



Mode of Action (MOA) Assignment Classifications for Ecotoxicology: An Evaluation of Approaches

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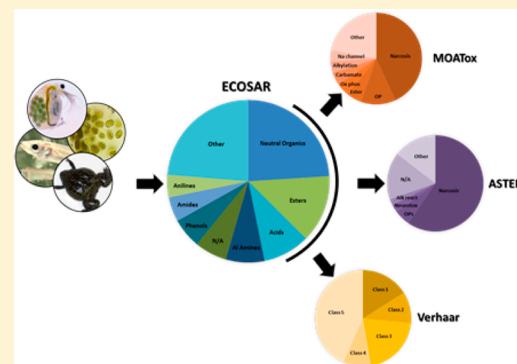
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Supporting Information

ABSTRACT: The mode of toxic action (MOA) is recognized as a key determinant of chemical toxicity and as an alternative to chemical class-based predictive toxicity modeling. However, MOA classification has never been standardized in ecotoxicology, and a comprehensive comparison of classification tools and approaches has never been reported. Here we critically evaluate three MOA classification methodologies using an aquatic toxicity data set of 3448 chemicals, compare the approaches, and assess utility and limitations in screening and early tier assessments. The comparisons focused on three commonly used tools: Verhaar prediction of toxicity MOA, the U.S. Environmental Protection Agency (EPA) ASsessment Tool for Evaluating Risk (ASTER) QSAR (quantitative structure activity relationship) application, and the EPA Mode of Action and Toxicity (MOAtox) database. Of the 3448 MOAs predicted using the Verhaar scheme, 1165 were classified by ASTER, and 802 were available in MOAtox. Of the subset of 432 chemicals with MOA assignments for each of the three schemes, 42% had complete concordance in MOA classification, and there was no agreement for 7% of the chemicals. The research shows the potential for large differences in MOA classification between the five broad groups of the Verhaar scheme and the more mechanism-based assignments of ASTER and MOAtox. Harmonization of classification schemes is needed to use MOA classification in chemical hazard and risk assessment more broadly.



INTRODUCTION

There has been a shift in the risk assessment paradigm toward a more mechanistically based understanding of the mechanism and mode of action of chemicals.¹ Improved understanding of toxicological mechanisms would reduce reliance on traditional animal testing, allow chemical grouping to increase assessment efficiency, and improve toxicity extrapolations.^{2,3} These outcomes would increase the potential use of animal alternative methods, which is a long-term policy goal of most chemical management programs.^{4,5}

Various structure-based classification schemes have been developed to categorize chemicals based on the mode of toxic action (MOA). Thousands of chemicals are in commerce and most have little available information other than structure. Understanding how the available MOA schemes were devised, what information they are based on, and the limitations of each approach are critical. The various MOA classification schemes provide information on a key determinant of chemical toxicity

and as an alternative to chemical class-based predictive toxicity modeling. Direct regulatory application of these MOA classifications has been seen most directly in chemical classification and in the use of Quantitative Structure–Activity Relationship (QSAR) models in risk assessment.⁶ MOA also plays a role in mixture risk assessment, where both the grouping of chemicals and the choice of the concentration addition or independent action hypothesis can be based on knowledge of the MOA.⁷

MOA is an operational term that has been loosely defined in both human health and ecotoxicology as a functional change at the cellular level, in contrast to the mechanism of action or molecular initiating event. The definition of MOA is critical in

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the context of Adverse Outcome Pathways (AOPs). Generally, MOA describes key events at various levels of biological organization, starting with cellular interaction and leading to functional and/or anatomical changes.⁸ MOA is important in classifying chemicals because it represents an intermediate level of complexity between molecular mechanisms and physiological or organismal outcomes and provides an organizing scheme for chemical classification.⁹ MOA has also been used to classify pesticides by the interaction with the receptor of the target species¹⁰ and to describe the toxicology of human cancer and noncancer agents by key cytological and biochemical events.^{11,12} The definitions of MOA used are unique to each of the classification schemes investigated: Verhaar used five broad categories based on general toxicological responses,¹³ whereas the U.S. Environmental Protection Agency (EPA) ASsessment Tool for Evaluating Risk (ASTER) QSAR (quantitative structure activity relationship) application¹⁴ and the EPA Mode of Action and Toxicity (MOAtox) database¹⁵ have a high degree of specificity based on fish behavioral responses or weight of evidence classification, respectively. It is also important to note that both within and between classification schemes, different levels of biological organization may be represented (e.g., at the molecular, cellular, and organism-level).

Research to develop screening and early tier methods to more easily classify or assess chemicals using approaches such as the ecological threshold of toxicological concern (eco-TTC)¹⁶ and chemical activity¹⁷ is ongoing. Evaluation of these approaches and determination of their domain of applicability is partly dependent on the MOA classification used to group chemicals.

The objectives of this study were to critically evaluate available MOA classification methodologies using a set of unique chemicals from a large aquatic toxicity data set, compare the various approaches, and evaluate their utility and limitations in screening early tier assessments.

OVERVIEW OF MOA SCHEMES

The comparison of classification approaches focused on three currently available schemes: the Verhaar scheme for prediction of toxicity MOA,¹³ the EPA ASTER QSAR application,¹⁸ and the EPA MOAtox database¹⁵ (Table S1 of the Supporting Information, SI). The Verhaar scheme classifies organic compounds into one of four categories: inert chemicals (Class 1), less inert chemicals (Class 2), reactive chemicals (Class 3), and chemicals acting by a specific mechanism (Class 4). Chemicals in Class 1 exhibit nonpolar narcosis or baseline toxicity and can only be predicted if they have log octanol:water partition coefficient (K_{ow}) values between 0 and 6 (e.g., benzenes).

Chemicals in Class 2 are more toxic and cause polar narcosis, and typically possess hydrogen bond donor acidity (e.g., phenols and anilines).¹³ Chemicals in Class 3 demonstrate enhanced toxicity as compared to baseline toxicity and react nonspecifically with biomolecules (e.g., epoxides) or are metabolized into more toxic species (e.g., nitriles).¹³ Chemicals in Class 4 cause toxicity through a specific mechanism such as acetylcholinesterase (AChE) inhibition by carbamate insecticides. The assignment of a chemical to a class is based on a decision tree that utilizes the presence or absence of certain chemical structures and moieties. The Verhaar classification scheme has been further modified by changing the order of some classification rules in the decision tree and by adding a

fifth class to which chemicals are assigned if the chemical could not be placed in Class 1–4.¹⁹

Verhaar and modified Verhaar schemes have been implemented into online tools in the public domain such as the Organization for Economic Cooperation and Development (OECD) QSAR Toolbox (<https://www.qsartoolbox.org/home>) and Toxtree (https://eurl-ecvam.jrc.ec.europa.eu/laboratories-research/predictive_toxicology/qsar_tools/toxtree).²⁰ The OECD QSAR Toolbox is a software application used in the grouping of chemicals and in filling (eco)toxicity data gaps for assessing the hazards of chemicals. Chemical profiling is based on the Chemical Abstracts Service numbers (CAS) and/or SMILES (Simplified Molecular Line Entry System). Toxtree, an open-source application, groups chemicals into categories using several schemes based on SMILES notation and decision trees. A postprocessing filter encoded as a KNIME (Konstanz Information Miner) workflow adds amendments to the classification rules²¹ to improve the Toxtree classification. The postfilter addresses three types of misclassification: Class 5 of some aliphatic alcohols, some halogenated compounds that should both be assigned to Class 1 (rule 1.5.2 and rule 1.7.1, respectively, of the Verhaar classification scheme), and non- or weakly acidic phenols (rule 2.1). Fewer compounds are misclassified as outside of the model domain, further improving the prediction accuracy of the scheme.

ASTER is a rule-based expert system that screens a chemical structure for structural fragments to determine MOA.¹⁸ The tool uses acute toxicity of over 600 chemicals in fathead minnows. Nonpolar narcosis is the default MOA if no MOA specific structural fragments are identified. If fragments satisfy requirements for more than one MOA, then the QSAR for the most potent toxicity prediction is selected.¹⁸ ASTER is based on the MOA categories in Russom et al.²² and contains information on over 50 000 molecular structures. ASTER was primarily developed with neutral industrial organic chemicals acting through narcosis and has a more limited representation of pesticides and other chemicals with more specific modes of action. The tool is not publicly available.

MOAtox is composed of a database of MOA assignments for 1208 chemicals including metals, organometallics, pesticides, and other organic compounds.¹⁵ The categorization scheme was based on earlier work determining chemical modes of acute toxic action in fish and encompasses six broad and 31 specific MOAs.²³ The resulting data set used a combination of high-confidence MOA assignments, including biological responses in fish acute toxicity assays,²² pesticide classifications schemes [e.g., Insecticide Resistance Action Committee (IRAC)], QSAR predictions (e.g., ASTER), and weight of evidence professional judgment incorporating an assessment of chemical structure (e.g., analogous structure; moiety/functional group presence) and available information on MOA, mechanism of action, and toxicity pathways. Chemicals with an uncertain MOA assignment and MOAs specific to invertebrates were excluded. Specific MOAs were developed as subcategories of the broad MOAs based on either chemical structure or known mechanism of action.

MATERIALS AND METHODS

Figure S1 provides an overview of the materials and methods used in this analysis.

Database Construction and Curation. A master data set was constructed using predetermined inclusion criteria (Figure

Table 1. Comparison of the Three MOA Classification Schemes^a

| Verhaar (Modified) | ASTER MOA | MOAtox Broad | MOAtox Specific |
|---|--|--|--|
| Class 1 (narcosis or baseline toxicity) | Non-polar narcosis | | Non-polar |
| Class 2 (less inert compounds) | Polar narcosis | Narcosis | Polar |
| | Ester narcosis | | Ester |
| | Diester toxicity | | |
| | Reactive | | |
| | Chloro-diester-based reactivity | | |
| | Carbonyl (C=O)-based reactivity | Reactivity | Carbonyl |
| | Carbonyl reactivity | Reactivity | Carbonyl |
| | Alkylation/arylation-based reactivity | Reactivity | Alkylation |
| | Acylation-based reactivity | | |
| | Sulfhydryl (-S-H)-based reactivity | | |
| Class 3 (unspecific reactivity) | Reactive dinitroaromatic group | Reactivity | Di/trinitroaromatic |
| | Nitroso-based reactivity | | |
| | Quinoline reactivity | | |
| | Acetamidophenol reactivity | | |
| | Reactive diketones | | |
| | Acrylate toxicity | Reactivity | Acrylate |
| | N-halogenated acetophenone inhibition | | |
| | Hydrazine-based reactivity | Reactivity | Hydrazine |
| | | Reactivity | Chromate |
| | | Reactivity | Phosphide |
| | | Reactivity | Other |
| | Isocyanate (-N=C=O)-based reactivity | Reactivity | Cyanate/nitrile (OCN-; C≡N) |
| | Pyridinium compounds | | |
| Class 4 (compounds and groups of compounds acting by a specific mechanism) | Neurotoxicant: DDT-type | Neurotoxicity | Sodium channel blocking |
| | Neurotoxicant: pyrethroid | | Diphenyl sodium channel modulation |
| | Neurotoxicant: cyclodiene-type | | Pyrethroid sodium channel modulation |
| | | | Alicyclic GABA antagonism |
| | | | Pyrazole GABA antagonism |
| | | | GABA agonism |
| | Neurotoxicant: strychnine | | Strychnine |
| | Neurotoxicant: nicotine | | Nicotinic acetylcholine receptor agonism |
| | | | Other |
| | Organophosphate-mediated AChE inhibition | AChE inhibition | Organophosphate |
| | Carbamate-mediated AChE inhibition | | Carbamate |
| | Uncoupler of oxidative phosphorylation | | Uncoupling oxidative phosphorylation |
| | Respiratory blocker: azides and cyanides | Electron transport inhibition | Arsenical respiratory inhibition |
| | | | Oxidative phosphorylation inhibition |
| | | | Metallic ion/osmoregulatory impairment |
| | | | Methemoglobinemia |
| | | Iono/osmoregulatory/circulatory impairment | Anticoagulation |
| | | | Other osmoregulatory |
| | | | Unknown |
| Class 5 | Unknown mode of action | Unknown | Unknown |

^aAChE, acetylcholinesterase; ASTER, assessment tool for evaluating risk; MOA, mode of toxic action; MOAtox, mode of action and toxicity database.

S2) following the Stepwise Information-Filtering Tool (SIFT) of Beasley et al.²⁴ An initial data set of approximately 200 000 studies and chemical property information was compiled, including MOA classification. Approximately 110 000 toxicity data records meeting inclusion criteria were prepared to inform the MOA assignment objectives.

Records in the final data set were subjected to additional curation steps:

- Harmonization of data (e.g., taxonomic name, duration, test statistic units)

- Spot verification of source information, durations, test organism
- Determination of acute/chronic test type and toxicity end point

CAS numbers were extracted from the working data set to inform the MOA assignment investigation. A total of 5638 unique CAS were subjected to further reconciliation, including identification of duplicates and mismatches.

The CAS list was processed with OECD QSAR Toolbox 3.4 to obtain the related SMILES code, the EPA Ecological Structure Activity Relationships (ECOSAR)²⁵ chemical classi-

fication, and the aquatic MOA according to the modified Verhaar classification. The SMILES are essential as the two-dimensional representation of the molecule processed by the MOA assignment tools. The CAS list was also employed to obtain the ASTER and the MOAtox classifications.

257 CAS were not recognized (no match, no name, and no SMILES were retrieved in the various databases) and therefore could not be processed. 151 CAS were linked to a chemical name without a corresponding SMILES and therefore resulted as “blank” or “non-applicable” regarding the MOA assignment. For some CAS, several SMILES matched, which was either linked to the presence of a salt form of the chemical or to an error in the SMILES. In some rare cases, the same SMILES linked to two different CAS, which was mainly related to different enantiomeric forms of the chemical.

Once the CAS list was scrubbed to remove incorrect or unrecognized CAS, 3448 of the original 5638 chemicals remained (61%) for which there were CAS matched to a name and a correct SMILES notation.

MOA Assignment. Two different tools (OECD QSAR Toolbox 3.4 and Toxtree 2.6.6²⁰) were used to compare the MOA assignment results for the modified Verhaar scheme, as Bhatia et al.²⁶ showed that discrepancies might exist between the tools. The CAS was used as an input in the OECD QSAR Toolbox, which also retrieves the name and SMILES for each chemical. The SMILES were then processed with Toxtree 2.6.6, to obtain the Verhaar classification. The results obtained with the Toxtree software were then processed through a KNIME postfilter workflow,²¹ which aims at lowering the number of compounds being classified as outside of the model domain and further improving the prediction accuracy of the scheme.

To obtain ASTER classifications, the SMILES corresponding to the listed CAS were first processed through the proprietary ASTER tool. If SMILES was not present or returned an error, then the corresponding CAS was submitted to maximize the number of CAS given an ASTER assignment. When the SMILES represented a structure described as “impossible,” ASTER returned an error message describing the structure discrepancies, and no MOA was assigned. When the SMILES represented a mixture, no MOA was assigned, as mixtures are outside the ASTER applicability domain. When no match was found from the SMILES or CAS, no MOA assignment was returned.

MOAtox assignments were obtained from previously published tables,¹⁵ rather than from model predictions. The individual CAS numbers of all chemicals in the database were cross-referenced to the curated CAS-linked MOA classifications in Table S2.¹⁵

Along with the MOA, the ECOSAR classes of the chemicals also were extracted to characterize the data set and to identify the chemicals and classes present in the data set. The ECOSAR classification distinguishes up to 111 classes of organic chemicals, including neutral organics, organic chemicals with excess toxicity, and chemicals that are determined to be surfactants.

Analysis. The Verhaar MOAs obtained with the OECD QSAR Toolbox and the Toxtree software for each chemical were compared to identify potential discrepancies ($n = 3448$). The result of the use of the KNIME postfilter on the classification given by Toxtree was also evaluated for discrepancies.

Mapping the three classification schemes shows how they correspond and where they overlap (Table 1). The subset of

chemicals for which at least three classifications were obtained using Verhaar, OECD QSAR Toolbox, ASTER, and the MOAtox classifications ($n = 432$) was further analyzed to compare the MOA assigned according to the various schemes, specifically where those classification assignments overlap. The complete data set is given in Table S2.

RESULTS

Chemical Classes in Data Set. The data set of 3448 chemicals was evaluated using ECOSAR to provide an overview of the chemical class coverage. For chemicals where multiple classifications were generated by ECOSAR, the first reported was used. These 89 categories were further collapsed into 46 more general categories. Collapsed ECOSAR classifications that each represent $>4\%$ of the total data set are shown in Figure 1.

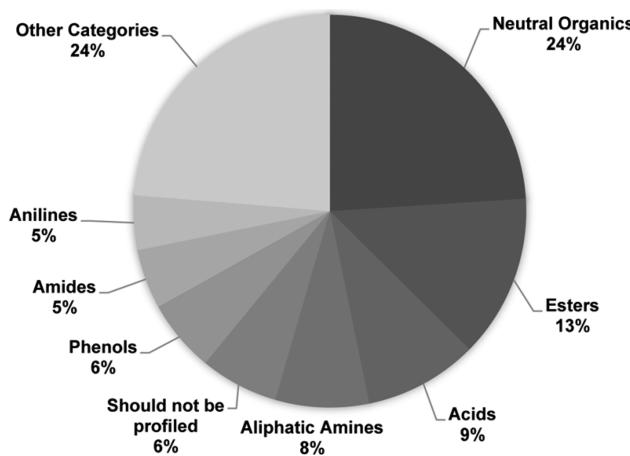


Figure 1. Pie chart illustrating ECOSAR chemical classes represented in this data set/analysis ($n = 3448$). ECOSAR, Ecological Structure Activity Relationships.

Figure 1 includes the seven major categories in which the most chemicals were classified (e.g., those individual categories in which $\geq 4\%$ of the total number of chemicals were classified). 24% of chemicals in the data set were classified as neutral organics, with the second most common category being esters (13%). Importantly, approximately 6% of chemicals were classified as “should not be profiled” and therefore were not assigned a chemical category. The “Other” category (24%) represents other nonmajor chemical categories that are each represented by $<4\%$ of the total chemicals. The complete list of ECOSAR classifications, the collapsed assignments, and the number of CAS within each category is provided in Table S3.

Overview of MOA Assignments. Verhaar assignments were made for 3448 chemicals, of which 1165 were assigned an ASTER classification (including NA; 981 excluding NA) and 802 were assigned a MOAtox broad classification. Within those 802 chemicals, six chemicals (classified as narcosis) did not get a MOAtox specific classification. For a subset of 432 chemicals (including the six chemicals missing the MOA specific classification), all three classifications were available.

Figure 2 illustrates the MOA assignments for the three classification schemes, with the total number of unique CAS classifications noted below. For all classification schemes, the category with the largest proportion of chemicals is narcosis (with Verhaar Class 1 and Class 2 both representing narcotic modes of action; Class 2 = polar narcosis).

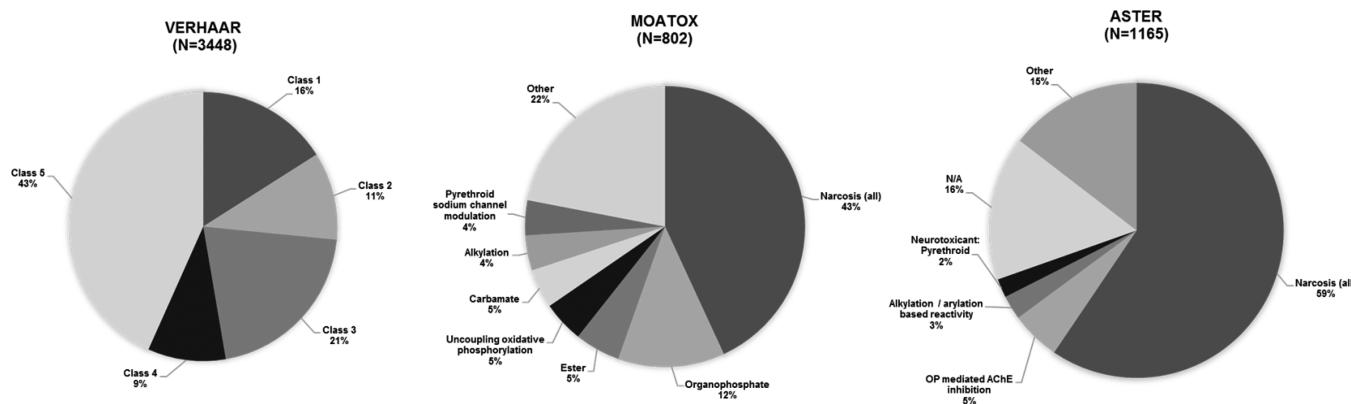


Figure 2. MOA assignments for unique CAS (represented by “N”) for each of the three classification schemes. AChE, acetylcholinesterase; ASTER, ASsessment Tool for Evaluating Risk; CAS, Chemical Abstracts Service number; MOAtox, Mode Of Action and Toxicity database; N/A, Not Applicable; and OP, Organophosphate.

Overlap Among Classification Schemes. The subset of 432 chemicals for which the three classifications were available was further analyzed to compare the MOA assigned according to the various schemes based on their overlap (**Table 1**). For 41.7% of the chemicals, the three schemes were in agreement. For 51.2%, one scheme gave a different classification than the two others. For 7.2% of the chemicals, a different class was provided by the three classification schemes.

Analysis of the agreement patterns according to the MOA (**Figure 3**) shows that the best agreement is obtained for

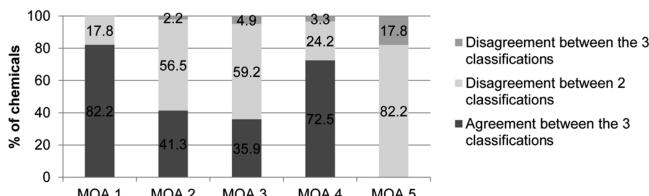


Figure 3. Agreement across the three classification schemes according to the MOA. MOA, mode of toxic action.

nonpolar narcosis chemicals (MOA-1), for which the three classification schemes agree in 82.2% of the cases, and for specifically acting chemicals (MOA-4), for which 72.5% of agreement across the three schemes was obtained. Regarding MOA-2 and 3 chemicals, there is agreement between the three schemes in 41.3 and 35.9% of the cases, respectively.

Classification Methods Using Different Tools: Level of Concordance in MOA Verhaar-Based Classification Tools. The coherence of assessments using different classification tools was checked by detailed cross-comparisons of the Verhaar modified classification obtained by the OECD QSAR Toolbox and Toxtree. Results are shown in **Table S4**.

A minor number of chemicals were classified into two classes by the QSAR Toolbox (16 chemicals of 3448). In total, 2466 chemicals of the data set are classified in the same class by both tools (71.5%), and 28.5% of them are classified into different classes.

Of the 3448 chemicals, 1862 and 1490 chemicals are classified as Verhaar MOA-5 by Toxtree and the OECD QSAR Toolbox, respectively (i.e., 54 and 43.2% of the data set). These chemicals are either recognized as outside of the domain of the model or should be classified in one of the previous classes but are not recognized by the currently implemented rules.

The highest concordance is found for MOA-4 and MOA-1 chemicals: respectively, 87.2 and 77.7% of those chemicals are similarly classified by both tools. The majority of MOA-4 and MOA-1 chemicals that are not recognized as such by Toxtree are classified MOA-5 (9 and 16%, respectively).

The lowest concordance is obtained for MOA-2 and MOA-3 chemicals, as only 47.5% and 45.4% of those chemicals, respectively, are similarly classified by both tools. The majority of MOA-2 and MOA-3 chemicals that are not recognized as such by Toxtree are classified as Class 5 (39.4 and 46.6%, respectively).

The use of the KNIME postfilter allowed the reattribution of 168 chemicals of the 3448 chemicals assessed into more appropriate classes (**Table S5**). For 139 of those 168 chemicals, the classification given by the KNIME postfilter is the same as the classification given by the QSAR Toolbox. The use of this filter decreased the apparent discrepancies found when using those two different tools. The complete list of the chemicals reassigned by the KNIME postfilter is given in **Table S2**.

Disagreement Between Two Classification Schemes.

For 221 chemicals, there was a disagreement between two classification schemes. Among those, eight were classified as MOA-1 chemicals by the OECD QSAR Toolbox, 26 as MOA-2 chemicals, 62 as MOA-3 chemicals, 29 as MOA-4 chemicals, and 96 as MOA-5 chemicals.

Among the eight MOA-1 chemicals, three were similarly classified by ASTER and MOAtox as neurotoxicants and one was classified as reactivity/quinoline. The other four were also classified as nonpolar narcosis by ASTER but were classified differently by MOAtox (one neurotoxicant, two reactivity, and one iono/osmoregulatory/circulatory impairment).

Regarding the 26 MOA-2 chemicals, the majority ($n = 18$) were classified as nonpolar narcotics by both MOAtox and ASTER. Four others were similarly classified as reactive (two because of a ditrinitroaromatic group and one because of an alkyl group) or as uncoupler of oxidative phosphorylation (one chemical). The last four were also classified as polar narcosis by ASTER but were classified either as uncoupling of oxidative phosphorylation (two chemicals) or nonpolar narcosis by MOAtox (two chemicals).

Regarding the 62 chemicals classified as MOA-3 by the OECD QSAR Toolbox, 35 were classified as nonpolar narcotics by both ASTER and MOAtox. Ten chemicals were classified as uncoupler of oxidative phosphorylation ($n = 7$), polar narcosis ($n = 2$), or neurotoxicant ($n = 1$) by both ASTER and

MOAtox. Nine chemicals were also classified as reactive chemicals by ASTER but classified differently by MOAtox (among those, five were classified as neurotoxicants) and one chemical was also classified as a reactive chemical by MOAtox but as nonpolar narcosis by ASTER. Finally, five were classified as diester toxicity by ASTER but ester narcosis by MOAtox; two were classified as reactive due to sulfhydryl moieties, whereas their classification as reactive by MOAtox was based on a cyanate/nitrile group. For these last seven chemicals, there is therefore no true contradiction in the classification obtained across the different schemes, but they rather reflect slight differences due to the way the schemes have been built.

Among the 29 MOA-4 chemicals for which a discrepancy was found between two classification schemes, 19 were classified as nonpolar narcotics, one as polar narcotic, and one as ester narcotic by both ASTER and MOAtox. Five chemicals were also classified as specifically acting by MOAtox but were classified as nonpolar narcotics by ASTER, and three chemicals were classified as specifically acting by ASTER but nonpolar narcosis by MOAtox.

For MOA-5 chemicals ($n = 98$), the discrepancies come for 63 chemicals from a nonpolar narcotic classification and for 18 chemicals from specifically an organophosphate (OP)-mediated AChE inhibitor by both ASTER and MOAtox.

Therefore, when there is a discrepancy between two of the three classification schemes, it is most often due to a nonpolar narcosis classification by ASTER/MOAtox, which is not recognized as MOA-1 in the Verhaar classification (this is the case for 134 chemicals of those 222 for which we have a discrepancy between two classification schemes; therefore, in 60% of the cases).

Disagreement Between the Three Classification Schemes. The majority of chemicals for which there is a discrepancy between all the classification schemes are MOA-5 according to the OECD QSAR Toolbox (21 of 31 chemicals). The others are MOA-3 ($n = 5$), MOA-4 ($n = 4$), and MOA-2 ($n = 1$). Three chemicals are inorganics, which are usually out of the domain of those classification schemes (Table 1) and therefore explain why they are not correctly classified. The remaining 17 of the MOA-5 classifications from the OECD QSAR Toolbox are identified as nonpolar narcosis by ASTER.

■ DISCUSSION

Suggested Modifications to "Harmonize" Classification Schemes. One of the most significant barriers to the use of MOA assignment tools is the high number of errors found in the database associated with the assignment of unique CAS numbers and their related SMILES. These types of errors can be due to database entry (CAS number or improper SMILE notations), which prevents the classification tool from recognizing the chemicals and, therefore, greatly reduces the reliability of results. However, this issue is more a function of the data quality in the database than to the various classification scheme/assignment tools themselves.

The different classification schemes were not built for the same purposes and did not respond to the same information needs. The Verhaar classification scheme aims at a broader classification, which is particularly interesting in ecotoxicity when dealing with various species for which the sensibility toward a specific mode of action might differ. The Russom classification scheme implemented within the ASTER MOA and MOAtox is more specific and addresses some particular mechanisms of action (e.g., uncoupler of oxidative phosphor-

ylation, pyrethroid sodium channel modulation, etc.). This might be of interest to identify the more sensitive species on which to base an ecotoxicological reference value in a risk assessment context. However, because there is an overlap between different classification schemes (Figure 2), especially for narcotic chemicals, the results should be coherent to provide a reliable interpretation.

Although narcotic compounds are chemicals for which there is a greater agreement between the three schemes, it was noted that the majority of the discrepancies found for the Verhaar MOA for MOA-2, 3, 4, or 5 were related to a nonpolar narcosis classification in ASTER. For the 63 MOA-5 chemicals for which this occurred, the discrepancy can be explained by the different logic underpinning the various classifications. In the Verhaar classification scheme, a chemical that could not be classified as Class 1–4 would be by default classified as MOA-5 as the last option, whereas in the ASTER classification scheme, a chemical for which the chemical moieties that trigger a classification as polar narcosis, ester narcosis, reactive chemicals, or specifically acting chemicals would be by default classified as nonpolar narcosis. Similarly, the majority of chemicals for which a discrepancy was found between the three classification schemes were classified MOA-5 according to the OECD QSAR Toolbox and nonpolar narcosis by ASTER.

Regarding the MOA-2, 3, and 4 chemicals that are classified nonpolar narcotic by ASTER, this probably comes from the fact that structural rules underpinning the Verhaar classification decision tree do not match with structural moieties looked for within the Russom classification. An in-depth analysis of what triggered a different classification for those chemicals within the different framework would be necessary for a better understanding of how to harmonize those various classification schemes.

Another inconsistency in the classification of narcotics is the presence of an ester subclass of narcosis within the Russom classification that is not present in the Verhaar classification. In ASTER, ester narcosis corresponds to chemicals with an ester structure that does not contain chlorodiesters, acrylates, or diesters (in which case they would fall under reactivity/ester).²² Similar to polar narcosis, ester narcosis elicits a narcotic-like anesthesia syndrome and is more acutely toxic than baseline nonpolar narcotics.²⁷ However, their toxicity seems to follow a different mechanism than other polar narcotics, which was the basis for creating an ester narcotics-subgroup.^{28,29} Ester compounds were also noted as narcotics in early papers.³⁰

Ester narcosis could also be considered a subgroup within the polar narcotic group.²⁸ In the Verhaar scheme, esters are not specifically considered and do not appear as such in the classification rules.^{13,19} They should be classified in Class 3 if they possess activated carbon–carbon double/triple bonds, enabling a Michael-type addition of nucleophiles across the double/triple bond or if they fulfill any other requirement for reactivity; or such chemicals could be placed in Class 4 as organophosphorothionate esters. In the present research, nearly all the chemicals classified as ester narcotics by ASTER were classified in Class 3 within the Verhaar scheme and thus were not recognized as narcotic chemicals. Moreover, diester toxicity is differentiated from ester narcosis in the ASTER classification scheme, whereas it is included in ester narcosis in the MOAtox classification.

The majority of chemicals for which there is a discrepancy between all the classification schemes are MOA-5 according to the OECD QSAR Toolbox (21 over 31 chemicals) and many

were classified as nonpolar narcotics by ASTER. Chemicals that could not have been identified as MOA 1–4 would be classified by default as MOA-5 by the Verhaar classification, but they were classified by default as nonpolar narcotics by ASTER if they could not be classified in any of the other class. Another classification discrepancy is linked to uncouplers of oxidative phosphorylation. Those chemicals should fall into Verhaar Class 4 due to the specificity of the underlying mechanism; however, those chemicals (with one exception) are systematically classified as MOA-2, 3, or 5. This issue was already highlighted by Enoch et al.,¹⁹ who identified several groups of chemicals which should fall into Verhaar Class 4 (chemicals acting via a specific [noncovalent] mechanism) and these typically include the weak acid respiratory uncouplers. This has partly been addressed by Ellison et al.,²¹ who have implemented in a postfilter KNIME workflow (to be run after Toxtree) some rules attempting to correct some misclassification of phenols, especially when they are wrongly assigned to Class 5 and should be classified either as Class 3 (reactive chemicals) or Class 4 if it contains an aromatic nitro group, in which case they would be an uncoupler. This postfilter allowed the reclassification of four chemicals (over the 17 chemicals classified as uncouplers of oxidative by ASTER and MOAtox) to Class 4, whereas three others were reclassified in Class 2. Initially, those chemicals were classified MOA-5 by Toxtree and MOA-3 by the OECD QSAR Toolbox. Clearly, a need exists to better understand the determinants of phenolic compound MOA as polar narcotics, reactives, or uncouplers of oxidative phosphorylation, and to implement reliable classification rules accordingly.

In this work, Toxtree 2.6 was used and discrepancies discovered using different assignment tools were not a result of utilizing Toxtree 2.5 as implemented in the OECD QSAR Toolbox. The update reported between those two versions is only bug-fixing and modification of the Cramer scheme and does not concern the Verhaar classification scheme. A discrepancy between the automated Toxtree and the OECD QSAR Toolbox was previously noted for 16% of the profiled chemicals in a comparison with Cramer classifications.²⁶ Further work should be carried out to understand why the outcome is so different when based on the same classification rules and to identify critical checkpoints in the decision tree for various chemicals classes.

No tendency regarding the chemical group was found among the 31 chemicals for which there was a disagreement between the three classifications. There were six organic chemicals, six esters, five aliphatic amines, three amides, three inorganic compounds, two carbamates, and individual chemicals belonging to other chemical classes. Twenty-two of these were classified as nonpolar narcotics by ASTER. Additional research and development would be needed to determine why the three classifications differed in MOA assignments for the 31 chemicals, to develop a unifying approach.

Schemes Have Varying Degrees of Coverage. The three classification schemes have various degrees of coverage. The Verhaar classification scheme classified 100% of the 3448 chemicals, with 57% of the data set within Class 1–4, whereas 43.3% was attributed to Class 5 (i.e., equivalent to a nonclassification). Those Class 5 chemicals are either outside of the domain of the classification scheme (82 of those Class 5 chemicals are classified inorganics by ECOSAR) or should have been attributed to Class 1–4 but have not been recognized as such by the actual rules implemented in the system. A deeper

analysis would allow identifying the chemical groups that are not recognized when they otherwise should be classified.

ASTER attributed a classification to 33.8% of the data set, including the “N/A” chemicals (16%); therefore, only 28.5% of the whole data set could be truly classified. The “N/A” chemicals were recognized as outside of the domain of the classification scheme (mainly metals and inorganics), but it is unclear why the other 2283 were not classified or were not classified by default as nonpolar narcotics. 23% of the chemicals were founded in the MOAtox database, which has the advantage to include inorganic chemicals in its applicability domain. The coverage of MOAtox could be expanded using the predictive classification model of Martin et al.,³¹ which is available online through EPA’s Toxicity Estimation Software Tool (<https://www.epa.gov/chemical-research/toxicity-estimation-software-tool-test>).

Among the classified chemicals, 27% were classified as narcotics by Verhaar, whereas 50% and 59% were classified as narcotics by MOAtox and ASTER, respectively. This high degree of narcotic classification by ASTER can be explained by the structure of the ASTER classification system where a chemical for which no chemical moieties that trigger a classification as polar narcotics, ester narcotics, reactive chemicals, or specifically acting chemicals would be classified by default as a nonpolar narcotic chemical. This classification rule might introduce a bias in the overall class representativity.

Unfortunately, a very large number of chemicals were not classified. This might limit the use of animal alternative methods based on grouping of chemicals according to the MOA. Further analyses of chemical structures are needed to determine if they cannot be classified because they are outside of the applicability domain of each scheme, or if the compounds cannot be recognized under the current classification rules. There is also a need to make the existing rules more accurate (to avoid misclassification) and to add new rules covering other moieties. This is especially important as chemical management frameworks continue to expand compounds being assessed and read-across or other grouping methods become increasingly sophisticated. All those classification schemes are based on the knowledge of a given chemical’s toxicity as it relates to its structure. Since this knowledge is constantly evolving, those classification schemes should be regularly updated to integrate this new knowledge.

Applications. Determination of the mode of action is essential to an array of subsequent ecotoxicological assessment activities. For example, threshold of toxicological concern (TTC) and eco-TTC approaches rely heavily on knowledge of chemical and structure to provide appropriate groupings for subsequent analysis including mode of action. In the development of the eco-TTC concept,¹⁶ it became apparent that numerous tools and classification/grouping schemes were available and could result in different eco-TTC outcomes. From the analysis presented in this paper, it is quite clear that the assignment of MOA arising from different classification schemes could result in a range of eco-TTC outcomes. However, there is a large degree of overlap in assignment outcomes, especially for compounds with less specific modes of action. As revealed in this study, the practical importance and outcomes of different assignment options and eco-TTCs should be an area for future research.

The MOA classification systems have been developed in parallel with growing knowledge about specific chemicals, modes of action, and the enormous expansion of available

ecotoxicological information brought forward by international chemical management programs such as the Canadian Categorization of the Domestic Substances List³² and the European REACH regulation. These extensive and growing databases of these programs argue for harmonized classification schemes as part of weight of evidence determinations within the regulatory frameworks.³³ To clarify the utility of each classification system and to take advantage of the growing knowledge of chemical structure and toxicity driven by chemical management programs, a cross-cutting analysis of these existing MOA classification systems and tools is a necessary prelude to applying them in eco-TTC development.

Another potential application of the MOA classification systems is in AOP development.³⁴ Here the outcomes are of primary importance in the organization of chemical interaction with biological receptors and subsequent downstream biological responses. Consistent MOA determinations will allow for the most relevant grouping of like compounds assessed as acting by a specific mechanism. It may be possible to generalize the nature of adverse outcomes for groups of compounds in a coherent way as a result of improved classification of their expected MOAs.

To conclude, the use of MOA classification in chemical risk assessment implies that a reliable MOA framework and assignment tools for classification are available. The existing MOA frameworks have great potential to improve risk assessment and strengthen the use of alternative methods. However, these frameworks lack harmonization and therefore often give contradictory results. Harmonizing and updating classification frameworks (built for the variety of purposes) with emerging knowledge on ecotoxicity and toxicological mode of action will significantly improve chemical risk assessment outcomes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.est.7b02337](https://doi.org/10.1021/acs.est.7b02337).

Table S1. Description of key attributes of three MOA classification approaches. Table S2. Curated CAS-linked MOA classifications. Table S3. Collapsed list of ECOSAR categories. Table S4. Verhaar mode of action classification obtained with the OECD QSAR toolbox and the Toxtree software. Table S5. Re-classification of chemicals by the KNIME post-filter according to their MOA. Figure S1. Overview of methods and analysis process. Figure S2. The process of curating and developing the toxicity data set ([PDF](#))

SMILES; ECOSAR class; Aster MOA; MOAtox broad; MOAtox specific OECD; QSAR toolbox-acute aquatic toxicity; Verhaar scheme (modified); Toxtree-acute aquatic toxicity; Verhaar scheme (modified); KNIME workflow modification ([XLSX](#))

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Notes

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