Vision of a near future: bridging the Human Health – Environment divide. Toward an integrated strategy to understand mechanisms across species for chemical safety assessment

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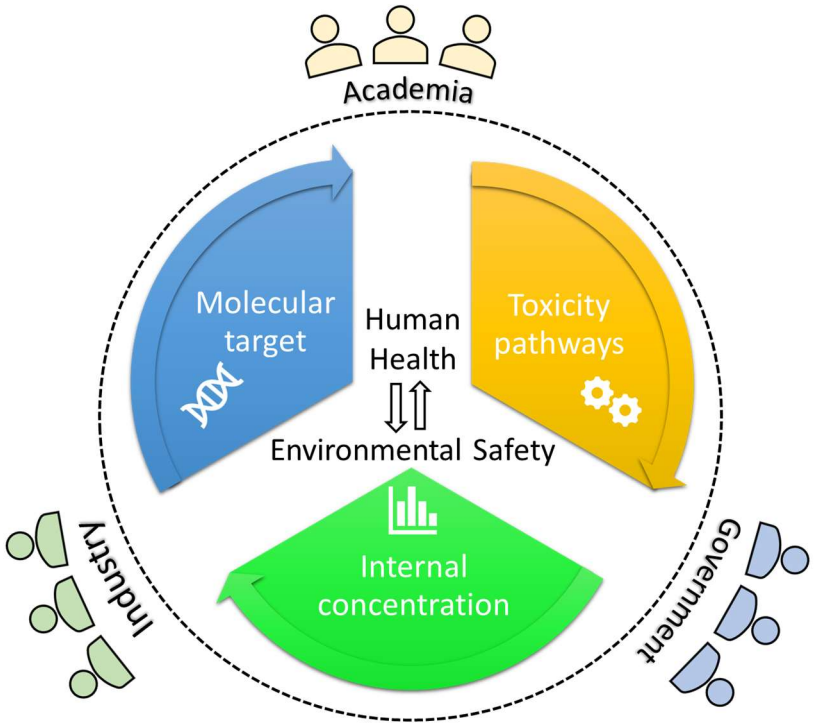
Graphical Abstract



1. Abstract

There is a growing recognition that application of mechanistic approaches to understand cross-species shared molecular targets and pathway conservation in the context of hazard characterization, provide significant opportunities in risk assessment (RA) for both human health and environmental safety. Specifically, it has been recognized that a more comprehensive and reliable understanding of similarities and differences in biological pathways across a variety of species will better enable cross-species extrapolation of potential adverse toxicological effects. Ultimately, this would also advance the generation and use of mechanistic data for both human health and environmental RA.

A workshop brought together representatives from industry, academia and government to discuss how to improve the use of existing data, and to generate new NAMs data to derive better mechanistic understanding between humans and environmentally-relevant species, ultimately resulting in holistic chemical safety decisions. Thanks to a thorough dialogue among all participants, key challenges, current gaps and research needs were identified, and potential solutions proposed.

This discussion highlighted the common objective to progress toward more predictive, mechanistically based, data-driven and animal-free chemical safety assessments. Overall, the participants recognized that there is no single approach which would provide all the answers for bridging the gap between mechanism-based human health and environmental RA, but acknowledged we now have the incentive, tools and data availability to address this concept, maximizing the potential for improvements in both human health and environmental RA.

Keywords:

Risk Assessment; Human health; Environment; Cross-species extrapolation; Mechanism of action

2. Introduction

It is recognised that new scientific improvements and their integration in risk assessment have the potential to improve human health risk assessments by enabling a mechanistic understanding of adverse effects and more accurate predictions of biological responses [1]. Current regulatory-accepted approaches to assess chemical safety are often based on a battery of *in vivo* methods and a limited number of accepted *in silico* or *in vitro* approaches. However, performing toxicity studies for all existing chemical substances using *in vivo* methods is not physically, ethically, or financially possible. Chemical or biological read-across approaches are being considered by industry and chemical management agencies as an alternative to reduce the reliance on these highly resource-intensive *in vivo* tests. There is an urgent need to improve current capabilities to perform chemical read-across and cross-species extrapolation (biological read-across) through an improved mechanistic understanding of the basic biology underlying toxicity and the chemistry-biology interactions involved. Development of descriptors and alerts that facilitate chemical grouping and a better understanding of the species hazard space (i.e. species that are sensitive to certain chemical classes) would also be highly beneficial. In this respect, there have been many efforts focused on the challenges involved in the development of chemical read across, improving its scientific justification and supporting documentation for use in both chemical hazard and RA. Chemical read-across and grouping approaches have become some of the most commonly used alternative approaches for data gap filling within analogue and category approaches [2]. These efforts have led to a wide recognition of the scientific validity of these and its regulatory acceptance and recently, ECHA has published a guidance document on how to perform and document chemical read-across under REACH (Read-Across Assessment Framework (RAAF) [3].

Over the last two decades, there has been a scientific and regulatory push towards the development of novel non-animal approaches for safety assessment [4]. There is a growing desire within the scientific community to achieve simpler, broader, faster and importantly, more predictive risk assessment (RA). To achieve the desired improvements in chemical RA, the current limitations concerning the generation, integration and interpretation of newer types of data proposed for use in RA need to be overcome. Recent developments in biotechnology and molecular biology have

given rise to New Approach Methodologies (NAMs) [5] that are greatly enhancing our ability to address some of the data gaps faced in both human and environmental toxicology. NAMs are a recently adopted concept to broadly refer to any non-animal approach, methodology and / or technology, aimed at providing information on chemical hazard and RA, including integrated approaches to testing and assessment, data interpretation, and performance-based evaluation of test methods [6]. NAMs open new opportunities to ensure RA is grounded in human biology rather than replicating the results of a prescriptive list of animal tests. This is especially important for mechanism-of-action-based RA. For instance, effect concentrations based on perturbations in signalling pathways in human cells will likely be different from those causing apical effects during rodent studies. The difference in species and level of biological organization considered in the example suggest that results of such tests cannot (and should not) be 'validated' against each other and should be compared with caution [7]. In this respect, the use of molecular-based, high content data has the innate potential to complement traditional human and environmental toxicology approaches [8-11]. Indeed, their use could catalyse a paradigm shift to more proactive pathway-based approaches, ultimately facilitating the development of *in silico-based* predictive toxicology [12, 13]. Available data on endpoints supporting traditional approaches to assess environmental and human safety, coupled with a growing weight of *in silico / in vitro* biological pathways-based data raise the question: are we already at a point where we can consider new types of data and incorporate them in a new or augmented approach to RA?

For this to happen, frameworks such as the Adverse Outcome Pathway (AOP) concept, which links the description of biological cascade from the insult at the molecular initiating event (MIE) to the adverse outcome (i.e. AO - the apical toxicological endpoint of concern), can be utilised [14-16]. In addition to the mapping of data, the AOP concept also allows for qualitative evaluation of a pathway and its overall reliability through a weight-of-evidence approach [17]. In some cases, for example in the regulatory assessment of endocrine disruption hazard, a weight-of-evidence-based approach has been advocated [18]. However, the next and ultimate step required for this approach to be fully implemented in RA is the development of its quantitative aspect [19, 20].

Use of cross-species extrapolation is a well-established concept for RA for environmental safety (e.g. using toxicity data from a reduced number of model species to represent the entire ecosystem biodiversity), but also for human health (e.g. using laboratory studies from rodents to infer effects on humans). However, an improved, more comprehensive and reliable extrapolation of biological pathways across species would facilitate the use of already available toxicity data across human health and environmental RA and allow for a more coherent and efficient characterization of overall hazard [21]. Whilst the potential of molecular-based, high-content data and mechanistic approaches has been recognized[5, 22], there are limited examples where molecular level data have been extrapolated across species, including human, to inform cross-species mechanistic understanding as part of the next-generation RA of chemicals[5, 23, 24]. There is an urgent need for new approaches to classify and (ideally) quantify inter-species similarities / differences based on mechanisms of action. However, there are some pragmatic first steps that can be taken using emerging and developing technologies (including OMICS) [25-27].

Motivated by these questions, a workshop was organized, entitled “Vision of a near future: bridging the Human Health - environment divide. Roles of molecular and data-rich approaches as part of an integrated strategy to understand mechanisms across species for chemical safety assessment”, held at Colworth Science Park (Sharnbrook, UK) on April 18th-19th, 2018. Representing academia, industry and government, thirty experts were brought together from diverse fields, including human and environmental toxicology and regulatory safety science, to foster this dialogue. The overall purpose was to discuss how existing data can be better exploited and how new data can be generated to improve mechanistic understanding across humans and environmentally relevant species to better inform chemical safety decisions.

3. Workshop outline

Participants were selected based on their domain of expertise as well as their affiliation, to ensure a broad coverage both in sense of background and areas of interest as action domain. Stakeholders from universities (U. Cambridge, U, California Berkeley, U. Birmingham, U. Liverpool, U. Exeter, U. Amsterdam, Brunel U.), private sector i.e. industries (Unilever, Astra Zeneca), and governmental / regulatory bodies (USEPA, EC-JRC and NC3Rs) were invited to discuss current problems and needs

concerning biological read-across and its implementation in current practices of RA. New strategies and solutions were also proposed.

In preparation for the workshop, delegates were asked to reflect and share their opinion on two key questions prior to the event:

(1) what are the main drivers to develop cross-species understanding of mechanisms of action in the context of RA?

(2) what approaches / techniques do we foresee as better suited to provide scientific evidence and increase confidence in cross-species extrapolation and what are the main limitations?

All feedback received was analysed and provided the basis for a focussed discussion in breakout groups. Based on their expertise and opinions shared prior to the workshop, delegates were divided into three work groups, each addressing a different level of biological organization: (WG1) target-level, (WG2) pathway-level and (WG3) physiological-level, including exposure. Each group was asked to discuss the current science and knowledge available and the scientific research needed to achieve full potential as outlined in Table 1, focusing on the main challenges, benefits and hurdles.

Table 1- List of the challenges considered by each of the three working groups

Challenge 1	Improve basic knowledge of Molecular Initiating Events (MIE) across species ● Improve knowledge of target homologue/orthologue characterization through evolutionary (and functional) conservation ● Develop an understanding of the chemistry of MIEs with a cross species perspective
Challenge 2	Develop basic knowledge of pathway conservation across species ● Increase scientific knowledge for pathways-based comparison to support extrapolation from a higher-tier perspective ● Develop species-specific pathway-to-phenotype association analysis in a chronic exposure scenario
Challenge 3	Refine understanding of biological processes impacting internal exposure ● Understand species-specific physiological processes (ADME) to predict chemical effective doses at the target site in different species ● Develop and refine PBK models for key species and link them to understand where there are common and species-specific processes

This report describes the proceedings of the workshop and presents the highlights of the discussion. All opinions were treated equally and were consensually accepted by all participants.

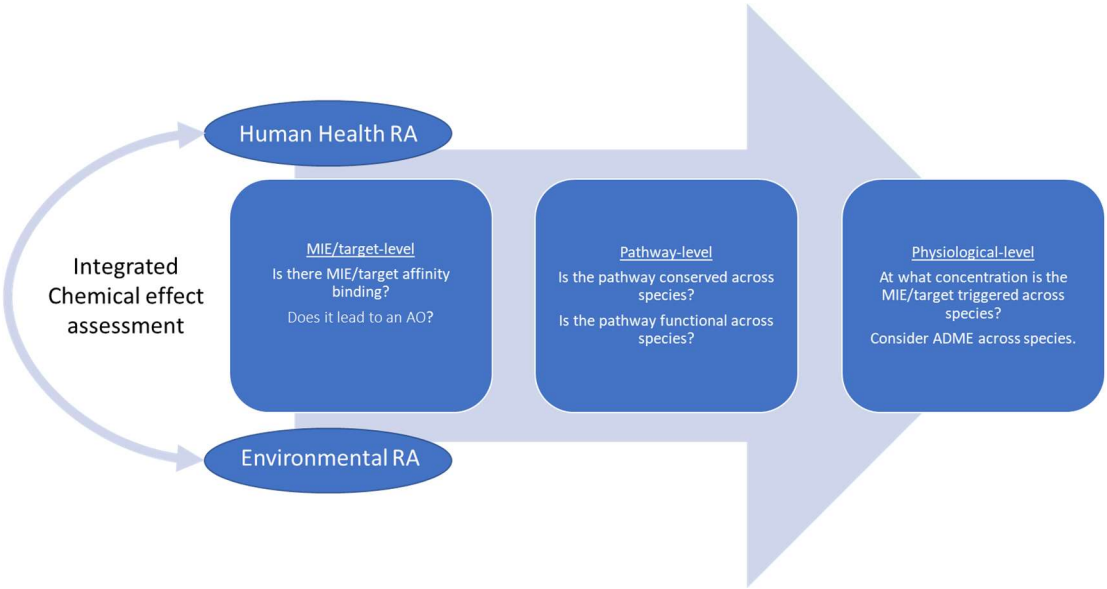


Figure 1-Holistic schematic of how to perform chemical risk assessment using mechanistic knowledge improving the cross-talk between human health and environmental safety.

4. Breakout Discussion group summaries

4.1 Work group 1: "The Challenge: Improve our knowledge of MIE across species"

Key shortcomings regarding the use of MIEs (the initial chemical–biological interaction that starts the AOP [28]) to identify and understand common pathway signal transduction for cross-species extrapolation were considered by WG1. When considering a MIE for RA across human health and the environment, it is important first to recognize that there are different aims and diverging protection goals. In fact, for humans the protection goal is optimally set at the individual level, aiming at protecting each individual against harm; for the environment, this is more often established at the population or ecosystem level [29]. Similarly, there may be varying layers of complexity to consider, for example general narcosis *vs.* specific Mode of Action (MoA), or a MIE with multiple interactions (e.g. skin sensitizers) *vs.* a MIE that leads to one specific adverse event (e.g. estrogenic receptor agonist). Challenges also differ substantially depending on the goal for extrapolation between datasets / species, i.e. to assess for a similar MoA / AOP (or toxicity pathway) across species or to extrapolate effect levels across species. If the aim is to assess for similar MoA / AOP across species (but not effect levels), current state of the art ortholog predictions (e.g.

OrthoDB [30] or EggNOG [31]) can provide a good starting point, provided the mechanism of toxicity is specific and the MIE limited to one (few) specific protein targets. However, there are many uncertainties associated with ortholog predictions. For example, uncertainty increases with evolutionary distance between species as well as for some types of protein families such as CYP450 or G-proteins [32]. In the pharmaceutical field this has been addressed by using a majority vote across three prediction platforms in a web-based application that looks for protein target conservation between human and a range of sequenced phyla (www.ecodrug.org) [33]. Furthermore to this, another tool facilitates summary, comparison and access to various sources of ortholog predictions and provides a comparison of 17 different tools and algorithms to increase the confidence in the orthologue prediction (http://www.flyrnai.org/diopt) [34]

To understand how a MIE could be used to inform RA and enable cross-species extrapolation, the extent of functional conservation of downstream effects across species also needs to be resolved. This could be achieved by deploying new functional *in vitro* assays, although this is expected to be time and resource intensive. However, some compounds will interact with multiple targets, and may lead to different downstream events. This highlights the importance of understanding the response of biological systems from a network perspective [20, 35]. Moreover, since chemical-target(s) interaction networks are often driven by internal exposure dynamics, it is also essential to enhance the understanding of adsorption, distribution, metabolism and excretion (ADME) processes, especially in lower species, where current knowledge is limited. This will allow enhanced consideration of chemical-target interaction networks that may occur following diverse exposure scenarios, thus simplifying and boosting the cross-species extrapolation process.

Furthermore, it was agreed that the pathway leading to the AO itself needs to be fully understood to prioritize testing needs. Understanding how gene / proteins relate to downstream functions through evolutionary relationships between protein families and super-families may also be informative and more meaningful than a one-to-one comparison. Therefore, it is important to understand the available data including substrate specificity and related potencies to discern how the level of gene / protein similarity influences the target affinity and the impact on potency.

While doing so, it is important to keep in mind the whole decision-making process, to better and more efficiently define what is the minimum but necessary information required to enable decision-making for RA purposes and thus reduce overall uncertainty. Pertinent to this, it will also be paramount to consider the different needs of different stakeholders (e.g. regulators vs. industry).

The need for a deeper understanding of the difference between receptor-mediated and more general stress responses was also discussed. Potential solutions included the idea of developing directed functional bioassays, as well as building a library of target-knockout systems encompassing several species. To ensure meaningful results and application, any of these approaches would need to make use of a broad selection of chemicals, representing a variety of chemical classes and MoAs / AOPs, as well as to cover different suitable exposure durations and time points (including life stages), ensuring coverage of potential sources of variability. Ultimately, these approaches would generate a repository which could then be interrogated for hazard characterization every time a new substance comes in for hazard evaluation. There are already ongoing efforts pointing to this same direction, including the Library of Integrated Network-Based Cellular Signatures (LINCS) Program (http://www.lincsproject.org/) [36], providing a first attempt to create a network-based library of biological signatures by cataloguing changes in gene expression and other cellular processes occurring when cells are exposed to a variety of perturbing agents. While this represents a powerful source of information, it is currently limited to human and more of these kinds of approaches are needed to support the evidence across species. Nevertheless, it is recognised that chemical exposure levels in the environment are often very low at a cellular level and producing assays with environmentally relevant cellular exposure becomes difficult (reiterating the need for cell level exposure considerations). Another discussed alternative, and potentially more efficient, way to test this concept would be to start using available data, comparing current existing human toxicity signatures (for instance, from the LINCS database, among others) to available historical toxicity records in the ecotoxicology literature. The proposed database would be used as a surrogate to define the biological target space and could be interrogated to identify potential consensus hazard signatures, based on effect conservation. However, this approach would also pose several other practical questions: what data types would that database include,

such as life-history, transcriptomics, metabolomics, etc.? On which species and chemicals? How would it be prioritized? All of these questions highlighted the recurrent need to increase the ecological realism by considering a larger number of species and thus, related delivered ecosystem functions. This also implies that thorough predictions of pathway-based signatures are urgently needed to better estimate risk, especially when trying to define the most relevant / appropriate species under each scenario. Increasing the two-way data flow (human health to ecotoxicology and vice-versa) would undoubtedly improve the understanding in this field.

The final note from the Work group 1 discussion was the recognition that environmental RA information is not currently being fully exploited within the human health arena (and vice-versa). In fact, there is still a great potential for developing additional biological read-across and extrapolation processes from human health to environmental safety science (and vice-versa), but their different needs and priorities need to be acknowledged. In this sense, generation of new data may not be a priority need, but rather the development of new / improved data mining tools to interrogate the wealth of data that is already available.

4.2 Work group 2: "The Challenge: Develop our basic knowledge of pathway conservation cross-species"

This group addressed how to tackle cross-species extrapolation at a pathway-level and discussed several key issues that need to be resolved to increase confidence before application. It was identified that gene function is the crucial aspect in this respect and the concept of "functional orthology" [37] was considered a beneficial approach to predict the conservation of the (adverse) outcome across species. To address and expand this concept, investigations on different levels are needed. (Re)defining and cataloguing the orthologs by function would help sorting and functionally annotating them into the relevant pathways. There is high probability that a number of genes and gene subfamilies are divergent across species, and additionally, multi-purpose enzymes found in lower species may replace their role and thus belong to multiple pathways. A first attempt in this same direction is provided by the new available software Gene2Function (http://www.gene2function.org/) [38] whose primary goal is to facilitate the development of new hypotheses regarding the function of a given gene based on what is known about the function of orthologs of that gene in other species.

Going beyond a better functional annotation, before stepping-up to a purely pathway-level analysis, the need for a more human and environmental toxicology-relevant gene annotation was also acknowledged. In fact, essential genes / gene families involved in human health (inferred by the many medical / pharmacological studies available) may not always be the same as those that are of ecotoxicological concern. Therefore, as a possible solution, it was suggested to map the human genome against existing ecotoxicology literature in a newly designed, fit-for-purpose database, thus re-annotating genes based on ecotoxicological needs.

The participants acknowledged attempts to define all known AOPs in human and environmental toxicology, however the data are currently far from complete and little is known about cross-species evaluation. The need to define a priority list of the most relevant pathways was discussed and agreed it would provide a good starting point for deeper exploration. One proposed hypothesis was that the “key” pathways that are essential for life are likely to be the more evolutionarily conserved. These could include pathways such as oxidative stress, Nrf2, the p53 DNA damage response, the unfolded protein response (UPR) and mitochondrial injury, among others [39]. Exploration here should be focused both on improving understanding on both an evolutionary scale and on an experimental level. For example, it was suggested the creation of a priority pathways screening panel across relevant species, including new *in vitro* assays for toxicity and stress responses coupled with Physiologically based Kinetic (PBK) models. However, improved insights on the level of pathway conservation is required to be able to interrogate their (potential) de-regulation to the initiation of apical effects (or the lack thereof). This would also serve to improve the functional annotation of the pathways themselves, as mentioned above, thus developing a new “apical functional ontology”. Though, even if some of the pathways are conserved between species, the apical endpoint might not be present, could be organ-specific or could manifest itself in a different (not directly identifiable) way. AOP-Wiki (https://aopwiki.org), the central repository for all AOPs developed so far, represents a good source of information to identify the known links between MIEs, and the cascade of key events (KE) leading to the apical endpoints / AO [15]. A good example of the former is AOP 150 “Aryl hydrocarbon receptor activation leading to early life stage mortality, via reduced VEGF” where the developing embryos of birds and fishes are most sensitive to the stressors activating this AOP, ultimately leading to embryo death and population

trajectory decline; mammals appear to be less sensitive, leaning towards cardiotoxicity that persists into adulthood, and increasing susceptibility to heart disease rather than embryo-lethality. It was also discussed that sub-pathway modules / key events might be more conserved, thus easier to track, and might give more information between different species than investigating the whole pathway. While it is appreciated that we cannot expect to unravel all AOs for all toxicity pathways of concern in all species, moving toward these kinds of approaches would help to increase confidence in toxicity predictions. Also, it would be a significant advancement to know when a pathway is conserved and disrupted across which species, thus defining with more confidence the space for environmental risk.

In this respect, it was acknowledged that the aim of research is always to advance science to serve society with the maximum knowledge possible. As scientists, it is appreciated that curiosity drives the understanding of every mechanism and interaction between a chemical and an organism. Regulatory pressures require data underpinning human health or ecological assessment to be consistent and robust, with the goal of ensuring safety to humans and the environment. This raises the challenge: when do we have enough data for RA? An admittedly complex question to which there is no easy answer. The ultimate goal is to achieve enough confidence to enable decision-making without endless laboratory testing and years of research. Starting from the point that it is not realistic to cover all aspects for assessment contexts, it was suggested that it may be easier to know when the data is not enough. It is a matter of increasing confidence and reducing uncertainty: for instance, one could hypothesize that enough data might mean having several pathways annotated to allow satisfactory toxicity predictions, although we don’t know all of them. Thus, to answer the question on how much investment is acceptable to reduce the uncertainty of risk, we first need to think of how current uncertainties are preventing decisions to be made and how much we are willing to invest (in time, money and effort) to improve this certainty. The current revolution in digital technology and machine learning approaches may well help to address both the question of how much data is required, and what information is retrievable from existing data [40] .

From an environmental RA standpoint, it will never be possible to consider all environmental species and all possible exposure scenarios (time, doses, frequency and ecological circumstances). As such, there is a need to derive sufficient evidence

allowing to build models and provide enough scientific basis to support reasonable predictions based on relatively small datasets. There is not one unique environmental relevant species that is better, more representative or more appropriate than others. A concept currently being explored by evolutionary biologists considers several species (5-8 species) covering the phylogenetic tree in its main branches. This could be considered the minimum number of species needed to reflect the main distinctiveness of evolution. In any case, it continues to be very challenging to include species-specific physiology into the equation as well as the ecological traits that are unique to each (sub-)species. Similarly, it is important to understand how to consider and account for genes that may exert different roles simultaneously, or different functions throughout the lifetime of the organism; how to deal with epigenomics and the knowledge that genomes are adaptive to environmental conditions and / or external stimuli; how to overcome the potential problem that genes may behave differently when tested in controlled lab conditions as compared to their native state. All of these remain open questions: although much work has been done to try to answer these questions, comprehensively addressing these and other concerns on a case-by-case basis are still far from application and beyond the current requirements for RA.

4.3 Work group 3: "The Challenge: Refine our understanding of biological processes impacting internal exposure"

Consideration of the main biological processes impacting internal exposure, and particularly, how species-specific ADME processes influence chemical concentrations at target sites, is critical to the application of mechanistically-based species extrapolation. The concept of "exposure" cannot usefully be discussed in isolation, but rather as an integrated part of the RA question. Without inclusion of exposure, any discussions on chemical-target interactions and species extrapolation of the responses remain theoretical, limited purely to the identification of similar hazards. In order to translate an identified hazard into a risk, considering and understanding exposure is essential. Being RA is driven by exposure, it is not relevant that the molecular target triggered by a given compound is conserved across species, if the exposure level is below the activation threshold of the MIE. Thus, it becomes essential to understand the RA question, and link to the specific exposure scenario, and the required level of confidence needed to make an early decision on risk. At sufficiently

high chemical exposure doses, organisms and cells often exhibit acute effects related to general membrane perturbation, e.g. narcosis, whereas at continuous but lower doses, different pathways may trigger measurable effects at various thresholds. In addition, complexity is also added by differences in sensitivity between different cell types as well as different rates of metabolism across tissues and organisms. Indeed, when extrapolating from *in vitro* to *in vivo*, or from species to other species, differences in both biokinetic and biodynamic properties are of central importance and neither can answer the question of risk independently.

Lessons can be learned from human health where for decades animal effect data have been utilised to extrapolate to potential human effects. More recently, research has been focused on extrapolating from *in vitro* to *in vivo* effects to eliminate and overcome the need for animal testing. The magnitude of the challenge of applying the same strategies to environmental RA is apparent: rather than dealing with one very well characterised organism, thousands of diverse, highly variable and poorly characterised organisms need to be considered. Given this complexity, the workgroup focussed discussion mostly around fish, where extrapolation approaches from existing data is key, given the desire to eliminate animal (i.e. vertebrate) testing.

In order to meet these needs, the MERLIN-Expo software (https://merlin-expo.eu/) was developed, which contains a library of models for exposure assessment coupling environmental multimedia and pharmacokinetic models, and aims to link environmental fate of chemicals and internal concentrations in humans, thus integrating environmental exposure assessment and human exposure assessment. Although it represents a very significant step in this space, it is centred over human health RA and does not cover the heterogenicity found in the environment in terms of species and ecosystems, that still need to be addressed further for its implementation in ERA.

Across the pharmaceutical industry as well as pesticides and biocides, chemicals of interest are designed for high levels of specificity and potency, and effective absorption. This combination of chemical attributes can often lead to measurable effects at realistic exposure scenarios for aquatic species despite being designed for low bioaccumulation potential [41]. As such, there has been a pressing need for a common strategy for environmental RA for these industries. In contrast, ingredients

used in Home and Personal Care (HPC) products are designed to be of low bioactivity as possible. As such, toxicological concern associated with these types of chemicals is reduced, though the volumes used are greater compared to pharmaceuticals, for example.

It is therefore understandable that the greatest examples forward in terms of MIE identification and species extrapolation come from the pharmaceutical sector. One of the most prominent strategies for environmental RA coming from this sector is the Hugget approach (Fig 1) [42-45]. This method presents an approach for biological read-across from human therapeutic doses to environmental species (e.g., fish). It rather simplistically compares internal concentrations in fish and human based on toxico-kinetic modelling and environmental fate calculations.

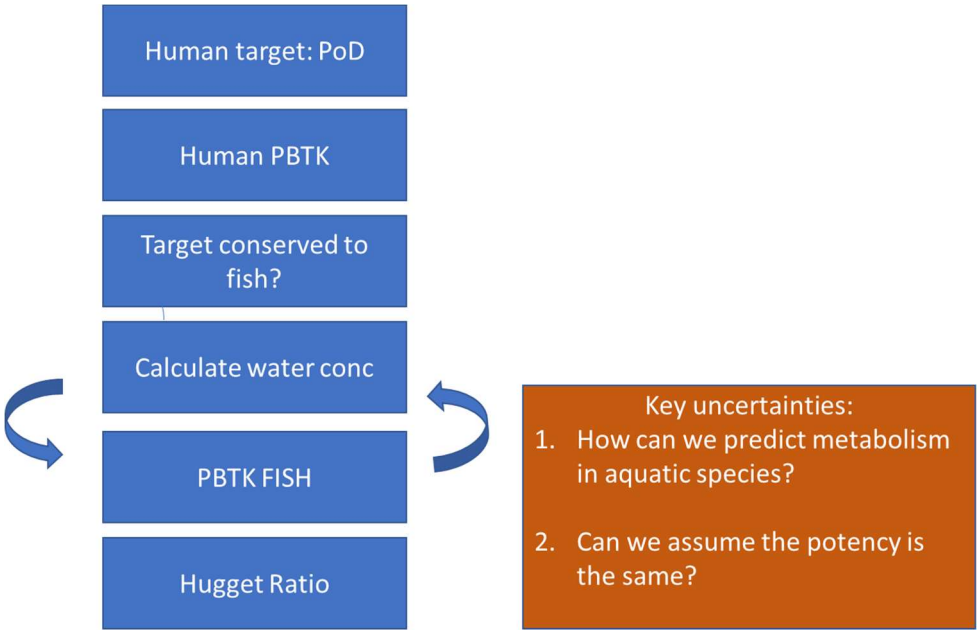


Figure 2 Hugget approach scheme. Acronyms PoD: Point of Departure, PBTK: Physiologically-based ToxicoKinetics

However, the application of this approach in other sectors is more challenging because, for instance, HPC ingredients rarely have a full package of ADME data associated with them, predominantly because they were not designed for biological interaction in the same way that pharmaceuticals are. This could suggest that for many chemicals the Hugget approach may be excessive due to its extensive data requirements. However, the exploitation of existing data from other compounds or from other species may obviate the need for extensive testing if the uncertainties surrounding these data can be addressed. These uncertainties result from 1) whether

we can assume metabolic machinery is significantly similar across species to predict metabolism in relevant species and 2) whether the potency of target effects is the same in different species.

Given the relative abundance of data generated regarding human metabolism, a consideration to be made regards how acceptable it is to read across from data generated to satisfy human safety needs (using *in vitro* assays) to fish and other species. This raises a number of research questions: a) Can we cover all the biological space for fish using existing human cell lines? b) Can we use existing cell line data sources (e.g. American Tissue Cell Collection or Cellosaurs) to define suitable cell lines to cover that biological space? c) have we performed extensive comparisons between these human-fish cell lines and if required can we establish new lines to fill the gaps? d) Can we use existing untargeted chemistry data e.g. a metabolomics study, to see and / or model metabolism patterns for selected chemical classes across human and model species? Can we take broader approaches for understanding metabolism in environmental species? For example, can we classify clearance in fish as high-medium-low and can we establish uptake rates for chemical classes? In this regard, the workgroup also discussed the possibility to perform computational simulations to address the uncertainty across a range of species and use randomness to generate draws from a probability distribution. Main strength of this approach is the possibility of considering any potential outcome of a process and thus, assessing the whole impact of risk and allowing for better decision making under uncertainty and deficiency of data.

When looking into the application of these approaches for RA, it was acknowledged that additional careful consideration is needed in designing a new exposure-driven environmental RA strategy. The conventional Predicted Environmental Concentration / Predicted No Effect Concentration (PEC / PNEC) strategy for assessing environmental risks is often criticised for its lack of environmental realism and its conservative nature. The strategies adopted by the pharmaceutical sector require extensive data generation that may not always be feasible for other products or ingredients. However, a strategy based on maximizing read-across data from other species principally focussing on understanding and characterising metabolism may negate the need for large-scale metabolism data generation. Additionally, this should enableto maximize the value of existing PBK approaches to establish internal

exposure concentrations as part of a broad modelling approach for environmental RA. Gaps in knowledge still exist in order to determine which approach is most appropriate and more species-specific PBK models and metabolism data is required to be generated to support. Overall, the group concluded that there is a need to develop further approaches to capitalize on the advances made in molecular target discovery and being able to determine internal exposure to a sufficient level of accuracy, whilst still maintaining a pragmatic approach.

5. Discussion and future outcomes

Over the last decade, there has been advancement in the way that chemical RA is performed and there has been an accelerating global shift toward animal-free methods. The International Cooperation on Cosmetics Regulation (ICCR) recently defined Application of 'Next Generation RA', as an exposure-led, hypothesis-driven RA approach that integrates *in silico, in chemico* and *in vitro* approaches, and provides an example of how this framework is becoming more embedded [7]. Nevertheless, current environmental RA standard regulation guidelines still rely on extrapolating largely *in vivo* data from a limited number of model species to a multitude of species of environmental concern using safety factors to account for uncertainties. This protective rather than predictive (or realistic) approach to biological read-across presents significant barriers to the broader use of the increasing wealth of NAMs-derived data for inferring impacts across organisms within a Next Generation RA framework. At present, biological read-across tends to be constrained by its large degree of uncertainty due to inherent physiological diversity (obvious at the organ to species-level, but less obvious at the sub-cellular MIE-level), the wide range of sensitivities to chemicals and the limited mechanistic understanding of toxicity in non-target species. In fact, the general lack of comparative cross-species sensitivity data (including human) limits the ability to make robust taxonomic extrapolations in support of RA. Overall, it is essential to increase trust in these methods, by building confidence among regulators and the broader scientific community that the necessary biology is comprehensively and adequately incorporated into the proposed animal-free strategies, so that they can be applied and used for both human health and environmental RA. To encourage this process and to reduce the associated uncertainty, it is imperative to identify and explain the relevant inter-species similarities

/ differences, to allow more evidence-based extrapolations and an improved assessment of uncertainty.

There are several ways to achieve this. A common concept is based on pure orthology, which relies heavily on sequence similarity and phylogenetic events, and is illustrated by SeqAPASS (Sequence Alignment to Predict Across Species Susceptibility, https://seqapass.epa.gov/) [46]. This tool attempts to answer the question whether or not a known protein target is present in another species for a chemical to act upon. Information from SeqAPASS, in concert with AOP descriptions can begin to inform the potential for cross-species effects propagating from a MIE to an AO. Another approach "Interspecies Correlation Estimates" (https://www3.epa.gov/webice/) [47], was developed by the USEPA with two aims: the estimation of acute toxicity from a surrogate species, and a species sensitivity distribution model which generates a prescribed hazard level. The main disadvantage of this approach is that it is based on statistical inference and modelling and is not inclusive of any biological or mechanistic information. While acknowledging the added value of these approaches in providing additional lines of evidence applicable to a WOE evaluation in a decision-making context, they presently still lack the underlying mechanistic understanding needed to improve safety decisions. Yet, the tools are not static and continue to evolve as the science advances in this area of bioinformatics.

The workshop discussed the opportunity for application and the limitations of the current approaches, along with proposing NAMs that could improve biological read-across while reducing the inherent uncertainty embedded in the process. At the same time, it would allow greater re-use of existing data sources. In this regard the main outcomes of the workshop can be distilled into an augmented concept of “functional orthology”, in which the common orthology concept should be merged with functional and mechanistic information, namely the information being generated by ToxCast, SEURAT-1, EU-ToxRisk projects among others [48-51], thus expanding the understanding of the underlying processes leading to toxicity. Although this is not a completely new concept, it is only now that the latest advances in technology and knowledge will allow “functional ontology” to be fully achieved. In this perspective, the most effective and unbiased way to determine gene function is through functional genomic studies, which usually involves (systematic) knockout of genes, followed by assessment of phenotypes such as lethality / viability, growth, development, etc.

These “genotype-to-phenotype” approaches can also provide insight into chemical mechanisms of action, helping to define more specific toxicological endpoints and informing the development of novel mechanistic-based toxicity bioassays. New powerful molecular techniques are now available to obtain targeted knockouts, such as homologous recombination, RNA interference (including siRNA and shRNA), engineered site-specific nucleases (i.e. zinc-fingers, TALEN, CRISPR). However, using only sequence homology (and / or orthology) as a basis for extrapolation may be somewhat limited (as discussed previously) and not a guarantee of the conservation of function. Thus, managing to overcome this assumption by considering the functional level would lower the ambiguity and reduce overall uncertainty. The translation of these new understanding into novel pathway maps could be used to better define the species-impact space for well-defined toxicity pathways. This is not a trivial task; though, if collective and coordinated efforts are brought forward, ensuring gaps are addressed a by all the relevant stakeholders and new knowledge is then translated into regulatory changes, the benefits for both human health and environmental RA are expected to be highly significant.

Since most of the currently generated NAMs data are designed to inform human health assessment, ecotoxicology has much to gain from an increased knowledge of pathway homology. There is high potential to benefit from a comprehensive and well understood mechanistic-based predictive science, addressing the long-standing issue of chronic (i.e. long term) sub-lethal exposure. Nevertheless, there are also potential advantages for human health RA, such as the concept of new PBK and dynamic models developed for chosen invertebrate species that may be the missing piece of the puzzle and provide the evidence of whole-organism function. This will offer human health researchers a new multi-dimension, fully functional biological model, helping better inform the processes involved in toxicity and / or disease. Human health researchers have recently developed several approaches to overcome the shortcomings of single cell line testing (e.g. lack of biological relevance, impaired metabolism) by using multicell plates, organotypic 3D models, among others. Although a big step forward and more relevant *in vivo* conditions, these approaches may not yet be sufficiently representative of the dynamics of a whole organism. In this sense, the development of a deeper biological / physiological knowledge of invertebrate species and how they deal with stress, can have a large impact. Existing vertebrate-based

PBK models can already provide valuable information on internal concentrations (particularly in fish) to support RA decisions, although broader applicability of such models may be obtained through closer investigation of data read-across potential for some input values and the associated uncertainties.For instance, until recently, when considering the traditional protection goal of environmental RA as the population level, decision-making, , has largely been based on the test chemical concentration able to disrupt apical processes, (i.e. development, growth, reproduction, and survival) in environmental species, which may ultimately alter population dynamics. In this regard, the issue of estrogenic chemicals in the aquatic environment provides an interesting example of the practical significance of this aspect. The intersex condition (i.e. presence of eggs in the testis) observed in male fish in the rivers of many western countries is probably one of the most dramatic phenotypic effects observed in freshwater wildlife associated with chemical contamination [52, 53]. The discovery of intersex in wild fish, in the 1990s, triggered an entirely new stream of research, endocrine disruption in the aquatic environment. The evidence produced by this large volume of research led, in 2015, to the inclusion of 17-Alpha-ethinylestradiol (EE2), 17-Beta-estradiol (E2), and estrone (E1) in the surface water Watch List under the Water Framework Directive. However, concurrently, a large study carried out in the UK demonstrated that populations of cyprinid fish are self-sustaining despite widespread feminization of males [54], raising a new challenge for the interpretation of the relevance of testicular intersex for decision-making in environmental RA. This suggests that more efforts should also be dedicated to the evaluation of whether implementation of the latest mechanism-based predictive toxicology approaches would bring significant benefits compared to the traditional approach. Regardless the aim of the RA, predicting adverse phenotypes triggered by exposure to chemicals remains an essential aim of modern toxicology. This challenge is particularly pressing if we consider that millions of animals would be required to test the potential toxicity of the thousands of chemicals currently in commerce. The application of the AOP concept to frame existing toxicological knowledge from a mechanistic perspective has been proven to provide a good platform to bridge the gap between human and environmental RA [20], and to support the regulatory acceptance of mechanistic considerations in an environmental RA context.

6. Conclusion and recommendations:

Overall, the workshop highlighted a clear and common motivation to progress towards the application of mechanistic-based animal-free chemical safety assessment methods. However, there is no single fit-for-all approach and it is clear that seldom will be possible to directly replace animal testing with NAMs, but rather coordinated efforts aimed at an integrative implementation of new approaches are required. As a first step, it was urged further exploitation and integration of the wealth of already available information. Better employment of existing data and tools may drive toward an improved cross-species extrapolation and lead to reduced reliance on animal data. This is particularly true for environmental toxicology where similar tests are traditionally required in multiple species to meet global regulatory requirements, but also useful to bridge the existing knowledge gap between human and environmental toxicology. Moreover, further targeted development of NAMs and generation of ad-hoc data would greatly increase confidence and scientific evidence for extrapolating MIEs, KEs, or entire pathways (e.g. MoAs / AOPs) between human and environmental relevant species, thus consolidating the shift to a more mechanistic-based predictive RA and support the use of a broader landscape of data across both human health and environmental RA fields.

The identified and prioritized research needs and key recommendations from the workshop cover both current technical challenges (i.e. required research & capability-build) and decision-making challenges (i.e. development & evaluation for RA), as follows:

- Research needs:
    - To setup collaborations between relevant stakeholders (academia, industry, regulators, NGOs) to define endpoints or AOs of concern across human models and environmental species, leading to prioritized testing needs.
    - Define a priority list of pathways of environmental toxicological concern, which are key to organism survival, growth and / or reproduction and describe the extent of their conservation across species.

- Build a database designed around functional gene annotation to enable full exploitation of data from all relevant species coupled with improved mining tools to adequately interrogate this data.
- Address uncertainties in orthology assignment by re-designing and deploying functional assays to report downstream / upstream pathway-based effects.
- To develop cross-species relevant screening panels for the identification of priority pathways able to predict AOs of concern.
- To develop approaches linking the environmental / external concentration to the cellular / internal concentration at the target tissue and the AO in order to understand the minimum level of perturbation necessary to trigger toxicity.
- Improve the understanding of ADME processes across key relevant biological classifications (e.g. family or species).
- Develop computational tools (i.e. models) to enable prediction or classification of chemical clearance rates for species relevant to RA, namely simplified PBK models.

- Decision-making challenges:
  - Identify current technology and application gaps which need to be addressed for the successful implementation of NAMs in RA.
  - Establish and promote confidence in extrapolating effects across species using mechanistic data, through the development of case-studies
  - Develop a new globally harmonized RA framework incorporating NAMs and making use of all the data available (using well established chemical read-across as well as cross species extrapolation where possible)
  - Improve the understanding of exposure scenarios, namely reducing its granularity, so that those considerations can be better incorporated into RA.
  - Ensure early engagement and maximize communication of the private sector (i.e. industry) and regulatory agencies to drive and ensure future fit-for-purpose in the scientific / technical development of NAMs.

As a last note from the workshop, it was remarked that it is imperative to continue the flow of these discussions to include the wider scientific community, regulators, and

industry, while continuing to progress the development of novel scientific approaches to fully explore the potential of NAMs. All relevant stakeholders should be involved in this discussion to ensure its proper development. Only by having developers and end-users discussing the advancement of new approaches together we can ensure that they are fit-for-purpose and meet the innovation and decision-makers' needs.

Disclaimer:

All authors are employees of their respective organisations. Their views expressed in this manuscript are their own and do not necessarily represent those of their institutions or companies.

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