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Alternative Vertebrate and Invertebrate Model Organisms Show Similar Sensitivity as Rodents to a Diverse Set of Chemicals

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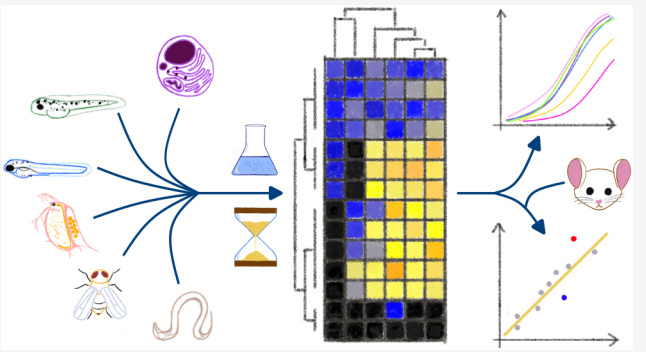
ABSTRACT: Current regulations aimed at safeguarding human health and the environment from chemical risks require toxicity testing, while regulatory bodies worldwide are phasing out the use of protected animals. Here, we systematically produced toxicity data from five alternative nonmammalian model organisms and a human cell line to demonstrate similarities and differences among distantly related species in their responses to chemical exposure (concentration range 1.8 pM to 2.1 M). We show that *Daphnia magna* and *Danio rerio* embryos are affected by more chemicals than *Xenopus laevis* embryos, *Drosophila melanogaster* and *Caenorhabditis elegans*. Lethality/immotility data assembled 72 diverse chemicals into 11 clusters, revealing species-specific outcomes and showing differential enrichment of the clusters for certain chemical structures and modes of action. The ranked relative toxicities of chemicals across the model organisms were highly correlated (Spearman's correlation, maximum rho = 0.86, *p*-value = 0; minimum rho = 0.57, *p*-value = 0.000312), and their average toxicity values closely matched published values for rodents (rat: rho = 0.88, *p* = 2.8e-06; mouse: rho = 0.88, *p* = 2.7e-06). Our findings suggest that chemical toxicity under standardized experimental conditions is broadly conserved across a phylogenetically diverse set of model organisms, providing a conservative estimate of mammalian toxicity.



KEYWORDS: *New Approach Methodology, chemical toxicity, Caenorhabditis elegans, Drosophila melanogaster, Daphnia magna, Danio rerio, Xenopus laevis, HepG2 cells*

INTRODUCTION

Regulatory toxicology faces the challenge of setting exposure limits to protect many animal species, including humans, from the harmful effects of chemicals based on experimental data obtained from a few standard test organisms,[1−4] such as mice and rats as surrogates for human health and ecologically relevant species (such as *Daphnia* and adult fish) as sentinels for environmental health. To improve protection of human health and wildlife, this pragmatic approach requires a better assessment of the similarity among distantly related animals in their responses to chemical exposure and of the extrapolation potential to other species. Furthermore, economic and ethical concerns regarding the use of legally protected animals, such as mammals, in regulatory toxicology have driven the search for alternative "New Approach Methodologies" (NAMs) for chemical risk assessment. They adhere to the 3R principle[5] to "replace, reduce and refine" the use of adult vertebrates in chemical testing procedures. NAMs include *in silico* and *in vitro* testing methods, as well as alternative organism-based techniques involving early vertebrate life stages (like zebrafish embryos) or invertebrates (such as *Daphnia* water fleas), which are suitable for high-throughput testing and are less ethically problematic than traditional laboratory animal tests.[6−10]

As part of its efforts to promote NAMs in chemical regulation, the European Commission's Horizon 2020 program funded the PrecisionTox project.[11] Among its activities, PrecisionTox explores the concept of "toxicity by descent" or "phylotoxicology", a cross-species, comparative approach for chemical hazard assessment.[12] Phylotoxicology

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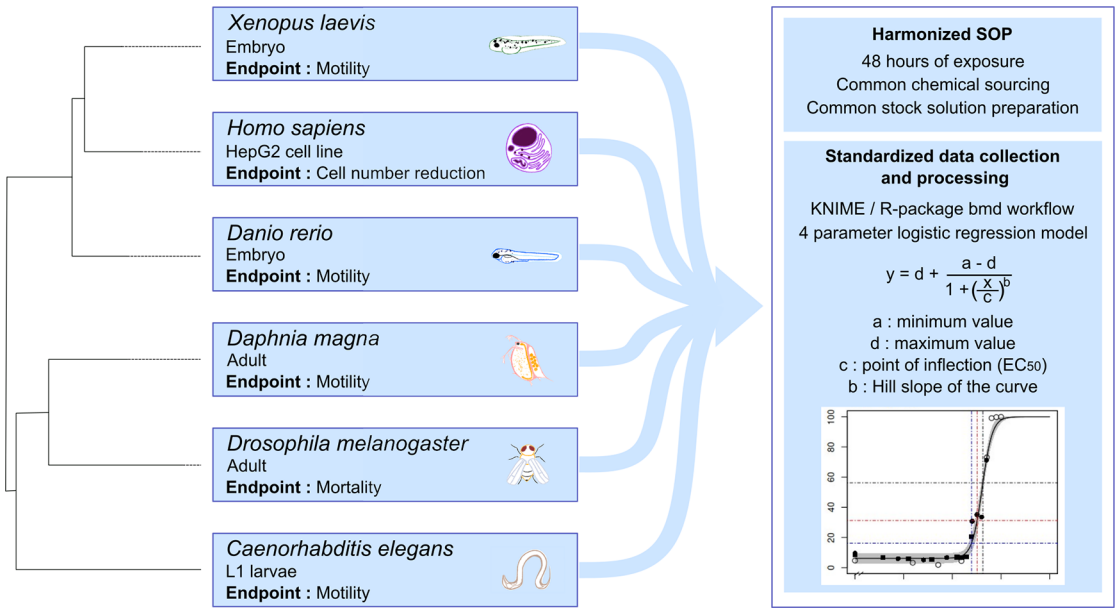

Figure 1. Overview of toxicity data acquisition. The indicated human cell line and five model organisms were exposed to centrally sourced chemicals for 48 h, and toxicity was evaluated based on the indicated endpoint following standard operating procedures (SOPs) for chemical stock preparation and exposure. A common R-based KNIME workflow was used to derive dose–response curves and calculate EC_{50} values (see text for details). Dose–response curves for each model and chemical are shown in Figure S1.

suggests that responses to chemical exposure, from the molecular to whole-organism levels, reflect traits with a genetic inheritance conserved by natural selection that dates back to common ancestors. If this concept holds for ancestors that predate the divergence of major taxonomic groups, toxicological information may be relevant to a much larger diversity of untested species. A primary objective in testing this idea is to examine whether there exists a high degree of species similarity in their toxicological responses to substances with diverse modes of action, particularly between vertebrates and invertebrates, using standardized toxicity data. This approach aims to predict chemical effects in mammalian species based on evolutionarily conserved, mechanism-based toxicity pathways.

However, toxicity data in current databases are not designed for a systematic comparison among model organisms across different branches of the phylogenetic tree, especially because data are usually available for only a subset of species for each chemical, and reflect a variety of different toxicity test protocols. Additionally, toxicity values for a chemical can vary by several orders of magnitude even when using the same testing protocols.[13,14] For example, a search of the pesticide ethoprophos in the Toxicity Reference Database[15] yields 275 Lowest Observed Adverse Effect Levels (LOAELs) for orally exposed mice ranging from 0.254 to 4.9 mg/kg-day (see Figure S5). These observations emphasize the need for toxicity data collection using standardized procedures over several organisms that enable data comparison across species.

In this study, we generated chemical concentration–response curves (i.e., Effect Concentration (EC)) across a range of phylogenetically diverse, widely used nonmammalian model organisms, following well-defined Standard Operating Procedures (SOPs) for chemical selection, test species maintenance and exposure (Figure 1). The test species include five biomedical and environmental model organisms along with a human liver cell line as an *in vitro* model, covering two primary animal clades: (1) vertebrate deuterostomes represented by *Danio rerio* embryos, *Xenopus laevis* embryos and human HepG2 cells, and (2) ecdysozoan invertebrates represented by *Drosophila melanogaster*, *Daphnia magna* and *Caenorhabditis elegans*. These models were exposed to a set of 72 well-characterized chemicals with high diversity in structure and mode of action, including 17 broad modes of action, 32 molecular target types and 32 chemical structure classes (Table S1A).[16] To help interpret results obtained from structurally similar chemicals, we introduced six acrylamides and eight imidazoles among the 72 substances. Immotility/survival was chosen as the toxicological endpoint to ensure comparability across species, and cell number was used for the cell culture model. Interspecies differences in response are expected to be shaped by taxonomic variation in molecular target availability/sensitivity, as well as by differences in Absorption, Distribution, Metabolism and Excretion (ADME), which remain challenging to comprehensively account for *in vitro*, even with advanced systems such as organ-on-a-chip devices or organoids.[17] We examined whether this type of multispecies data set can provide consistent cross-species extrapolation to mammals. Results from this study show that chemical toxicity under standardized experimental conditions is broadly conserved across a phylogenetically diverse set of model organisms. We provide empirical support for the cross-species extrapolation of chemical toxicity, despite biological target and ADME differences, paving the way for toxicity prediction by evolutionary descent.

RESULTS

A Unique Experimental Data Set for Comparative Toxicology. We acquired >17,000 data points and obtained 504 dose–response curves (Figure S1 and Table S1B), from which we extracted 374 effect concentration 50 (EC_{50}) values (see Materials and Methods for details on EC_{50} determination). 71 tested substances produced toxicity in at least 1 species (Pregnenolone/PTX030 showing no effect in all species), and 130 species-substance combinations did not

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Figure 2. Clustering of the chemicals based on EC_{50} values leads to 11 clusters most easily characterized by substances that produced no observed toxicity for specific species, showing enrichment for chemical class and mode of action features. (**a**−**k**) Heatmap indicating EC_{50} values in each model organism/substance combination clustered using the Manhattan distance metric and Ward's linkage. Black tiles indicate that no toxicity was observed. Orange boxes highlight the clusters indicated by letters **a**−**k**, and subclusters (**a1**−**a3**) of cluster **a** are indicated with dotted orange lines. Enriched annotations related to chemical and toxicity features of the substances are shown on the right part of the graph. A colored tile indicates that an annotation is associated with a compound, a white dot on a colored tile indicates that the annotation is significantly enriched in the

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Figure 2. continued

corresponding cluster (Fisher's exact test). A white star on a colored tile indicates that the corresponding annotation is significantly enriched in the corresponding subcluster of cluster **a**. A dendrogram showing the cluster relationships is shown in Figure S3. EC_{50} values used for clustering and the cluster assigned to each compound are shown in Table S1C. Enrichment scores and *p*-values for each annotation and (sub)cluster are shown in Table S1D and E.

result in toxicity when tested up to the chemical's solubility limit (Figure S1, Figure S2, and Table S1B). *Drosophila melanogaster* and *Caenorhabditis elegans* are the least susceptible species showing no toxicity for 47% and 39% of the tested substances, respectively.

Comparative Toxicity Assessment Identifies Substances with Similar Patterns of Species Susceptibility. Next, we aimed to discover the degree to which species varied in their relative susceptibility to chemicals and whether patterns of species-specific outcomes could be associated with toxicokinetics, such as exposure routes, certain chemical structures or modes of action. Therefore, we subjected the EC_{50} values to hierarchical clustering based on Manhattan distance. This analysis identified 11 chemical clusters (Figure 2, Figure S3 and Table S1C). These clusters (**a** to **k**) are most easily characterized by substances that produced no observed toxicity for specific species.

The largest cluster, **a**, consists of 25 chemicals that are toxic to all models. The substructure of this cluster comprises three main subclusters. Subcluster **a1** is characterized by relatively low toxicity across all models. It contains mainly acrylamides. In contrast, subcluster **a2** shows relatively high toxicity across most organisms. Chemicals in this subcluster have varied structures, including two metal salts and two metal−organic substances. They are associated with modes of action that target the nervous system, especially the cholinergic neurotransmitter system. Thus, nicotine (PTX036), chlorpyrifos (PTX026), chlorpyrifos oxon (PTX027), triethyltin bromide (PTX081) and tributyltin (PTX041) target cholinergic signaling, either as inhibitors of the acetylcholine esterase (AChE) or by interacting with acetylcholine receptors. Sodium metaarsenite (PTX006), cadmium chloride (PTX001) and caffeine (PTX022) are also neurotoxicants. The third subcluster **a3** contains mostly imidazoles and again shows relatively low toxicity across all models.

The next major cluster **b** includes 6 chemicals that show relatively moderate toxicity to all models yet no immotility/lethality in *X. laevis* eleutheroembryos. It comprises chemicals that affect the thyroid hormone system and PPAR signaling (PTX082 = perfluorooctanoic acid), an AChE inhibitor (PTX002 = ethoprophos), an inhibitor of DNA synthesis (PTX101 = hydroxyurea), and three imidazole derivatives, including methimazole (PTX098). Methimazole has been described as a developmental neurotoxicant that interferes with thyroid hormone synthesis, while the other two imidazole derivatives are poorly characterized.

The 10 chemicals in cluster **c** show relatively high toxicity in most organisms except in *Drosophila*, where they are nontoxic. Many chemicals (6 of 10) belong to the chemical class of benzenes and their substituted derivatives.

The neighboring clusters **d** and **e** contain 6 chemicals that show relatively high toxicity in most models except in *C. elegans*, where they are nontoxic. Additionally, one chemical of cluster **d** is nontoxic also to *Xenopus* embryos and the two chemicals in cluster **e** are nontoxic in *D. magna* as well. Chemicals in these two clusters mainly target the nervous system, in particular cholinergic and GABAergic neurotransmitter systems. Two chemicals in cluster **d** are azoles.

Cluster **f** is formed by 9 chemicals that show relatively high toxicity in most organisms, except in *C. elegans* and *D. melanogaster*, for which no toxicity was observed. Two features characterize this cluster: 1) Three chemicals are developmental neurotoxicants or have other effects on the nervous system, such as interfering with GABAergic (PTX021 = propofol) or serotonergic (PTX094 = citalopram) signaling. 2) Six of the 9 chemicals belong to the chemical class of benzenes and their substituted derivatives, which was also observed in cluster **c**.

Finally, five smaller clusters, **g** through **k**, contain chemicals associated with varied modes of action that show a lack of toxicity in three or more model species, primarily no toxicity in *C. elegans*, *D. melanogaster* and *X. laevis*. This may reflect their interference with processes that are not acutely lethal in many organisms, such as hormonal signaling (PTX030 = pregnenolone and PTX031 = dexamethasone in cluster **k**), PPAR receptor signaling (PTX003 = pirinixic acid and PTX041 = clofibric acid in clusters **h** and **i**, respectively) or immune system function (PTX024 = fingolimod and PTX039 = diclofenac in cluster **g**).

Overall, *Danio* and *Daphnia* are the most susceptible organisms to chemical toxicity, along with the HepG2 cells, showing immobilization or mortality from exposure to 88−97% of the substances. The relative resilience of two species (*Drosophila* and *Caenorhabditis*), which showed no toxicity from exposure to a substantial number of substances (55%) that define most clusters, requires an explanation.

Statistical enrichment analysis (Fisher's exact test) for features of chemical class, molecular target/mode of action or organ toxicity of the chemicals in each of the 11 clusters was performed to determine if the clustering patterns reflected species-specific biological features that might be linked to toxicokinetics or toxicodynamics (Figure 2 and Table S1D). Among our findings, benzenes and substituted derivatives were enriched in clusters **c**, **f** and **i** (*p*-value = 0.0125, 0.0059 and 0.0458, respectively) that do not show toxicity in *Drosophila*. Benzene rings of bitter substances have been implicated in generating binding affinity to *Drosophila* odorant-binding receptors and vertebrate taste receptors.[18−21] This chemical structural feature might thus partly explain the lack of toxicity of these substances in the flies, which are potentially repelled by these substances, leading to a decrease in food-mediated uptake. To test this explanation, a blue dye feeding assay which measures nutrient uptake from toxicant-containing media was employed.[22−25] For 11/17 benzenes, flies took up nutrient media and additionally showed blue dye accumulation at the proboscis, which is indicative of regurgitating behavior (Figure S4).[22] These observations are consistent with an aversive response to these and possibly other similar substances.

On the other hand, *C. elegans* possess a relatively impermeable cuticle[26] that may explain, in part, the lack of toxicity to substances in clusters **c** and **f**-**k** because of this species-specific aspect of toxicokinetics. However, the enrichment analysis of cluster **d** (composed of substances that

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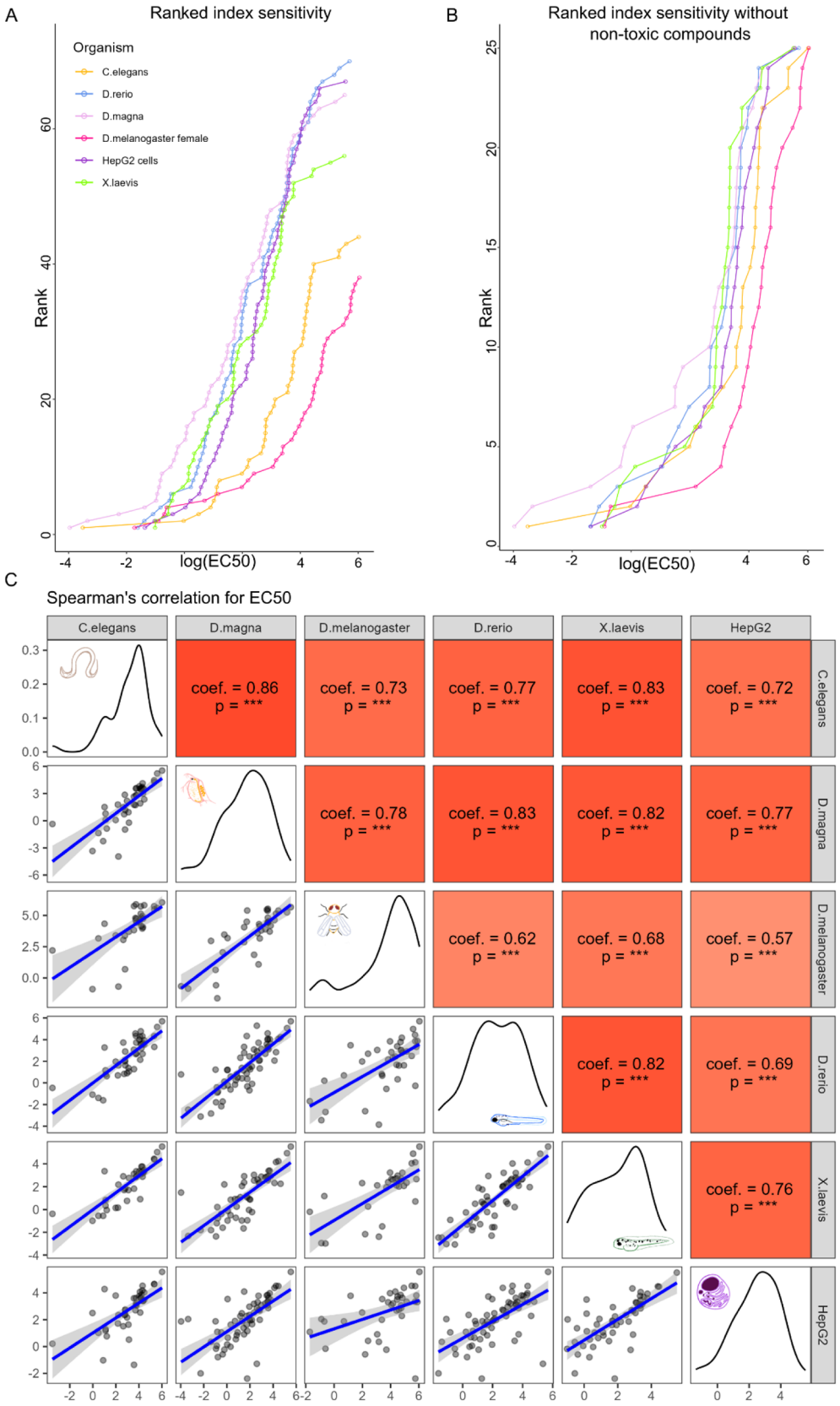

Figure 3. Relative sensitivity to the tested substances is strongly positively correlated across PrecisionTox model organisms. (A) Rank-indexed sensitivity distribution for all tested substances for each of the PrecisionTox model organisms. Within each model organism, chemicals were ranked according to their EC_{50} values. Plotted rank values are colored by model organism as indicated. (B) Rank-indexed sensitivity distribution for those chemicals showing toxicity in all PrecisionTox model organisms. Plotted rank values are colored by model organism as indicated in panel A. (C) Correlogram showing Spearman's rank correlation between pairs of PrecisionTox model organisms. The lower triangle shows scatterplots comparing EC_{50} values for each pair of model organisms. A regression line is indicated in blue, with the gray shades highlighting the 95%

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Figure 3. continued

confidence interval. The upper triangle shows the correlation coefficients of the respective comparisons and their associated significance level, with higher red background color intensity indicating higher correlation coefficients. The diagonal shows density curves of the EC_{50} levels for each model, which are used to estimate the normality of the EC_{50} value distribution within each model organism.

uniquely showed toxicity in all species except in *C. elegans*) also revealed an enrichment in immunotoxicants (p-value = 0.027) and substances targeting cholinergic signaling (p-value = 0.033). This finding is indicative of toxicodynamic differences contributing to species-specific susceptibility as well. In addition to the cuticle there are two differences in culture conditions between *C. elegans* and the other species that may influence toxicity. *C. elegans* are cultured in a saline buffer and salinity is known to influence the solubility and bioavailability of metals[27] and organic compounds.[28] Likewise, the presence of bacterial food in the culture media during testing has been shown to affect toxicity.[29]

It is currently unknown why *Xenopus* eleutheroembryos appear more resistant to the chemicals of cluster **b** under the tested exposure conditions. Future transcriptomics and metabolomics studies might help to shed light on mechanistic differences between the organisms, e.g., regarding substance uptake and metabolism or biological targets.

Enrichment analysis of the chemicals in cluster **a**, containing substances toxic in all species, may provide hypotheses for their relatively similar toxicity among species. For example, cluster **a** shows enrichment for membrane-disrupting chemicals (p-value = 0.038; Figure 2 and Table S1D), highlighting a physicochemical mode of action. Among its three largest subclusters, acrylamides forming a structurally related group are enriched in cluster **a1** (p-value = 0.032, Figure 2 and. Table S1E), while neuronal signaling as a mode of action is enriched in cluster **a2** (p-value = 0.0055), overlapping with an enrichment for cholinergic signaling (p-value = 0.024). Consistently, cardiotoxicity and neurotoxicity also are enriched in cluster **a2** (p-value = 0.022 and 0.022, respectively).

Taken together, these observations suggest that the clusters obtained from comparative toxicology EC_{50} values can reflect toxicokinetics and structural similarities of the substances, associated with shared modes of action and organ toxicity.

Relative Toxicity Is Highly Correlated across Taxonomy. To determine systematic differences across taxonomic groups in the relative sensitivity of species to chemicals as opposed to their susceptibility, the rank-indexed toxicities of the 72 test chemicals based on their EC_{50} values were plotted for all models (Figure 3A, for details see Materials and Methods section). The results indicate that some species are overall more sensitive to chemical perturbation than others. To assess whether the inclusion of chemicals that were nontoxic for some species contributed to differences between the rank-indexed sensitivity curves, we reanalyzed only those substances that induced a toxic response in all organisms (i.e., chemicals of cluster **a** of Figure 2). This analysis shows that, for this subset of chemicals, the difference of sensitivities is reduced between the organisms (Figure 3B), indicating a large influence of nontoxic chemicals on the rank-indexed sensitivity curves. Furthermore, a comparison of the EC_{50} values of cluster **a** between organisms revealed that they are significantly different only between *D. melanogaster* and the organisms other than *C. elegans* (Kruskal−Wallis test, Dunn's posthoc test, p-values indicated in Table S2C). Thus, *D. melanogaster*, but not *C. elegans*, is indeed overall slightly less sensitive than the other models to the chemicals of cluster **a**.

To examine whether the relative toxicity of each chemical is correlated across the different species, we determined the rank correlation values between pairs of models and found a significant positive rank correlation between all the organisms (Spearman's correlation; maximum Spearman's rho = 0.86, p-value = 0; minimum Spearman's rho = 0.57, p-value = 0.000312; Figure 3C and Table S2A). This correlation is similar when only analyzing the chemicals of cluster **a** (Spearman's correlation; maximum Spearman's rho = 0.89, p-value = 2.21e-6; minimum Spearman's rho = 0.55, p-value = 0.00523; Table S2B). Therefore, not only does the relative sensitivity to chemicals follow a similar distribution across the species, but also the relative rank order among the chemicals is broadly conserved.

Distantly Related Model Organisms Show Similar Sensitivity to Chemicals as Rodents. To assess the utility of this comparative toxicology data in predicting toxicity in mammals, they were compared to toxicity data for rat and mouse. Data derived from oral exposure for 24 h were collected from ToxValDB v9.4[30] (Table S1F,G). LC_{50}/EC_{50} equivalence for rat or mouse was approximated by averaging all values available for each chemical (Figure S5). These rodent values were compared to averaged values of the PrecisionTox models (Figure 4A−B, for comparisons of individual species see Figure S6A,B). Overall, the chemical sensitivity of the PrecisionTox models was significantly correlated to that of the two rodents (Spearman's rank correlation analysis; rat: rho = 0.63, p = 7.3e-08; mouse rho = 0.55, p = 5.4e-05). Except for three substances (PTX004 = DMSO, PTX013 = methacrylamide [mouse only] and PTX065 = *N*,*N*-dimethylformamide), the PrecisionTox models demonstrated similar or higher sensitivity than mammals. The chemicals showing higher sensitivity in PrecisionTox models belong to different EC_{50} clusters and, therefore, do not show a systematic bias across chemical classes or molecular targets/modes of action.

We next examined whether limiting the data used for prediction to only a subset based on phylogeny or chemical grouping influences the sensitivity correlations. Spearman's rank correlation did not improve using only vertebrate model data (rho 0.61 vs 0.63 for rat, 0.56 vs 0.55 for mouse, Figure 4C−D) and was slightly reduced when limited to invertebrate data (rho 0.59 vs 0.63 for rat, 0.52 vs 0.55 for mouse, Figure 4E-F). In contrast, excluding chemicals that were nontoxic to one or more species from the data set and using only those assembled in cluster **a**, improved sensitivity correlation (rho 0.88 for both rat and mouse, Figure 4G−H. Additionally, Figure S6C−H shows separate comparisons for each individual model species).

Thus, toxicity data from the PrecisionTox model species and HepG2 cells show high correlation with rodent toxicity data extracted from literature databases, and the correlation is further improved in those cases where toxicity is present in all of the model species. Taken together, our results demonstrate that toxicity testing in a taxonomically diverse array of ethically

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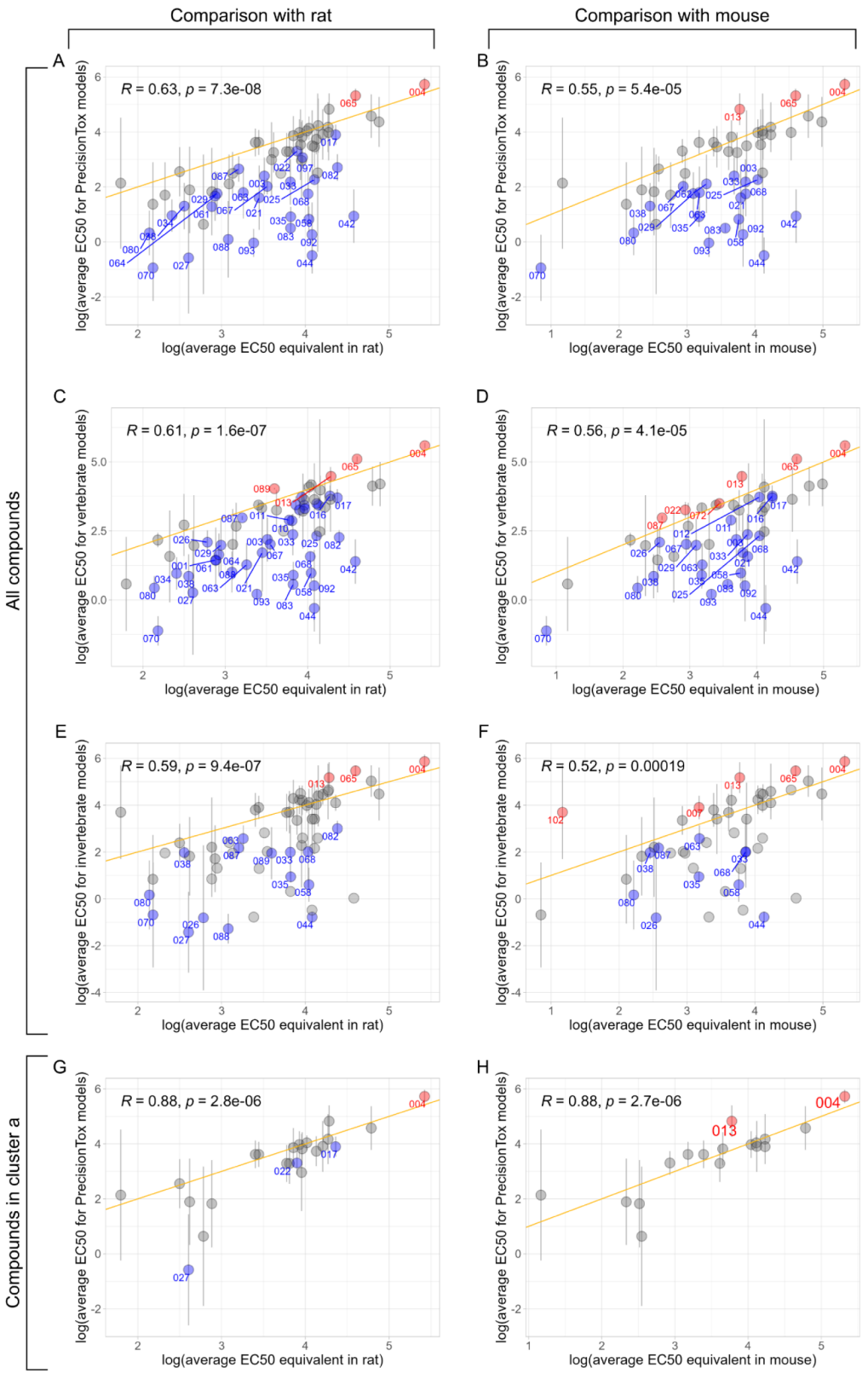

Figure 4. Correlation of EC_{50} equivalents between rodents and PrecisionTox species is improved when only substances leading to a toxic response in all PrecisionTox models are used. Scatterplots comparing EC_{50} value averages of the PrecisionTox models with the averages of EC_{50} equivalents in rodents (rat or mouse) as retrieved from the ToxValDB data set. The identity line is indicated in dark yellow. Red dots indicate higher toxicity to the rodent model than to the PrecisionTox models (=rodent value was one standard deviation lower than the average of the PrecisionTox model value). Blue dots indicate lower toxicity in rodents (=rodent value was one standard deviation higher than the average of the PrecisionTox model value). Numbers indicate PTX numbers of chemicals, as listed in Table S1C. Spearman's rho correlation coefficients and the associated *p*-values are shown for each comparison. Scatterplots displaying the nonaveraged PrecisionTox values are depicted in Figure S6. Panels A, C, E and G show comparisons with rat values, B, D, F and H show comparisons with mouse values. A and B show comparisons using all substances and all

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Figure 4. continued

PrecisionTox models. C and D show comparisons using all substances, but with EC_{50} averages calculated only based on the vertebrate PrecisionTox models (HepG2 cells, *X. laevis* and *D. rerio*). E and F show comparisons using all substances, but with EC_{50} averages calculated only based on the invertebrate PrecisionTox models (*C. elegans*, *D. melanogaster* and *D. magna*). G and H show comparisons using only those substances showing toxicity in all PrecisionTox models (i.e., substances of cluster **a** in Figure 2).

unproblematic model species can provide toxicity information relevant to mammalian species.

DISCUSSION

Standardized toxicity profiling across diverse model organisms and a human cell line demonstrates significant differences among species in their relative susceptibility to 72 tested substances. However, the dose−response toxicity data obtained by measuring immotility or mortality among the taxonomically diverse species show broad conservation in their relative sensitivity, thus permitting cross-species extrapolation to mammals. This is particularly evident in the 35% of chemicals that are toxic in all the models, yet also apparent in an additional 46% of chemicals that produced no observed toxicity in only one or two species. Thus, these results are indicative of potential widespread conservation in the relative sensitivity of species to toxic substances across the animal kingdom. Combined with known or expected environmental or human exposure concentrations, these data can inform risk evaluation. At the same time, variations in the toxicity response among the species can be exploited to indicate the hazard class connected to chemical substances.

One explanation for the broad conservation of relative sensitivity could be that many tested chemicals have a wide and nonspecific target spectrum of effects, which is connected to chemical structure features and physicochemical properties, such as membrane accumulation of lipophilic substances or activation of cellular stress signaling due to high chemical reactivity.[31,32] Indeed, chemicals in cluster **a** of Figure 2, which are toxic in all models, include all six structurally similar substances of the reactive chemical class of acrylamides. Acrylamides act primarily as electrophiles and thereby might damage DNA, but also proteins.[31] Furthermore, this cluster shows an enrichment for "membrane disruption" in the mode of action category, which is linked to substances with solvent (PTX004 = DMSO) or surfactant (PTX045 = butoxyethanol) properties. These observations would be consistent with the idea that recently developed "baseline toxicity" models, which employ quantitative structure−activity relationships (QSARs) related to lipophilic properties of chemicals and species-specific lipid content data, can help predict toxicity of chemicals,[33,34] and our data set can serve to test such models. However, not all substances of cluster **a** fall into these physicochemical mode of action categories. The eight structurally similar imidazoles, for example, are suspected developmental toxicants and endocrine disruptors with a poorly defined mode of action.[35] Their grouping in subclusters of cluster **a** indicates that read-across approaches for these substances may prove fruitful. Furthermore, the cluster also contains neuroactive substances and metals, equally associated with more specific modes of action.

We made use of the standardized toxicity profiles of each substance across the taxonomically diverse models to cluster chemicals to identify similar features, including chemical structure, molecular target/mode of action and adverse outcome/type of toxicity. These substances might trigger similar molecular events in different species, which are then linked to different taxon-specific downstream consequences, with different species showing different abilities to compensate adverse molecular events. For example, the differential sensitivities of terns and chicken to dioxins have been linked to differential binding affinities of their aryl hydrocarbon receptors, leading to reduced transcriptional activation of target genes in the tern,[36] and neonicotinoids such as imidacloprid (PTX087) and thiamethoxam (PTX089) have higher affinities for insect nicotinic acetylcholine receptors than for those of mammals, matching the lower toxicity seen in mammals.[37] Also, differences in toxicokinetics linked to the biology, life history and ecological adaptations of the organisms will shape the susceptibilities and sensitivities to toxicants, as exemplified by the low toxicity of several benzene derivatives to *Drosophila*, apparently connected to avoidance-related reduced uptake. Further data on Absorption, Distribution, Metabolism and Excretion (ADME) and on the molecular networks involved in the response to the substances in different organisms will help to distinguish between these possibilities. Our observations are consistent with the idea that species-specific similarities and differences in these interactions shape the susceptibility and sensitivity profiles.[38] Importantly, our comparative toxicology approach does not require precise *a priori* knowledge of these mechanisms, but exploits the standardized toxicity profiles of a simple adverse outcome (e.g., lethality) to hypothesize the likelihood of pathways leading to toxicity being broadly shared among species for specific groups of chemicals.

The adverse outcome used for our approach is highly integrative and may result from complex combinations of modes of action of the chemicals. A potential application of the comparative assessment would be the identification of adverse outcome pathways (AOPs) targeted by the test substances. The AOP concept has garnered significant interest due to its potential to refine chemical testing procedures.[39] AOPs link molecular interactions of chemicals with biomolecules to changes in gene regulatory and metabolic networks, which in turn are causally connected to adverse outcomes at the organismal and population levels. Combined with OMICS methods, the AOP concept could enable the identification of biomarkers that represent early indicators of an adverse outcome triggered by exposure to a chemical substance.[40,41] Importantly, a large body of research on gene regulatory networks has shown the conservation of crucial elements and design principles across taxonomic groups during evolution.[42−45] This observation of conserved gene networks is consistent with the widespread conservation of sensitivity to toxicants across our diverse model species. One future application of standardized comparative toxicology will be the use of such phylogenetic conservation of biomolecular networks to predict AOPs across animal phylogeny based on the analysis of OMICS data.[12,46−49]

In previous attempts at predicting mammalian toxicity based on nonmammalian toxicity data, *in silico* methods have been developed to calculate species sensitivity distributions (SSDs)

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Table 1. Exposure Condition Overview

Model	Exposure media	Phenotype	Exposure	Reference
C. elegans	K-media	Motility	48 h, L1 stage larvae	62
D. melanogaster	Yeast extract, sucrose and sterile Milli-Q water	Mortality	48 h from 8 day to 10 day old adult flies	22
D. magna	Borehole water	Motility	48 h from 4 to 6 days	63
D. rerio	E3 medium	Motility	48 h from 3 to 5 days post fertilization	64
X. laevis	Glass bottled Evian water	Motility	48 h from Nieuwkoop-Faber (NF) stage 45 to 47	65
HepG2 cells	DMEM medium	Cell number	48 h after seeding	66

for ecological risk assessment (reviewed in ref. 38). SSDs are used to determine a likely maximal value of sensitivity to a toxicant in a set of related species from a limited set of available species sensitivity values. Interspecies Correlation Models (ICM) have been employed to generate SSDs. These models predict toxicity values for untested species using the sensitivity relationship between measured toxicity values of two species.[50,51] However, often these approaches did not include a similarly phylogenetically diverse selection of models as our data set, or did not attempt to predict values relevant for human toxicity evaluation. Other methods aim to explicitly incorporate phylogenetic information into the extrapolation to unknown toxicity values in species of interest. For example, phylogenetic eigenvector maps combined with linear regression modeling have been used to predict tolerance of aquatic species to pesticides based on database sources.[52−54] One problem with these approaches is the availability and quality of the data used for extrapolation. Standardized data sets such as ours now provide a solid basis for further exploration of such methods.

Our data correlate well with rodent toxicity values extracted from the literature, despite the highly diverse nature of our models in terms of substance uptake, general lifestyle, and developmental stage, as well as the differences in exposure routes, duration, and endpoint determination compared to the rodent data. The data correlation is not improved when using only data from the vertebrate models for extrapolation to mammals. This observation, together with the strong correlation of relative toxicities across the entire data set, suggests that a large part of the response to toxic substances is taxonomically conserved, despite modulation by ecological adaptations that will affect, for example, toxicokinetics. Furthermore, data correlation with rodents is particularly high for chemicals showing toxicity in all PrecisionTox species. In the remaining set of chemicals, our models tend to show higher toxicity values than those observed in rodents, potentially reflecting differences in exposure route and treatment duration. Only very few substances show less toxicity than in the rodents. These findings suggest that data from the model species set employed in this study may be helpful to inform acute toxicity testing in mammals, for example, by indicating initial dose levels for Acute Oral Toxicity—Fixed Dose Procedure tests.[55] For chemicals showing toxicity in all PrecisionTox species, the initial dose can be chosen with high confidence; for those showing toxicity in a subset of the species, a conservative lower boundary for the dose is indicated.

Here, we presented results from a uniquely large and diverse experimental data set obtained from standardized testing of 72 chemicals using ethically acceptable, 3Rs-compliant model organisms. We demonstrated a high degree of correlation among distantly related species in their responses to chemical exposure, providing a conservative estimate of mammalian toxicity with favorable safety margins.

MATERIALS AND METHODS

Substance Selection and Classification. A collection of 72 chemicals was selected that included substances with extensive toxicological information as well as data-poor ones.[16] Other parameters used in the creation of this library included: structure (e.g., imidazole, acrylamide), target organ (e.g., liver, nervous system, endocrine system), use classification (e.g., pesticide, pharmaceutical) and phylogenetic bias. Chemicals with phylogenetic bias or taxonomic-specific toxicants are predicted to target different parts of the phylogenetic tree (e.g., toxic to invertebrates but not vertebrates). A detailed description of the chemicals, based on currently available data, is presented in Table S1C. Included in this table are chemical identifiers and toxicity information (e.g., molecular targets, target organs, modes of action and predicted species-specific toxicities). Molecular target and mode of action information was manually retrieved through searches in the databases DrugBank (https://go.drugbank.com/),[56] and PubChem (https://pubchem.ncbi.nlm.nih.gov/),[57] and in the Comptox Chemicals Dashboard (https://comptox.epa.gov/dashboard/),[58] Chemical class information was retrieved using the ClassyFire application for automated structural classification of chemical entities (https://bitbucket.org/wishartlab/classyfire_api/src/master/),[59] Annotations and their sources are available in Table S1C.

Establishment of Dose−Response Curves. For each substance, model organisms and cells were exposed for 48 h to at least seven concentrations, arranged in a geometric series that included the predicted BMD10/BMD25 (BMD = Benchmark Dose[60,61]). If BMD10/BMD25 were unavailable, the concentration range started with the highest soluble concentration. The spacing between concentrations did not exceed a 3-fold difference. Ideally, the highest exposure concentration should cause 100% effect, while the lowest concentration should show no observable effects. The pH was maintained between 6.8 and 7.2 throughout the exposure. If pK_a values indicated a dissociation of substances and a neutral fraction proportion <100%, the test media were supplemented with HEPES buffer (final concentration 5 mM for zebrafish, 10 mM for the other models exposed in water). When DMSO was used as a stock solvent, the maximally accepted DMSO concentration in the exposure medium was 0.1% (0.5% for *Drosophila*). Each experiment was conducted at least in triplicate. Exposure conditions are shown in Table 1.

***Caenorhabditis Elegans* Husbandry and Toxicity Assay.** Nematodes (N2 strain) were obtained from University of Minnesota *C. elegans* Stock Center and cultured on Nematode Growth Media (NGM) agar plates with 0.5 mL of concentrated *E. coli* (variant UVRA) as food for 2 days at 25 °C, in a 16 h light: 8 h dark cycle. They were then transferred

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to larger (14 cm) NGM agar plates containing 2.5 mL of concentrated *E. coli* and allowed to grow for an additional 2 days at 25 °C. On day 4, embryos were isolated as described previously.[67] The isolated embryos were cultured overnight in liquid M9 media without food to hold them in their first larval stage (L1). The next morning the L1 nematodes were centrifuged at 1300 g for 2 min, the nematode pellet resuspended in K-media, and their density was determined by counting using a microscope. The counted and synchronized L1 nematodes were used for all toxicity tests.

Toxicity assays were conducted using a modified protocol described by ref. 62. In brief, 48 h assays were performed in 96 well plates in which 8 test concentrations (including controls) were replicated 6 times (totaling 48 wells). Each well contained 25 L1 nematodes, K-media (containing only KCl and NaCl), individual chemical treatments (controls contained 0.1% DMSO if used as a carrier for that chemical) and concentrated UV killed and frozen *E. coli* (0.5 μL) for a total volume of 100 μL per well. After a 48 h incubation period at 20 °C in a 16:8 h light:dark cycle, the number of immobilized nematodes (i.e., nematodes without any movement that are dead or indistinguishable from dead) and phenotypic changes (i.e., reduced growth) were measured and recorded using a microscope.

***Drosophila melanogaster* Husbandry and Toxicity Assay.** OregonR (BDSC 25211) male and female *Drosophila* were maintained on Bloomington Drosophila Stock Center (BDSC) Cornmeal Food (15.9 g/L inactive yeast, 9.2 g/L soy flour, 67 g/L yellow cornmeal, 5.3 g/L agar, 70.6 g/L light corn syrup, 0.059 M propionic acid), at 25 °C, 55% humidity, and under a 12 h:12 h light-dark cycle. For all experiments, flies were only handled during the light cycle.

Flies were aged and exposed using the protocol described in Holsopple et al.[22] Briefly, starved adult female flies (20 each) were placed in exposure vials (n = 4−6) containing 750 μL yeast/sucrose medium (4× stock: 16% sucrose and 6% yeast extract (m/v) dissolved in sterile purified water) and the desired concentration of test chemicals on filter paper at the bottom. After 48 h of exposure, the number of dead flies was recorded. For feeding assays, an FD&C Blue No. 1 solution (CAS 3844-45-9; 100× stock: 1 g in 10 mL sterile purified water) was included in the exposure medium. After 24 h flies were examined for the presence of blue dye in the abdomen (indicative of substance uptake) and for any signs of abnormal feeding behavior, such as regurgitation or crop distension.

***Daphnia magna* Husbandry and Toxicity Assay.** The *Daphnia magna* genotype used in this study was previously resurrected from the sedimentary archive of Lake Ring, Denmark (55°57′51.83″. 9°35′46.87″E).[68] The stock culture is maintained at the University of Birmingham *Daphnia* Facility under laboratory conditions (10 ± 1 °C; 16:8 light:dark photoperiod) in borehole water collected from a deep aquifer well and showing stable physicochemical properties. Prior to experiments, LRV_0_1 clones were acclimated to 20 ± 1 °C for three generations. *D. magna* cultures were cultured at a density of 20 individuals per 1 L growth medium and fed *Chlorella vulgaris* (strain CCAP 211/11B) daily, to a total of 0.5 mg carbon between days 0−6 and 0.8 mg carbon from day 7, with media replacement twice weekly. Randomly selected 24-h-old neonates from broods 2−6 were assigned to experimental conditions.

The protocol for exposure experiments was adapted from OECD Test Guideline No. 202.[63] Briefly, immobilization tests were conducted on three biological replicates of 10 *D. magna* juveniles (4 days old) across 8 test concentrations and a control for a period of 48 h. Replicates were exposed in 50 mL borehole water containing the test substances, with a maximum DMSO concentration of 0.1%. Immobilization was recorded at 24 and 48 h and compared with control values.

***Danio rerio* Husbandry and Toxicity Assay.** All zebrafish husbandry was performed in accordance with the German animal protection standards and approved by the Government of Baden-Württemberg, Regierungspräsidium Karlsruhe, Germany (Aktenzeichen 35-9185.64/BH KIT). Zebrafish (*Danio rerio*) of the AB2O2 strain were raised and maintained at standard conditions (28 ± 0.5 °C and 14/10 h light/dark cycle) as described previously.[69,70] Embryos were raised in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4),[70] at 28.5 °C, under constant darkness. Staging of embryos was performed on a dissecting microscope according to Kimmel et al.[71]

Exposure experiments were conducted as described.[64] Briefly, 10 embryos (72 h post fertilization, hpf) per concentration and replicate were exposed in a 6 cm tissue culture dish with 10 mL of E3 medium containing the test substances or controls for 48 h, then vibration-induced escape movements were recorded with a custom-made device. Embryos were subjected to a vibrational stimulus (500 Hz, duration 1 ms) by an electrodynamic transducer below the plate and ensuing movements were recorded with a highspeed camera at 1000 frames per second (fps). Videos were manually scored for the number of immotile embryos (i.e., those not responding with an escape movement to the stimulus).

***Xenopus laevis* Husbandry and Toxicity Assay.** *Xenopus laevis* adults were maintained in accordance with institutional and European guidelines (2010/63/EU Directive 2010).[72] Briefly, they were housed in 60 L tanks with 16 females or 25 males per tank and a 12:12 light:dark cycle. Clean, dechlorinated, charcoal filtered water at a constant temperature of 21 °C was provided constantly by a flow through system. Adults were fed two times per week with dry pellets (Gouessant) and once per week with minced veal heart. *Xenopus* embryos were obtained by hormonally induced natural breeding between adult animals. Adults were injected with either GnRH (males) or hCG (females) to induce breeding, doses of hormones were adapted depending on the size of the animals (project number: APAFIS #36464-2022021411529365). Clutches of eggs were dejellied with cysteine solution and dead or unfertilized embryos were removed. Embryos were raised to Nieuwkoop and Faber (NF) stage 45.[73]

Exposure experiments were conducted based on procedures specified for the XETA test.[65] Briefly, eight *Xenopus laevis* eleutheroembryos (NF stage 45) per 8 mL exposure solution/well of a 6-well plate (Greiner Bio-One 657160) were exposed for a duration of 48 h at 26 °C. At 24 h of exposure, exposure solutions were replaced with fresh solutions. At 48 h of exposure, the number of immotile eleutheroembryos was recorded.

Maintenance and Exposure of HepG2 Cells. The human epithelial-like, hepatocarcinoma cell line HepG2 was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) FCS, 100 U/mL penicillin and 100 mg/mL streptomycin in 5% CO_2 at 37 °C. At 80−90% confluency, cells were harvested after

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washing with PBS using 0.25% Trypsin/EDTA (6−10 min, 37 °C). Cell numbers were determined using a Neubauer chamber according to the manufacturer's instructions and 1.5 × 10^6 cells were seeded per flask (75 cm^2).

For chemical exposures, cells were seeded into 96-well plates at 5 × 10^3 cells/well in 200 μL of culture medium for 24 h hours, after which medium was aspirated and 100 μL of treatment or control solution was added for 48 h. One hour prior to the end of exposure all wells were treated with 50 μL of a Hoechst/Propidium Iodide mixture prepared in culture medium at final concentrations of 0.15 μg/mL/well of Hoechst and 0.25 μg/mL/well of Propidium Iodide (PI). After incubation, the stained cells were assessed with an automated fluorescence microscope to record cell numbers and cell death as described previously.[66] Data analysis results (expressed as percentage values "x" %) were transformed to "100-x" % for calculation of effect concentrations.

Calculation of 50% Effect Concentration (EC_{50}). BMD calculations were performed with an established R script,[64] using the R packages plotrix, drc and bmd[74,75] and R version 3.6. For user convenience the R-script was embedded in a KNIME workflow (KNIME analytics 4.7),[76] and was provided via a KNIME server. The KNIME workflow itself is available via GitHub (https://github.com/precisiontox/range-finding-drc). Curves were fitted using a four-parameter log−logistic model: $y = d + (a - d)/(1 + [x/c]^b)$, with "$a$" the minimum of the curve, "d" the maximum of the curve, "b" the slope of the Hill curve and "c" the point of inflection. To account for differences in background mortality between species and assay types, curves were fitted to a fixed maximum response level of 100%, while the offset was fitted to the background level. In the case of substances not leading to 100% of affected individuals in any of the tested concentrations, the substances were scored as "non-toxic", except if the percentage of affected individuals in the highest tested concentration exceeded 50% or if the two highest tested concentrations led to percentages of affected individuals higher than 20% and showed a dose dependent increase. BMDs, BMDLs (the lower limit of the 95% confidence interval of the BMD) and the slope of the Hill curve were automatically calculated for all substances and end points. The BMD50 value (termed EC_{50} throughout the manuscript) was then used for clustering and comparative analysis, since its determination is more robust than that of those for lower effect levels.

Clustering. Clustering of the model organisms and of the chemicals was performed using the EC_{50} values. Nontoxic values were assigned an EC_{50} of 100 M. Hierarchical clustering was applied using the Manhattan distance metric and Ward's linkage (ward.D method) using the R packages FactoMineR and factoextra.

Enrichment Analysis. For each cluster and each annotation of the substances, we build a contingency table with the number of substances labeled with the annotation of interest present in the cluster of interest and outside of it and the number of substances lacking the annotation in the cluster and outside of it. We then tested for enrichment by conducting a one-sided Fisher's exact test on the contingency table. The expected number of substances labeled with the examined annotation in the cluster of interest was estimated by multiplying the total number of substances linked to the examined annotation with the total number of substances in the cluster of interest divided by the total number of substances (72). The enrichment score of the annotation of interest was defined as the ratio between the observed and expected number of substances in the cluster of interest.

Rank-Indexed Sensitivity Curves. Chemicals were ranked based on their EC_{50} for each species. Rank 1 was attributed to the substance with the lowest EC_{50} value (=the most toxic substance for the organism) and the highest rank was attributed to the substance with the highest EC_{50} value (the least toxic substance for the organism). Ranks were plotted against EC_{50} values to create the rank-indexed sensitivity curve.

Correlation Analysis. Normality of the distribution of the EC_{50} values for each model organism was assessed visually by plotting a density curve for each organism (Figure 3). Since the EC_{50} values were not normally distributed, the correlation between all pairs of model organisms was assessed by performing Spearman's correlation, using the R packages corrplot and ggcorrplot.

■ ASSOCIATED CONTENT

Data Availability Statement

All data generated or analyzed during this study are included in this published article (and its Supporting Information files).

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.5c10177.

Figure S1: Dose−response curves for all substances and all PrecisionTox models; Figure S2: Range of concentrations used to establish dose−response curves; Figure S3: Heatmap of Figure 2 including dendrogram of clustering result; Figure S4: Pictures of *Drosophila melanogaster* tested for food intake and regurgitation; Figure S5: variability of rodent (rat and mouse) toxicity data in the ToxValDB database; Figure S6: correlation of EC_{50}/LC_{50} equivalents between rodents and PrecisionTox species (PDF)

Table S1: raw data and EC_{50} values for each of the compounds and organisms, annotation of the compounds, enrichment analysis results, and EC_{50} equivalents for rat and mouse (XLSX)

Table S2: Spearman correlation analysis results and Kruskal−Wallis test results (XLSX)

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Notes

The authors declare the following competing financial interest(s): John Colbourne is one of the Founders and Directors of Michabo Health Science (MHS) Ltd., a spin-out company of the University of Birmingham. MHS also operates as a trading division of University of Birmingham Enterprise Ltd., a wholly owned subsidiary of the University of Birmingham. MHS provides scientific consultancy services in New Approach Methodologies (NAMs) specializing in omics technologies and computational toxicology.

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