids and amino acids. However, some herbicides act specifically on either monocotyl or dicotyl terrestrial weeds. Therefore, we broadened the group of aquatic plants to cover green algae (mostly *R. subcapitata*), duckweed as

a monocotyl macrophyte (*Lemna* sp.), and a dicotyl macrophyte *Myriophyllum* sp., as far as acceptable data were available in regulatory dossiers. Unfortunately, no toxicity data could be retrieved for 4 herbicides on *Lemna*, and for only 6 herbicides endpoints on *Myriophyllum* were available. The endpoints on aquatic primary producers are indicated by different green symbols in Fig. 6.

As expected, for 21 of the 25 herbicides with complete toxicity data on duckweed and green algae, the herbicides do seem to impair growth by a specific toxic MoA ($TR > 10$) for at least one of these two aquatic plant species (Table 6). When comparing the two plots of Fig. 6, it appears that most acidic herbicides are more toxic to duckweed than to green algae, while most neutral herbicides affect green algae at lower concentrations than duckweed.

For several anionic synthetic auxins, *Lemna* and algae appeared to be affected only by baseline toxicity, with TR of less than 10 for 2,4,5-T (algae), 2,4-DB (algae and *Lemna*), 2,4-DP (*Lemna*), MCPB (*Lemna*). *Lemna* does show a high specific effect for MCPA with a $TR > 4000$. *Myriophyllum*, however, appears to be a much more sensitive primary producer than *Lemna* and *R. subcapitata*, with a TR of 6.5×10^4 for 2,4-D.

For the herbicide 2,4,5-T, only an algal toxicity endpoint was retrieved, which showed a $TR < 10$. For 3 out of the 29 herbicides the TR was < 10 for green algae, but TR was > 10 for one or both of the aquatic plants. For the neutral herbicides targeting lipid synthesis (ethofumesate and prosulfocarb), microtubule assembly during mitosis (trifluralin), and lycopene cyclase to block provitamin A carotenoid synthesis (clomazone), duckweed appeared to be affected only through baseline toxicity ($TR < 10$). For ethofumesate, however, *Myriophyllum* showed a higher



TR of 59, and green algae appeared to be specifically affected by clomazone (TR of 290).

The herbicides most toxic to aquatic plants in our data set are the acidic *N*-sulfonylurea chemicals bensulfuron-methyl and triasulfuron. In target plants, these herbicides inhibit plant-essential amino acid synthesis (acetohydroxyacid synthase AHAS). Particularly duckweed growth is affected by this (or related) specific MoA, at a TR of 3.4×10^5 and 7.8×10^7 , for bensulfuron-methyl and triasulfuron respectively. Triasulfuron has been banned for use in the EU since 2017, while bensulfuron-methyl is approved in several European countries, according to the University of Herfordshire's PPDB.

Chemical set 2: do herbicides only induce a non-specific effect on aquatic invertebrate and vertebrate organisms? For rainbow trout (*O. mykiss*), none of the neutral herbicides had a TR higher than 13, suggesting that these herbicides do not induce toxicity *via* a specific MoA. For 18 of the 29 herbicides, the 48 h immobilization EC_{50} for *D. magna* also showed a TR < 10. The CMB approach combined with IAM-HPLC based on D_{MLW} values indeed seems to be a valuable method to define acutely toxic aqueous concentrations at non-specific baseline levels.

However, the CMB-approach also demonstrates that several herbicides affect non-target aquatic species other than aquatic plants *via* a specific MoA. Since some of the acidic herbicides were classified as having an uncoupler MoA already in the FHM database, as well as in PPDB (Table 5: DNOC, dinoseb), it was expected that some other acidic herbicides may also demonstrate specific toxicity to daphnids and fish. For 11 of the 17 acidic herbicides the TR was indeed higher than 10, ranging up to 4050. For some of these acidic herbicides fish were the most sensitive species, although no data for *Lemna/Myriophyllum* were available for both DNOC and dinoseb to evaluate over multiple aquatic plant species. The herbicides most toxic to fish were indeed the phosphorylation uncouplers DNOC and dinoseb, with respective TR values of 4050 and 319. Dinoseb is even considered highly toxic to birds, and while it was once widely used, it has therefore been banned as a pesticide in most countries. DNOC has no longer been approved in the EU and North America since 1991.

Simazine is the only neutral herbicide example in the current selection with a specific toxicity to daphnids, with a TR of 83, while it is not acutely toxic *via* a specific MoA to rainbow trout. The acids 2,4,5-T, DNOC and dinoseb also appear to affect daphnids by a specific toxicity, with a TR of 53, 77, and 73, as well as the photosystem inhibitors bromoxynil and ioxynil (TR of 19 and 25, resp.).

4. Synopsis

The CMB_{narc} approach for determining acute fathead minnow toxicity and acute effects of herbicides on non-target organisms shows that IAM-HPLC is able to predict baseline toxic concentrations and distinguish between chemicals that exert baseline narcosis and toxicity due to another more specific MoA, for neutral, cationic and anionic organic chemicals. A Toxic Ratio (TR) of observed toxic concentrations against the predicted

LC_{50,narc} of >10, corresponding to a CMB <14 mmol kg⁻¹, is a good indication that the chemical exerts toxicity *via* a specific MoA for the purposes of screening chemicals as part of a consensus MoA approach. The fact that IAM-HPLC is a simple experimental assay makes it a valuable alternative to octanol-water based predictions for chemicals that fall outside the applicability domain of the log K_{OW} -log K_{MLW} relationships. The limiting factor of IAM-HPLC is that the maximum apparent log K_{IAM} that seems experimentally feasible is about 6, which translates to retention times of ~24 h with an eluent flow rate of 1 mg min⁻¹. At a higher retention capacity factor, higher solvent fractions are required, which may not linearly extrapolate to fully aqueous eluent, particularly for ionogenic chemicals. Still, a series of high solvent fractions in the eluent could provide for a rough value of components in complex (technical) mixtures, or provide a lower limit to the tested chemical of log D_{MLW} ~6, which would already indicate strong sorption to cell membranes, a potentially high bioaccumulation factor, and toxic baseline concentrations <0.1 mg L⁻¹.

Author contributions

The experimental work was performed by SD. The manuscript was written by SD with contributions of all authors. All authors have given approval to the final version of the manuscript. The conclusions expressed in the paper represent the expert judgement of the authors, but not necessarily the opinion of their affiliation.

Conflicts of interest

There are no conflicts of interest to declare. The contribution by SD is based on his experimental work during employment at Utrecht University, which ended on September 30th, 2016, and at the University of Amsterdam, which ended on December 31st, 2019.

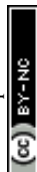
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