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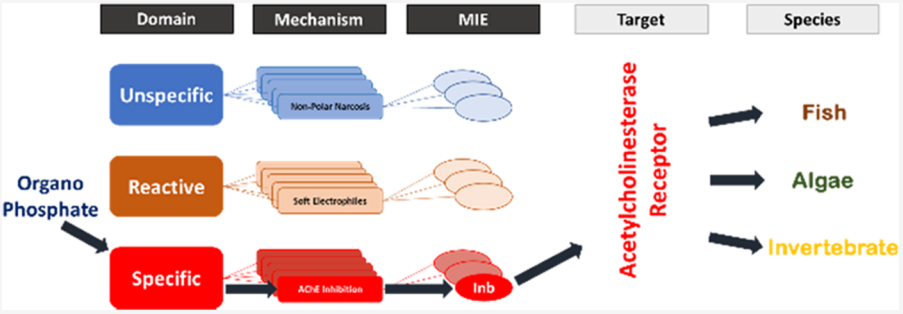

ABSTRACT: This study developed a novel classification scheme to assign chemicals to a verifiable mechanism of (eco-) toxicological action to allow for grouping, read-across, and *in silico* model generation. The new classification scheme unifies and extends existing schemes and has, at its heart, direct reference to molecular initiating events (MIEs) promoting adverse outcomes. The scheme is based on three broad domains of toxic action representing nonspecific toxicity (e.g., narcosis), reactive mechanisms (e.g., electrophilicity and free radical action), and specific mechanisms (e.g., associated with enzyme inhibition). The scheme is organized at three further levels of detail beyond broad domains to separate out the mechanistic group, specific mechanism, and the MIEs responsible. The novelty of this approach comes from the reference to taxonomic diversity within the classification, transparency, quality of supporting evidence relating to MIEs, and that it can be updated readily.

INTRODUCTION

Understanding and ameliorating the environmental impact of chemicals is seen as an urgent need in society, with computational methods at the forefront to provide information to determine risks.[1] Traditionally, quantitative structure−activity relationships (QSARs) have been used widely as a means to predict toxicity and have been particularly useful for acute effects on fish, algae, and daphnid species. Software such as ECOSAR from the United States Environmental Protection Agency (USEPA) has been widely applied for nearly three decades.[2] Much of the success of the using QSARs can be attributed to the availability of many high-quality data for key endpoints and that toxic potency can be related to simple and easily available physicochemical properties.[3,4] A particular strength of QSARs for environmental toxicity has been the development of QSARs based on chemical classes and/or mechanisms of action, with robust models being available for modes of action such as narcosis and unspecific reactivity.[5] In addition, there are a number of well-used classification schemes to allocate chemicals to modes, or mechanisms, of action. However, some are based exclusively on insights from toxicological studies in fish.

Of the classification schemes to assign compounds to modes or mechanisms of action, Verhaar et al.[6] and Russom et al.[7] proposed the most prominent and regulatory well-endorsed schemes for acute aquatic toxicology. The Verhaar scheme assigns compounds into one of the four mode of action (MOA) classes or to a fifth class when no assignment can be made. Since its conception, the Verhaar scheme has been challenged, modified, extended, and further validated.[8−10] The Russom scheme[7] assigns compounds into one of the eight mechanistic classes and is a part of the ASsessment Tools for the Evaluation of Risk (ASTER) expert system, used in-house within the USEPA for environmental risk assessment purposes. Both classification schemes assign compounds to MOA classes broadly associated with narcosis, nonspecific reactivity, and

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specific toxic action.[6,7] Later, Barron et al.[11] proposed the MOATox classification database as a high-quality training set for model development with MOA classifications based on expert judgment with, however, no further supporting evidence or explicit justification. More than 1200 compounds were assigned by Barron[11] into one of the six broad, and 31 specific, modes of action. While the Verhaar[6] and Russom[7] schemes were derived using fish toxicity data, the MOATox database[11] is applicable to both fish and daphnids. The strengths and weaknesses of the schemes have been evaluated and discussed by Kienzler et al.[12,13] In addition, a recent study by Bauer et al.[14] outlined the development of a revised classification scheme for MOAs, which assigns compounds into one of the six broad mechanistic classes with a further 23 subclasses. Interestingly, the rules associated with the Bauer[14] scheme were developed from a variety of species giving a broad range of taxonomical applicability, including fish, mammals, plants, unicellular organisms, and *Daphnia* sp. (with a strong emphasis on fish and mammals). The development of such methods relies on the use of high-quality data and knowledge to identify mechanistic information accurately.

The role and understanding of mechanistic knowledge throughout toxicology, and to computational modeling in particular, has been bolstered by the rapid uptake of the Adverse Outcome Pathway (AOP) paradigm.[15] An AOP is a framework that organizes mechanistic information in key events (KEs) from the molecular level (molecular initiating event—MIE) to the organ and population level (adverse outcome—AO) with the potential to inform decision making.[16] The novelty of the AOP concept lies in the introduction of a consistent framework for risk assessment for both environmental and human health, empowering risk assessors to integrate and cluster diverse information and identify research needs.[17] The MIE is of particular use in the development of classification schemes and, through an AOP, provides direct linkage to mechanistic knowledge. For instance, knowledge of AOPs can support the development of mechanistically derived QSARs.[18] Thus, AOPs, and knowledge of the MIE explicitly, hold the possibility of extending classification schemes not only to further chemical domains but also across more species and taxa.

A significant advantage of the application of knowledge from the MIE to support the classification of chemicals is the possibility to link the MIE to an adverse outcome[19] or to create an AOP map from multiple MIEs and adverse effects.[20] Another advantage of taking the focus away from the AO to the biochemical interactions at the MIE level is that the additive action of one or multiple substances could potentially be predicted and quantified.[16,21] Accounting for the action of multiple chemicals may require using networks to fully explain interactions and allow for true quantification.[22] While networks for AOPs are in their infancy, they are increasingly being seen as a solution to address issues such as endocrine disruption[23] and specific initiating events, such as ecdysone receptor agonism[24] and those leading to neurotoxicity.[25] A further key component of MIEs and AOPs is the well-defined taxonomic applicability domain, enabling decision making at the population level as well as at the species/taxon level.

To gain benefit from the extended mechanistic knowledge and opportunities provided by AOPs and other sources of information, *in silico* methodologies are required to capture the chemistry associated with MIEs. These computational, chemistry-based methods typically classify compounds based on the presence of structural features (termed structural alerts). The Verhaar[6] scheme was published as actionable structural alerts, while that of Russom[7] required some translation to form a set of usable alerts. The alerts have been coded into many pieces of software including, for instance, the OECD QSAR Toolbox and Toxtree.[12] These provide a useful resource to assist in the classification of chemicals to allow for the application of QSARs to predict the toxicity or grouping, leading to read-across. While they are widely applied, it is acknowledged that the schemes have limited domains in terms of the chemicals they represent, the diversity of species, and mechanisms of toxic action (with a particular focus on specific mechanisms of action). As such, there is an opportunity to unify the current approaches, as well as to update and extend the classification schemes utilizing robust mechanistic knowledge across various species and chemical domains. Assessment of the current knowledge related to classification schemes[6,7,11−14] indicates that mechanisms of toxic action (both acute and chronic) fall into one of the three broad domains. The first domain is that of narcotic chemicals, typically associated with unspecific, reversible effects related to membrane disruption,[26] with nonpolar narcosis dominating and forming a baseline toxic effect. The second domain is of unspecifically reactive chemicals, which can form covalent bonds with biological macromolecules by a variety of electrophilic and nucleophilic reactions.[27] The third domain is for specifically acting chemicals that bring about adversity by a specific interaction such as binding to a receptor or interference with physiological processes.[28]

The aim of this investigation was to provide a unified, mechanistically driven scheme across a broad range of species for the classification of environmental toxicants, bringing together and enhancing current knowledge. The updated scheme is based on the collection of knowledge of the key MOA for environmental toxicity derived with a view of capturing the MIE, such that chemical domains may be defined. At its starting point, the scheme and data/information collection exercise considered populating three broad MOA domains, namely, narcosis, nonspecific reactivity, and specific mechanisms of toxicity to be consistent with existing knowledge and classification schemes.

2. MATERIALS AND METHODS

2.1. Review of MIEs Relevant to Environmental Toxicity. MIEs relevant to environment effects were compiled on the basis that an MIE was assigned to an identifiable mechanism of action. In this regard, the OECD[29] definition of an MIE "A specialized type of key event that represents the initial point of chemical/stressor interaction at the molecular level within the organism that results in a perturbation that starts the AOP" was taken as the starting point and evidence gathered as proposed by the OECD[29] guidance. MIEs were considered to be the initiating interaction of the molecule with the biological macromolecule for which plausible biological evidence in at least two aquatic species was provided. The only exemption to requiring evidence from two species was made for chemical domains designed to have a species-specific effect, e.g., herbicides targeting photosynthetic machinery. The starting point for the compilation of MIEs was existing knowledge covered by the mechanisms described in the existing schemes.[6,7,11−14] The mechanistic information was organized into one of the three broad modes of toxic action (unspecific reversible toxicity, unspecific reactivity, and specific

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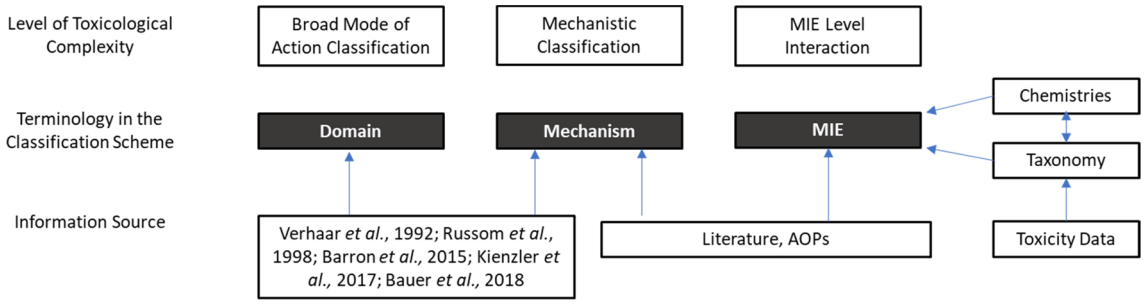

Figure 1. General structure for the classification scheme for environmental toxicants summarizing the three levels of information, sources of knowledge and information, as well as where chemistries and taxonomy are captured.

toxicity) around which the current scheme is based. The presence of a mechanism of action in an existing scheme was deemed evidential for the new scheme, although the overall weight of evidence for individual MIEs was not assessed. After the organization of existing mechanisms of action into broad modes, MIEs were assigned with reference to the source publication, other existing knowledge, or the AOP wiki.

From the outset, it is clear that existing schemes are incomplete in terms of chemical coverage, especially with regard to specific mechanisms of action and taxonomic applicability. To gain the widest possible source of mechanistic information related to environmental mechanisms of action, a literature search was conducted in two stages: (a) evaluation of mechanistic environmental toxicology literature and (b) assessment of mechanistic information from publicly available peer-reviewed studies.

Evaluation of the environmental toxicology literature allowed for the retrieval of mechanistic information from Di Guilo and Hinton[28] related to mechanisms of toxicity action for industrial organic compounds, from Casida[30] for specific mechanisms of toxic action of organic plant protection products, and from the AOP wiki (aopwiki.org) for MIEs that have been formally evaluated and are open for citation.

For the peer-reviewed literature search, the key phrases "aquatic toxicology," "mechanistic toxicology," and "acute toxicity" were used and refined further for all combinations using the keywords "fish," "daphnia," and "algae" from the online resources PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) and Web of Science (http://webofknowledge.com) leading to more than 5000 studies for assessment.

Due to the disparate nature of the information retrieved, it is hoped that all published mechanisms have been captured. However, it is accepted that further information may become apparent in the future. The focus on broad modes leading to individual MIEs should allow for update and expansion of the scheme, as required. The literature retrieved was organized as being either associated with mechanisms of toxic action with known MIEs[6,7,11−14] or recorded as a new MIE entry. The review focused on capturing biochemical information for MIEs with defined stressors, i.e., chemicals. The information was sought for adverse effects regardless of species, life stage, or sex; however, with a defined taxonomic applicability, which focused primarily on aquatic species, taken as the starting point.

The starting point for the compilation of MIEs was existing knowledge covered by the mechanisms described in the existing schemes.[6,7,11−14] The mechanistic information was organized into one of the three broad modes of toxic action (unspecific reversible toxicity, unspecific reactivity, and specific

toxicity) around which the current scheme is based. The presence of a mechanism of action in an existing scheme was deemed evidential for the new scheme, although the overall weight of evidence for individual MIEs was not assessed. After the organization of existing mechanisms of action into the broad modes, MIEs were assigned with reference to the source publication, other existing knowledge, or the AOP wiki.

2.2. Capturing the Taxonomic Domain of Applicability. The MIE-based classification scheme is intended to be relevant across multiple environmental species and taxa. While this information is not currently available beyond a few species, capturing molecular events leading to adversity was crucial for as broad a range of species as possible. At the current time, most information is available for the species and taxa required for regulatory purposes, i.e., fish, invertebrates, and algae. To gain an understanding of the taxonomic applicability domain, information was captured based on the literature, toxicity databases (USEPA ECOTOX database)[31] and Pesticide Properties DataBase (PPDB),[32] and, when biologically relevant, from the reported cross-species extrapolation using tools such as SeqAPASS (Sequence Alignment to Predict Across Species Susceptibility),[33,34] OrthoDB,[35] Homologene,[36] and Conserved Domains by NCBI.[37,38] Specifically, the species to which the MIEs are relevant was recorded. The key to recording this information was that there was evidence that the relationship to a species was deemed biologically plausible from experimental data. The information on species was organized as part of Supporting Information Table S1 according to accepted classification and taxonomic rank of organisms—this included the possibility of attaching common names to species names.

2.3. Organization of the Classification Scheme. The classification scheme was developed from the MIEs retrieved. The information was organized into an ontology covering the three broad domains of toxic action as described above and then by mechanistic groups and finally individual mechanisms of action. Each mechanism of action comprises one or more MIEs. Evidence is reported for the mechanisms of action including the specific molecular interaction, chemicals causing the effect, and target species.

3.1. RESULTS AND DISCUSSION

This investigation aimed to develop a scheme for the classification of chemicals with regard to their environmental toxicity MOA unifying and extending existing knowledge through new information and mechanistic understanding. The novelties of the analysis undertaken were not only the organization of the mechanisms within biological and chemical space but also the anchorage of MOA classifications to MIE,

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Table 1. General Scheme for the Classification of Environmental Toxicants

	Mechanism		
Domain	Mechanistic Group	Individual Mechanism	MIE
1. Non-specific	1.1. Narcosis	1.1.1. Nonpolar	accumulation in, and disruption of, membrane-based phospholipids
		1.1.2. Polar	accumulation in, and disruption of, membrane-based phospholipids
		1.1.3. Ester	accumulation in, and disruption of, membrane-based phospholipids
		1.1.4. Amine	accumulation in, and disruption of, membrane-based phospholipids
2. Reactive	2.1. Electrophilic	2.1.1. Soft	alkylation
			alkylation (quinones)
			Michael addition
		2.1.2. Hard	reactive oxygen species (ROS) formation (liver)
		2.1.3. Pre-reactive	alkylation
			alkylation (quinones)
			DNA adduct formation
			ROS reactivity mediated by episulfonium ion
			ROS reactivity mediated by nitrenium
			ROS formation (liver)
	2.2. Nucleophilic	2.2.1. Nucleophilic	nucleophilic reactivity
	2.3. Free radical generation	2.3.1. Radical damage of tissues	redox cycle activity (cyanin-like)
			redox cycle activity (Fenton-like generation of hydroxyl radical)
		2.3.2. Production of oxidative stress	inactivation of CYP1A leading to oxidative stress that may influence the toxicity of planar polyaromatic hydrocarbons
		2.3.3. Redox cycling	redox cycle activity/redox cycle activity (quinones)
			redox cycle activity (paraquat-like)
3. Specific	3.1. Enzyme inhibition	3.1.1. Acetylcholinesterase (AChE) inhibition	acetylcholinesterase inhibition (organophosphate/carbamate)
			nicotinic acetylcholine receptor (nAChR) agonism
			nAChR allosteric agonism
		3.1.2. Photosynthesis inhibition	inhibition of photosystem II (PSII), triazine site
			inhibition of PSII, nitrile site
			inhibition of PSII, urea site
			electron diversion (paraquat-like)
	3.2. Ion channel modulators	3.2.1. Modulation of ion channels	modulator leading to deactivation
			noncompetitive antagonism leading to channel deactivation
			glutamate-gated chloride channel (GluCls) activation
			binding leading to cytosolic Ca^{2+} transients
			covalent binding to calmodulin (CaM) leading to inhibition
	3.3. Cellular function disruption	3.3.1. Amino acid biosynthesis disruption	enolpyruvylshikimate 3-phosphate synthase (EPSP) inhibition
			acetolactate synthase (ALS) inhibition
			glutamine synthase inhibition
			inhibition of the conversion of 4-aminobenzoic acid to 7,8-dihydropteroate
			inhibition of protein synthesis
		3.3.2. Cell structure disruption	interaction leading to cell wall (cellulose) synthesis inhibition
		3.3.3. Fatty acid biosynthesis disruption	fatty acid synthesis inhibition, acetyl-CoA carboxylase mediated
			inhibition of fatty acid synthesis
			inhibition of very long chain fatty acid synthesis and disruption of cell division
			up-regulation of enzymes in peroxisomal and mitochondrial fatty acid catabolic pathways leading to disruption on lipid degradation
		3.3.4. Nucleic acid biosynthesis disruption	disruption of nucleic acid biosynthesis
			inhibition of nucleic acid biosynthesis
		3.3.5. Steroid biosynthesis disruption	inhibition of sterol biosynthesis
			binding leading to decreased activity, inhibition of ergosterol biosynthesis
			inhibition of ergosterol and cholesterol biosynthetic pathways
		3.3.6. Carotenoid synthesis disruption	disruption of carotenoid synthesis (phytoene desaturase)
			disruption of carotenoid synthesis (lycopene-β cyclase)
			disruption of carotenoid synthesis (4-hydroxyphenyl pyruvate di-oxygenase)
		3.3.7. Developmental disruption	mimicking effect leading to increased male neonate production
			ecdysone receptor agonistic effect leading to premature molting
			disruption of chitin biosynthesis leading to premature molting
			cell division disruption (microtubule 1)
			cell division disruption (microtubule 2)
			inhibition of mitosis and cell division

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Table 1. continued

Domain	Mechanism		MIE
	Mechanistic Group	Individual Mechanism	
	3.4. Mitochondrial	3.4.1. Mitochondrial electron transport chain inhibitors	inhibition of mitochondrial respiratory chain (succinic dehydrogenase)
			inhibition of mitochondrial respiratory chain (ubiquinol oxidase at Qo site)
			inhibition of ATP synthesis step
		3.4.2. Nonspecific mitochondrial ET chain inhibitors	dissipation of proton gradient across inner mitochondrial membrane by the action of protonophores, leading to uncoupling of oxidative phosphorylation
	3.5. Hormonal function disruption	3.5.1. Binding to nuclear receptors (estrogen, androgen, thyroid receptors, etc.)	covalent binding leading to activation
			agonistic interaction
			antagonistic interaction
			inhibition of thyroid synthesis
			interference with electron transfer via the cytochrome P450 heme group of the aromatase enzyme

the definition of chemistries associated with MIEs, and the capture of the cross-species taxonomic applicability. It is intended to capture information relating both to acute lethality and chronic effects in a flexible and adaptable way such that further information can be included. In addition, it is intended that the scheme is fully referenced and openly available such that it can support activities including grouping and read-across.

3.2. General Framework for the Classification Scheme. A generic scheme for the classification of environmental toxicants is shown in Figure 1. A key differentiator of the proposed scheme with those in current use is the detail at the third level (termed MIE in Figure 1), providing explicit linkage to chemistry and taxonomy. Of the other levels, the top level is intended to include a very broad consideration of the different types of modes of toxic action. As such, the top (domain) level is analogous to the Verhaar classes. The second (mechanistic) level is a more in-depth characterization of the mechanisms of action and could be considered to be analogous to the mechanistic detail provided by Russom[7] and Bauer.[14]

In the current scheme, three "domains" or top-level categorizations are considered as a means of organizing the MIEs. Considering each domain in turn, mechanisms were identified, defined, and categorized in terms of MIEs. The existing knowledge was supplemented with that from the literature and AOPs published on the AOP wiki and other sources.[28]

The literature review identified chemical classes associated with MIEs. These were relevant to more than 50 environmental species as reported in Supporting Information Table S1. The capture of information in this manner enabled the applicability domain of an MIE to be associated with and characterized by taxonomic and chemical information.

3.3. Detailed Development of MIEs for Environmental Toxicity Effects. The classification scheme for environmental toxicants is summarized in Table 1, with more details and supporting data in Supporting Information Table S1. The three domains for the classification scheme are discussed in more detail below, both in terms of their mechanistic basis and the MIEs associated with them. The scheme includes eight distinct mechanistic "groups" across the three domains, each group being distinguished by one or more individual mechanisms with more than 25 in total at this time. Each mechanism of toxic action is associated with an MIE; this has enabled a stronger association with chemistry and taxonomic applicability as part of the domain allowed for the creation of a strong and highly specified mechanistic basis for category formation. In terms of chemistry, a large number of industrial chemical classes are covered (e.g., representative of high-production volume chemicals) as well as biocidal products. Nanoparticles and other materials, such as microplastics, are not included in the scheme at this time, but the scheme could be extended to them in the future. Inevitably, the nonspecific domain (i.e., the narcosis mechanisms) has a broad coverage of chemical classes, while the reactive and specific domains are much narrower chemistries. In total, the scheme captures information about more than 50 species representing the taxonomical kingdoms of bacteria, plants, animals, and fungi. Species representing the main regulatory endpoints, i.e., toxicity to algae, invertebrates, and fish, are captured as well as other potentially sensitive species, e.g., snails, frogs, etc. Generally, the MIEs within the nonspecific unreactive and nonspecific reactivity domain are prominent in the majority of aquatic species—with the exception of metabolic activation such as with the esters,[39] whereas the specific-toxicity MIEs display species specificity and defined taxonomic applicability.

3.4. Nonspecific and Nonreactive Mechanisms including Narcosis. The first major domain includes mechanisms that are nonspecific and nonreactive and are analogous to Verhaar Classes 1 and 2. With regard to acute toxicity, since the seminal publications by Meyer[40] and Overton,[41] extensive research has focused on the definition of the underlying mechanism behind aquatic baseline toxic effects, known as narcosis. This mechanism of action is dependent on a compound's ability to move out of the aqueous environment and into, or through, cellular membranes.[26] Chemicals acting by narcosis are thus characterized by their diffusion into membranes based on their lipophilicity, often described by their octanol−water partition coefficients ($\log K_{ow}$).[42] However, while this established relationship has led to the development of predictive QSAR models to determine species-specific acute toxicity, it does not facilitate increased understanding of the links both between KEs and population relevant AOs, which are needed for risk assessment.[43] Narcosis has been studied in multiple biological matrices (e.g., rainbow trouts,[44] guppies,[45] daphnids,[46] algae,[47] *Tetrahymena pyriformis*,[48] zebrafish embryos[49]). In all biological matrices, the well-known correlation between hydrophobicity and median effective concentration holds and pinpoints the lipid compartments as the target of toxic action. With regard to chronic toxicity, these effects may be related to nonspecific organ toxicity, although these are seldom defined explicitly.[50,51]

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Narcosis has been studied *in vivo*, with McKim et al.[44] describing the behavioral observations associated with narcosis and Veith and Broderius[52,53] suggesting *in silico* approaches to distinguish among different types. Based on the evidence of the narcosis chemical applicability domain, classification schemes have been proposed that accurately assign compounds as narcotics (e.g., Verhaar scheme and its implementations,[6,9,10] Acute Aquatic Toxicity profiler by OASIS in the QSAR Toolbox[7]). For the current classification scheme, information for the definition of the narcosis domain was taken from schemes by Verhaar et al.[6,9,10] and Ellison et al.[48] For instance, Verhaar et al.[6] described the chemical domain of narcosis to include aliphatic halides, hydrocarbons, ethers, alcohols, ketones, aliphatic primary and secondary amines, phenols, anilines, and pyridines. It has been hypothesized that narcotic effects, dictated by chemistry, manifest as nonpolar, polar, ester, and amine narcosis.[7]

Despite the compelling evidence on narcosis, identification of MIEs relating to narcosis is complicated and not yet well defined due to the unspecific nature of the interaction.[26] Ankley et al.[16] discussed the importance of including low-confidence mechanistic information when backed by a plethora of evidence, specifically in the context of narcosis defining the MIE as membrane perturbation. Since this definition of the MIE for narcosis is generic, it must be realized that the exact MIE of narcosis has yet to be determined. Antczak et al.[54] indicated events such as interference with calcium uptake in invertebrates may be significant in the initiation, albeit after membrane disruption. Further, Vinken and Blaauboer[55] proposed an AOP for what they termed "basal cytotoxicity," which provides evidence of a nonspecific effect that is likely to be prevalent across all species. Three mechanisms that may elicit cell damage are proposed, namely, disturbance of plasma membrane integrity, interference with the subcellular architectural organization (or so-called compartmentalization), and by negatively affecting cellular energy supplies, in particular, by targeting mitochondria.

3.5. Nonspecific Reactivity. The broad domain of mechanisms referred to as "reactive" is nonspecific and chemistry-based. These rely on a mechanism of toxicity that can be rationalized and interpreted from organic chemistry. They are analogous to Verhaar Class 3 and may result in elevated acute toxicity relative to baseline effects or chronic effects such as tumor formation.[56] MIEs associated with aquatic toxicity within the reactivity domain have been well studied and outlined in publications and classification schemes.[6,7,11,14] Information from these sources as well as the analysis of high-quality MIE-related reactivity datasets was used to derive the MIEs within the reactivity domain.[57−59] MIEs were split into three mechanistic classes within the reactivity domain: electrophilic, nucleophilic, and free radical. Electrophilic chemistry included soft nucleophilic−electrophilic interactions, hard nucleophilic−electrophilic interactions, proreactivity (biotic), and prereactivity (abiotic), which have been described in general terms with regard to protein binding.[27]

The soft electrophile mechanism comprises a large number of chemical classes. Common among this group were compounds that act through a Michael addition mechanism such as polarized alkanes, their alkyne equivalents, and quinone groups. In addition to compounds that form covalent bonds through Michael addition, other soft electrophiles were identified such as compounds that form covalent bonds through the S_N2 mechanism. Various publications have studied the relationship between reactivity and toxic potency to ciliate *T. pyriformis*. Importantly, these studies highlight that the rate of reactivity and toxicity are linearly related. Such studies have been carried out on various Michael acceptors such as α,β-unsaturated aldehydes, esters, amides, nitro, sulfonates, sulfonyl, sulfinyl, and quinones.[57,58,60−62] Similar studies have been carried out on compounds that react through an S_N2 mechanism such as sulfates, sulfonates, and epoxides.[59,63,64] Proreactive compounds are compounds that themselves are not reactive but become reactive through either abiotic oxidation or biotic metabolism. Similarly, compounds that can be transformed into quinone type compounds through oxidation can then react through the Michael addition reaction.[57]

Redox cycling can occur in compounds that can undergo single-electron reduction, resulting in the generation of reactive superoxide radical species ($O_2^{\bullet-}$) while reproducing the parent compound. This typically occurs with quinone-like structures. These ROS cause a toxicological response by depleting the antioxidant stores within the cell such as glutathione (GSH) and can cause lipid and protein peroxidation and DNA oxidation.[65] It has been suggested that the ability of these chemicals to be reduced to produce radical species can cause interruptions within the electron transport chain. These radical species can transport an electron directly from complex I to complex IV within the mitochondria in the electron transport chain. This disruption could ultimately lead to a reduction in the mitochondrial membrane potential, resulting in a reduction in ATP production[66] and potentially cell death. Finally, compounds were identified that generate free radicals and can be extremely reactive, for instance, aliphatic tertiary amines, sulfur and nitrogen mustards, and cyanidins.

3.6. Specific Mechanisms. The third broad domain of modes of action includes those usually referred to as specific mechanisms of toxic action and are analogous to Verhaar Class 4. They are classified here as being "specific" since the interaction disrupts a (specific) physiological pathway or process causing adversity. In particular, specific toxicity refers to a mechanism of action with a defined molecular target, leading to a specific AOP and thus a well-defined adverse outcome. The MIE literature revealed more than 50 such MIEs with defined taxonomy and chemical space (see Supporting Information Table S1). All specific effects involved binding to molecular targets, leading to enzyme inhibition (e.g., reversible AChE inhibition), ion channel disruption (e.g., modulation of voltage-gated sodium channels), cellular function inhibition (e.g., amino acid biosynthesis disruption), mitochondrial toxicity (e.g., mitochondrial electron transport chain inhibitors), or hormonal function disruption (e.g., interference with aromatase). A comprehensive summary of the MIEs as found in the literature along with chemical and taxonomic applicability domain can be found in Supporting Information Table S1.

3.7. (Regulatory) Application of the MoA Classification Scheme. Currently, those MOA classification schemes that are applied routinely, namely, the chemistry-based Verhaar scheme[6,8,9] and the expert knowledge-based Acute Aquatic Toxicity MOA by OASIS,[67] are most readily accepted by regulators and industries to support regulatory submissions. However, both schemes come with limitations, especially with respect to the coverage of the chemical applicability, mechanistic transparency, taxonomic applicability (i.e., pre-

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dominantly based on fish studies). The classification scheme developed in this investigation aimed to address these limitations using MIE information as the basis for class formation and relating this to the corresponding chemistry of the chemical applicability domain in a transparent fashion with defined taxonomic applicability ranging among multiple aquatic species.

Mechanistic classification schemes have played a vital role in the regulatory assessment of chemicals' ecotoxicological endpoints.[68] Specifically, they have found use in the grouping of similar chemicals with similarity in terms of common mechanisms of action. Such a grouping has enabled the better application of QSARs and read-across to fill data gaps and provides a means of increasing the probability of regulatory acceptance.[3]

As a new generation of computational tools is required to ensure sustainability, this investigation goes some way to provide broader and more adaptable frameworks for chemical classification.[69] Currently, many regulatory agencies are investigating mechanistic knowledge when prioritizing and assessing chemicals for further evaluation. The Organization for Economic Cooperation and Development (OECD) program on Integrated Approaches for Testing and Assessment (IATA)[70] brings together regulatory agencies, chemical industries, and nongovernment organizations (NGOs) to exchange ideas and approaches for integrating alternative data into regulatory and nonregulatory frameworks. Accelerating the Pace of Chemical Risk Assessment (APCRA), led by USEPA, is another multigovernment collaborative effort that examines how "new approach methodologies" (NAMs) can be integrated into regulatory schemes for prioritizing and assessing chemicals.[71,72] There are a number of common themes that run through the potential regulatory use of the novel classification scheme. From one side, it is an implementation of the practical utilization of AOPs to support hazard identification, i.e., moving from a framework to organize information to usable tools and approaches. The linkage between MIEs and computational modeling is well established,[15] and the scheme begins to fulfill the potential of AOPs to support regulatory assessments. From a practical point of view, the scheme will assist in two distinct approaches that are, or will be, applied to fill regulatory data gaps for algae, invertebrates, and fish, for chemical legislations such as the European Union's Regulation, Evaluation, Authorisation, and restriction of CHemicals (REACH), Water Framework Directive, etc., namely, the identification of analogues for read-across and assignment of chemicals to individual QSARs. The scheme will provide a clear means to assign chemicals to toxicologically relevant and species-specific categories, allowing for read-across—the application of these techniques is clearly recognized as one way of reducing *in vivo* testing.[73] The concepts of "Next-Generation Read-Across" (NGRA), whereby there is a greater emphasis on the collection of evidence to support the justification of similarity, will benefit from the transparency of the new scheme, the data underpinning it, as well as the linkage to AOPs, which could support the collection of NAM data. The utility of NGRA is becoming established for mammalian toxicological endpoints[74] and could be the basis of Integrated Approaches to Testing and Assessment (IATA) as proposed to OECD.[70] The novel scheme could assist in the development of better chemical classes, as are sought through initiatives to map the chemical universe to address substances of concern.[75] In addition to read-across, the novel classification scheme presented in this study will assist in the better allocation of chemicals to appropriate QSARs with the potential to increase acceptance of QSAR predictions through transparencies of their domains. This is particularly relevant for QSARs for unspecific effects such as nonpolar narcosis, where high-quality QSARs are available for a number of species and endpoints.[3,5] Application of the information from the scheme will allow for better justification of the use of QSARs as well as identification of where further information, e.g., to support NGRA, will be required for the reactive and specific mechanisms. This increased understanding of specific mechanisms will support the vision of QSARs for data gap filling in other areas such as plant protection products.[76]

The above international collaborative efforts are forward-thinking and have focused on the use of alternative data for read-across purposes, forming chemical categories and to some degree prioritizing chemicals for further regulatory evaluation. In Canada, however, many of the mechanistic concepts discussed in this paper were incorporated into computational models for organic chemical prioritization via two versions of the Ecological Risk Classification (ERC) approach.[77−79] Developed by Environment and Climate Change Canada (ECCC), ERC introduced 21st-century science concepts to reprioritize 640 organic substances on the Canadian Domestic Substances List (DSL) for the third phase of the Chemicals Management Plan (2016−2021). ERC uses a chemical profiling approach to provide evidence for classifying hazard, exposure, and risk. The mode of action is one descriptor used for hazard classification in ERC and is determined using data consensus weighting between critical body residue (CBR)-derived toxicity ratios (TRs) and QSAR classifications of the mode of action. 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 ERC.[80]

In 2018−19, ECCC developed the second version of ERC (ERC2) to prioritize approximately 12 200 organic chemicals on the DSL not originally categorized as a persistent or bioaccumulation and inherently toxic priority in 2006. The output from ERC2 will provide ECCC with information for further post-2020 work planning considerations.[77,78] Built on the back of ERC, ERC2 is a weight of evidence logical model relying on data consensus to determine the risk classification, risk confidence, and risk severity of organic chemicals for further regulatory consideration. Mechanistic profiling is enhanced by integrating both MIE information and modes and mechanism of action in four of the five descriptors used to classify hazard potency. ERC2 increases the number and types of tissue residues and QSAR approaches used for consensus determinations of specific and nonspecific modes of action including many of those discussed in this paper. Key chemical (molecular) interactions (e.g., steric, covalent, nonspecific, specific) in target tissues (e.g., lipids, proteins, nucleic acids) are examined that can plausibly be linked to adverse outcomes.[81] Most of this information is generated or collected from *in silico*, *in chemico*, and *in vitro* sources and is combined with *in vivo* data to form weight of evidence for hazard classification. The ERC2 approach relies on a high degree of biological read-across (cross-species susceptibility), accepting that many biological pathways are conserved across species. It therefore combines mammalian laboratory data with aquatic species data for many endpoints. The degree of cross-species susceptibility is checked where feasible (e.g., receptor-mediated interactions) using USEPA's Sequence Alignment to Predict

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Across Species Susceptibility (SeqAPASS) Tool.[33,34,82] Finally, hazard information collected or generated in ERC2 is organized according to the adverse outcome pathway (AOP) concept[16] according to specific biological targets of regulatory concern (e.g., neurological, reproductive, and developmental effects). Using the AOP in such a manner provides plausible reasoning for an observed adverse outcome, thereby supporting hazard classification. Data gaps or lack of data consensus in the conceptual ERC2 AOPs results in a lower confidence assignment to the hazard classification and helps target key areas for further research and development. The AOP approach is therefore very useful even during high-throughput priority settings for identifying chemicals with common MIEs and modes or mechanisms of action.

3.8. Further Novelty of the MoA Classification Scheme: Taxonomical Applicability. MIEs are associated with more than 50 species (Table S1), which are classified according to their taxonomic rank. This goes beyond what is currently available in the existing schemes. A comparison of the MOA classification scheme presented in this investigation with the mechanisms previously identified shows overlap.[11,14] However, Barron et al.[11] proposed an expert judgment-based MOA assignment with accompanied experimental and calculated acute toxicity data for three fish species and daphnid species, with no possibility for *de novo* classification, limiting the predictability or expansion of the chemical applicability domain. Bauer et al.[14] extended the whole concept in a structured and transparent fashion with mechanistic information being the basis for the classification scheme and explored the overlap between environmental and human health at the molecular level. The relevance of the MIEs in this investigation has, however, not yet been explored for mammalian species, and the proposed taxonomic applicability is not exhaustive. However, the design of the scheme allows for further expansion and exploration of effects on other environmental and mammalian species.

The increased taxonomic applicability of the classification scheme presented here provides additional benefits to the existing suite of classification schemes. Adopting an inclusive approach to define the taxonomic applicability domain for an MIE, it was considered appropriate to demonstrate the presence of the MIE targets with all evidence that could be derived from the literature. Thus, supporting evidence was captured on all available lab- and field-based studies, alternative testing methods (i.e., *in vitro*, *in chemico*, *in silico*) or homology/orthology of target structures. The taxonomic applicability of the majority of the specific (biologically based) MIEs spans more than one taxon (commonly fish and crustacean). The remaining specific (biologically based) MIEs are algal-specific, covering MIEs with causal links to photosynthesis inhibition effects and inhibition of carotenoid synthesis. Specifically acting compounds are used extensively in *in vitro* assays as inhibitors of biomolecular targets of interest (e.g., PCBs for inhibition of CYP1A enzymes), so for the corresponding aquatic species cell lines, it is safe to assume that the targets were present in the origin species.

The number of species for which definitive knowledge of MIEs is available is, of course, very small in comparison to the total number of species in the natural world. However, with the exception of specific mechanisms of action, which may show species specificity, there is likely to be a broad similarity in MIEs across all species. Ultimate differences in the adverse effect or potency will be a result of events downstream in the AOP or relate to toxicokinetics and adaptive stress responses. Therefore, the scheme can be considered to be more broadly applicable than to the relatively limited number of species noted in Table S1. To provide additional confidence in the extension of the taxonomic applicability domain in the new scheme, a number of online tools could be applied that can capture the sequence similarities and orthologous/homologous structures among protein targets for multiple species. The potential of *in silico* resources to establish taxonomic applicability and provide evidence on biological plausibility is significant, for instance, focusing on the role of AOPs.[83] Key among these tools are Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS),[34] OrthoDB,[35] Homologene,[36] and Conserved Domains by NCBI.[38] For instance, LaLone et al.[33] demonstrated it is possible to compare empirical toxicity data to cross-species predictions based on the similarity in key MIEs using the examples of 17α-ethinyl estradiol on the human estrogen receptor, permethrin on the mosquito voltage-gated paralike sodium channel, and 17β-trenbolone on the bovine androgen receptor. Separately, LaLone et al.[33] demonstrated how *a priori* mechanistic knowledge of an MIE can facilitate cross-species extrapolation using the example of the antagonistic effect of spironolactone to androgen receptors as a conserved mechanism of toxic action in mosquitoes and fish species. This concept has been extended further to demonstrate how information on the MIE can be extrapolated among species; for instance, Mellor et al.[84] demonstrated how SeqAPASS could provide knowledge about the capability to bind to the ecdysone receptor across species.

In summary, a novel classification scheme has been presented to support the grouping of potential environmental toxicants. It unifies existing knowledge into three broad mode of action classifications, which are then broken down into individual mechanisms and MIEs. The approach provides additional benefits over existing schemes in that it provides further evidence for mechanistically based MIEs over a broader taxonomic space, providing a scheme that is anchored to MIEs with the supporting chemical information. This approach also allows for the taxonomic diversity of MIEs to be expanded, captured, and applied. As such, it is intended to be a flexible, transparent, and updatable scheme and is the most comprehensively published so far in terms of coverage of mechanisms and species.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c06551.

Details of the mechanism-based classification scheme and related citations (xlxs)

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