Cross-species extrapolation of biological data to guide the environmental safety assessment of pharmaceuticals – The state of the art and future priorities

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ABSTRACT

The extrapolation of biological data across species is a key aspect of biomedical research and drug development. In this context, comparative biology considerations are applied with the goal of understanding human disease and guiding the development of effective and safe medicines. However, the widespread occurrence of pharmaceuticals in the environment and the need to assess the risk posed to wildlife has prompted a renewed interest in the extrapolation of pharmacological and toxicological data across the entire tree of life. To address this challenge, a biological 'read-across' approach, based on the use of mammalian data to inform toxicity predictions in wildlife species, has been proposed as an effective way to streamline the environmental safety assessment of pharmaceuticals. Yet, how effective has this approach been, and are we any closer to being able to accurately predict environmental risk based upon known human risk? Here, we discuss the main theoretical and experimental advancements achieved in the last ten years of research in this field. We propose that a better understanding of the functional conservation of drug targets across species, and of the quantitative relationship between target modulation and adverse effects, should be considered as future research priorities. This pharmacodynamic focus should be complemented with the application of higher throughput experimental and computational approaches to accelerate the prediction of internal exposure dynamics. The translation of comparative (eco)toxicology research into real-world applications, however, relies on the (limited) availability of experts with the skill set needed to navigate the complexity of the problem; hence, here we also call for synergistic multi-stakeholder efforts to support and strengthen comparative toxicology research and education at a global level.

Introduction

Patient access to effective and safe medicines relies on the successful completion of a complex 4-dimensional drug discovery and development process (Wagner *et al*., 2018). Typically, this requires many years of research (average 8.3 years; range 3.6-16.6 years) and has an average cost estimated to be between $1.3 and $2.8 billion per drug (Di Masi *et al*., 2016; Wouters *et al*., 2020). This process generates large volumes of *in silico, in vitro, and in vivo* data across multiple mammalian species, including preclinical rodent and non-rodent species and clinical (human) data (Namdari *et al*., 2021). The

interpretation and extrapolation of this multi-dimensional data drives human safety assessment and related decision making (e.g. project closure *versus* progression to clinical phases). Although the potential value of this knowledge to support the environmental risk assessment (ERA) of human pharmaceuticals (a legal requirement in Europe since 2006) has been recognised by the European Medicine Agency's guidelines (European Medicine Agency, 2006), the lack of a formalised process to guide the extrapolation of mammalian data to wildlife species has represented an important factor hampering this ambition (Winter *et al*., 2010).

The ERA of human pharmaceuticals is typically considered a stand-alone tiered process, and relies on the experimental determination of adverse and non-adverse exposure concentrations using environmentally relevant model species representing three trophic levels (e.g. fish, invertebrates, algae). The assessment is conducted using standardised tests designed to detect apical effects on development, growth, reproduction, and survival (e.g. US Food and Drug Administration, 1998; European Medicine Agency, 2006; OECD, 2011a, 2012a, 2013). Considering the biological differences between mammalian species and other taxa, this experimental approach may appear like a pragmatic and effective strategy to generate empirical data, while minimising uncertainties associated with the extrapolation of toxicity data across distant species. However, a recent large-scale analysis of ecotoxicity data available for 975 approved small molecule drugs revealed that a complete set of regulatory compliant multi-species ecotoxicity data (e.g. across all three levels) is lacking for 88% of compounds (Gunnarsson *et al*., 2019). Similar conclusions were reached by Burns *et al*. (2018) by considering the 1,912 Active Pharmaceutical Ingredients (APIs) registered in the UK. Filling that data gap with experimental data would require decades of work (typically 2-3 years per compound) and, in the case of fish, the use of many thousands of protected animals. Specifically, Burns et al. (2018) found that only 11% of the 1,912 APIs registered in the UK have ERA data. This data coverage decreases further if we consider the 332 APIs identified as priority compounds for ERA in 76 different prioritisation exercises published in the scientific literature, as only 3% of those compounds have ERA data. Using this UK data coverage as a proxy, we could conclude that approximately 1,700 APIs available on the market lack ecotoxicity data. The lack of data, by itself, does not automatically imply that regulatory-relevant *in vivo* fish testing is needed. To estimate possible realistic testing requirements for the 1,700 untested APIs, we can extrapolate the available information already available for the 208 APIs that do

have ERA data. In this case, the data collected by Gunnarsson et al. (2019) indicate that ~50% of those compounds triggered a Fish Early Life Stage Test (OECD, 2013), 10% a bioconcentration assessment (OECD 2012a), and ~5% a Fish Full (or Reduced) Life Cycle Test (OECD, 2011, 2012b; OPPTS, 2016). Applying these proportions to the full 1,700 APIs that lack data, this would conservatively translate into the use of more than 300,000 fish and would require a (likely unavailable) testing capability in contract laboratories able to accommodate >800 Early Life Stage Tests, and 85 Life Cycle Tests. With this consideration in mind, there is an urgent need to develop more intelligent, efficient, and cost-effective approaches to prioritise compounds of concern, and better predict potential adverse effects associated with the presence of human drugs in the environment.

This ambition could be realised, at least partially, by developing a battery of novel scalable and, most importantly, predictive *in vitro* and *in silico* methods (e.g. Artificial Intelligence (AI)-powered Quantitative Structure-Activity Relationship (QSAR), *in vitro* organoids, etc.) for the detection of species-specific toxicity in the species of interest (e.g. fish) (reviewed by Langan *et al*., 2023). However, even in a scenario in which this novel battery of 3Rs friendly methods were to become available, the volume of ecotoxicity data generated for human pharmaceuticals will always be much smaller than the volume of mammalian data generated for the purposes of human safety and efficacy assessment. Thus, framing ecotoxicity data within a wider data-rich cross-species knowledge base, which includes mammalian data, would bring significant advantages, facilitating data interpretation and Weight of Evidence (WoE) evaluations, and increasing the overall confidence of the decision-making process for ERA. This mammalian-data driven 'read-across' approach to streamlining ERA has been heralded for a number of years now (e.g. Huggett *et al*., 2003; Ankley *et al*. 2005; Winter *et al*., 2010; Berninger and Brooks, 2010; Rand-Weaver *et al*., 2013), yet are we any closer to accurately predicting environmental risk based upon known human risk? This review will discuss the advancements achieved in this field in the last 10+ years as well as future research priorities, with a special focus on fish and other aquatic species.

2. State of the art

The interpretation of the toxicological relevance of complex biological data relies on the integration of multi-dimensional evidence generated along a continuum of processes that link exposure, mechanisms, and adverse effects.

2.1 Pharmacodynamics – linking drug-target interaction to adverse effects

A key element needed to predict the potential toxicity of pharmaceuticals across species is the assessment of the evolutionary and functional conservation of drug targets. The higher the conservation between non-target species (e.g., fish) and humans, the higher the probability of target-mediated effects (Rand-Weaver *et al*., 2013). In the last ten years, this field of research has progressed at a very high pace, transitioning from the analysis of single targets (e.g. 5α-reductase, Margiotta-Casaluci *et al*., 2011, 2013) to the large scale evaluation of all known drug targets, and the generation of publicly available informatic tools that allow user-friendly data exploration within an ERA-focused context, such as ECOdrug (Verbruggen *et al*., 2018; Gunnarson *et al*., 2019) and SeqAPASS (LaLone *et al*., 2013, 2016). Importantly, these resources allow the assessment of the evolutionary conservation of drug target genes and proteins, in species of ecotoxicological relevance.

This systems-level understanding of the evolutionary conservation of drug targets has facilitated our ability to predict the hazard of pharmaceuticals in the environment (LaLone *et al*., 2013; Gunnarsson *et al*., 2019). A growing number of studies have demonstrated that mode-of-action-related effects can be accurately extrapolated from mammals to fish for several classes of pharmaceuticals (Table 1). These include antidepressants and other drugs targeting the central nervous system (e.g. Winter *et al*., 2008a; Valenti *et al*., 2012; Brooks, 2014; Margiotta-Casaluci *et al*., 2014; Huerta *et al*., 2016; Tanoue *et al*., 2017; Winter *et al*., 2021), cardiovascular drugs (Owen *et al*., 2007; Winter *et al*., 2008b; Giltrow *et al*., 2009; Margiotta-Casaluci *et al*., 2019;), adrenergic agonists (Weil *et al*., 2019), steroids and anti-steroids (Thorpe *et al*., 2003; Runnalls *et al*., 2013; Runnalls *et al*., 2015; Margiotta-Casaluci *et al*., 2013), steroidal and non-steroidal anti-inflammatory drugs (Mehinto *et al*., 2010; Margiotta-Casaluci *et al*., 2016; Marmon *et al*., 2021).

In some cases, the Adverse Outcome Pathway (AOP) framework (Ankley *et al*., 2010; Margiotta-Casaluci *et al*., 2016; Conollly *et al*., 2017) and AOP-informed data visualisation strategies have been used to synthesise the evidence mentioned above (e.g. Margiotta-Casaluci *et al*., 2016; Marmon *et al*., 2021), and have demonstrated the great potential of these data-driven approaches to support ERA and follow up ecopharmacovigilance strategies (Gunnarsson *et al*., 2019; Marmon *et al*., 2021). Moreover, meta-analyses have been used to generate quantitative comparative evaluations of mode-of-action-relevant responses across species (i.e. effect magnitude and direction), highlighting the value of this approach to support the WoE assessment of

toxicology data from a cross-species extrapolation perspective (Margiotta-Casaluci *et al*. 2019). The successes achieved with the mode-of-action-centred strategy discussed above holds great promise for the scaling up of this predictive approach to inform the ERA of groups of pharmaceuticals acting via common pharmacological mechanisms. For example, Marmon *et al*. (2021) demonstrated the value of network pharmacology methods and PK/PD data synthesis to support hazard and risk assessment of a mixture of 25 non-steroidal anti-inflammatory drugs (NSAIDs).

It is important to highlight that the experimental efforts explicitly aimed at characterising the cross-species concordance of mode-of-action-driven effects of pharmaceuticals (for ecotoxicology applications) have been largely focused on fish, whereas a relatively limited amount of research has been carried out on invertebrates. Some notable exceptions are represented by antidepressants (e.g. Campos *et al*., 2012; Fong and Ford, 2014; Rivetti *et al*., 2016), beta-adrenergic receptor agonists and other cardiovascular drugs (e.g. Villegas-Navarro *et al*., 2003; Dziawloski *et al*., 2006; Stanley *et al*., 2006), and steroid hormone agonists (e.g. Kaur *et al*., 2015, 2016), whose effects have been investigated also in molluscs and crustaceans. Larger sets of invertebrate data are available for apical endpoints, like those quantified during regulatory ecotoxicology testing (e.g. algal growth, *Daphnia magna* reproduction). Analysing this apical regulatory-relevant dataset, Gunnarsson et al. (2019) observed that the toxicological sensitivity of fish, *Daphnia*, and algae to pharmaceuticals may be comparable when the relevant drug target is conserved across these three taxa. Moreover, Coors et al. (2023) recently proposed that this scenario may even be leveraged to reduce the *in vivo* fish testing needed for the safety assessment of legacy pharmaceuticals, suggesting that the No Observed Effect Concentrations (NOECs) generated with *Daphnia* and algae may also be sufficient for the protection of fish.

2.2 Drug uptake and pharmacokinetics – the importance of internal exposure

The ERA of pharmaceuticals typically considers the concentrations of the compound of interest (either nominal or measured) in the relevant environmental matrices outside exposed living organisms (e.g. water, sediment, soil). From a practical perspective, this approach facilitates hazard and risk evaluations by providing a parameter that is easy to interpret, even without prior knowledge of the pharmacological features of the compound or the need for complex analytical chemistry. Indeed, the simple comparison of measured environmental concentrations and effect concentrations (e.g. Lowest Observed Effect Concentrations, LOEC) determined in ecotoxicity studies has been

proposed as a rapid method to prioritise chemicals, including pharmaceuticals, for ERA in the aquatic environment (Donnachie *et al*, 2015; Johnson *et al*., 2017). Despite its obvious advantages, this approach can only allow retrospective evaluations, whereas modern ecotoxicology should strive for high-precision predictions able to inform hazard and risk management strategies before the marketing of the compound, or at least when market penetration is still limited.

Considerable progress has been achieved in the measurement (Tanoue *et al*., 2020; Wilkinson *et al*., 2022) and modelling (e.g. Kapo *et al*., 2016; Oldenkamp *et al*., 2018; Wilkinson *et al*., 2022) of environmental concentrations of pharmaceuticals in rivers, and dedicated databases have now been created to collate exposure data published globally (Graumnzi and Jungman, 2021). However, from a pharmacological and cross-species perspective, predictive toxicology methods would benefit greatly from understanding uptake and pharmacokinetic (PK) profiles of pharmaceuticals in non-target (i.e. non-human, environmentally relevant) species, as it is the concentration of chemicals inside the organism (e.g. in the blood or other tissues or more precisely at the target itself) that ultimately drives toxicological risk (Huggett *et al*., 2003; Nyman *et al*., 2014; Hutchinson *et al*., 2014; Margiotta-Casaluci *et al*., 2016). The importance of this concept was highlighted by large experimental studies on synthetic glucocorticoids, in which compounds with comparable *in vitro* potency showed very different abilities to elicit *in vivo* effects, which were in line with their different uptake and PK features (LaLone *et al*., 2012; Margiotta-Casaluci *et al*., 2016).

The Read Across Hypothesis and the Fish Plasma Model theory (Huggett *et al*. 2003; Rand-Weaver *et al*., 2013) have provided a theoretical framework to interpret the pharmacological risk of pharmaceuticals in fish, based on a comparison with pharmacologically and toxicologically relevant measured blood concentrations in humans. The theoretical and experimental utility of this approach has been demonstrated in several large-scale prioritisation studies (e.g. Fick *et al*., 2010; Gunnarsson *et al*., 2019; Marmon *et al*., 2021; Sumpter and Margiotta-Casaluci, 2022) and in a growing number of experimental studies performed on a wide range of classes of pharmaceuticals, including beta-blockers (Winter *et al*., 2008b; Giltrow *et al*., 2009; Owen *et al*., 2009); beta-adrenergic receptor agonists (Weil *et al*., 2019); antidepressants (Valenti *et al*., 2012; Margiotta-Casaluci *et al*., 2014); anxiolytics (Huerta *et al*., 2016); opioids (Tanoue *et al*., 2017); synthetic glucocorticoids (Margiotta-Casaluci *et al*., 2016); and synthetic progestins and oestrogens (Runnalls *et al*., 2015). These

experimental studies have been instrumental in the validation of the Fish Plasma Model, and have helped to refine testing approaches to account for factors such as plasma protein binding, metabolic activation of prodrugs, and pH (Tanoue *et al*., 2017; Chang *et al*., 2021; Henneberger *et al*., 2022).

Although *in vivo* experimental studies are highly valuable for advancing this field, they often still require the use of protected animals; thus, there remains an urgent need to integrate all existing data and information to generate scalable modelling methods and databases that are in line with the 3Rs-driven vision of modern toxicology. In line with this, already in 2005, Reimschuessel and colleagues (US Food & Drug Administration) published a curated database (Phish-Pharm) that includes absorption, distribution, metabolism, and excretion (ADME) and other PK information extracted from over 700 articles (to date) on 191 aquatic species (Reimschuessel *et al*., 2005). This data centralization exercise was followed by the work of Berninger *et al*. (2016), who generated a database containing ADME data for 1,070 APIs, which can be used to drive the translation of PK considerations across species and inform the development of fish-specific PK models (Nichols *et al*., 1990; Brinkman *et al*., 2016). Although not widely used, the development of PK models in environmental toxicology has been growing slowly but steadily over the last decade, unsurprisingly, in parallel with rapidly improving analytical chemistry capabilities (e.g. Stadnicka *et al*. 2012; Larisch *et al*., 2017; Grech *et al*., 2019). In a recent example, Larcich and Goss (2018) assessed which physiological parameters drive interspecies differences in fish PK, and identified lipid content, ventilation rate, uptake efficiency from food, and metabolism rates as the most important factors. As PK models have only been developed for very few fish species, Wang *et al*. (2022) proposed to overcome this limitation by developing a generalised Physiologically Based-PK Model for fish that could facilitate the application of PK modelling for the ERA of pharmaceuticals more widely. Beyond fish species, a similar approach was used by Baier et al. (2022) to develop a generic avian PBPK model for avian species, validated in three bird species using a set of pharmaceuticals that included antibiotic, antiparasitic, antifungal, and sedative compounds.

One of the main challenges hampering the transition towards the wider application of PK modelling in ecotoxicology is the availability of species-specific ADME data; however, *in vitro* and computational methods can represent a valuable 3Rs-friendly alternative that builds on the advancements achieved in the human ADME and *in vitro-to-in vivo* modelling fields (Lombardo *et al*., 2017; Davies *et al*., 2020). Several fish *in vitro*

methods have been established for the characterisation of parameters such as, for example, drug uptake through gills (Stott *et al*., 2015; Chang *et al*., 2021) and intestine (Langan *et al*., 2017), and for hepatic metabolism (Baron *et al*., 2017) and clearance (Baron *et al*., 2017; Nichols *et al*., 2018).

The integration of the PK and PD considerations, as discussed above, could greatly accelerate the transition from hazard to risk assessment as it would allow us to predict whether, under a given exposure scenario, the compound of interest could achieve target-site concentrations high enough to activate a pharmacological response. Hence, the extrapolation of PK/PD across species could represent a powerful tool to generate accurate toxicity prediction, even in those cases when species-specific data availability is limited. An additional area of promise that could be leveraged into future PK/PD models is the expanding field working to better predict uptake into fish, invertebrates and terrestrial organisms (Miller *et al*., 2019; Carter *et al*., 2022). Combining these uptake models based on machine learning and mathematical models that include ionisation, with *in vitro* systems (listed above) that can determine metabolism and excretion, offers the prospect of a tiered approach to understanding the risk of these chemicals, but without the need for *in vivo* experimentation.

3. Priorities for future research

3.1 Assessing the conservation of drug targets - from structure to function

A central hypothesis of the read-across concept for the ERA of pharmaceuticals is that the higher the evolutionary conservation of human drug targets in other species, the higher the probability that the drug will induce target-mediated effects in those species (Rand-Weaver *et al*., 2013). This hypothesis prompted an intense programme of research that culminated in the development of the databases ECOdrug (Verbruggen *et al*., 2018) and SeqAPASS (LaLone *et al*., 2016) mentioned previously. These novel and centralised knowledge platforms have been important in accelerating the implementation of predictive approaches within a discipline that typically relies on experimental data. However, the interpretation of the biological significance of gene similarity data across species is far from being straightforward. Typically, a given biological target can play numerous functional roles in different cell types or tissues. Most of these functions may be highly similar between humans and other species, such as fish (Colbourne *et al*., 2022). However, others may not. For example, the gene (*n3rc1*) encoding the glucocorticoid receptor (GR) of many species of teleost fish displays a medium degree of similarity (approx. 48%) to the human GR (Verbruggen *et al*., 2018). Despite this

relatively low gene sequence homology, drug-induced GR modulation elicits similar effects in both fish and humans, including the perturbation of the immune system, reproductive function, and glucose metabolism (Margiotta-Casaluci *et al*., 2016; Hamilton *et al*., 2022). This apparent discrepancy is clarified once we consider the conservation of gene homology specifically in functional domains. Indeed, the DNA binding domain and ligand binding domain of the GR protein display, respectively, 98.4% and 85.5% similarity to the human protein (Schaaf, 2008). This example suggests that future emphasis should be focussed on assessing drug target conservation at the level of individual gene domains, rather than merely at the whole gene level (Kruger *et al*., 2012; Nitta *et al*., 2015). In this respect, precise genome editing approaches (e.g. using CRISPR/Cas) can be useful for targeting specific sequences within a given gene with relative ease, and allowing the measurement of the resultant functional impact in the species of interest (Cornet *et al*., 2018; Kroll *et al*, 2021; Winter *et al*. 2022). The progressive knockout of different coding regions could also help to determine the functionally important parts of a given gene where this is not known (Wright and Sanjana, 2016; Klann *et al*., 2017), a process that has been rendered achievable through the advent of CRISPR-mediated approaches. Given the rapid growth of genetic screening and functional genomics applied to drug discovery (Gianni and Farrow, 2020), this knowledge may also be extrapolated from existing mammalian data without the need for further experimental determination. An ecotoxicology application of this approach could focus on a priority set of drug targets, such as those most likely to be frequently modulated by pharmaceuticals in the environment.

The interpretation of the functional implications of a given-drug target interaction is challenged further by a duplication event that occurred in the genome of teleost fish 350-400 million years ago (Glasauer and Neuhauss, 2014), and by interspecies differences in resultant protein function. For example, many teleost species possess 2 GRs (GR1 and GR2) due to gene duplication, and the distribution of ligand binding affinity values to the various isoforms in different species varies greatly (Hamilton *et al*., 2022). This of course has important implications for the assessment of functional conservation across species. Furthermore, the GR plays an important additional osmoregulatory function in fish (but not in humans) (Bury *et al*., 2003; Prunet *et al*., 2006), which may directly affect the risk profile of GR modulating chemicals in aquatic species. This aspect of read-across, however, can only be addressed on a case by case or, rather, species by species basis and this requires expertise that are not widely available. This last point

raises the importance of the need for appropriate training of our next generation of comparative physiologists, pharmacologists and toxicologists to equip them with the knowledge to interpret the potential effects of chemical exposure between, often rather disparate, groups of species of relevance to ERA.

In all, these examples suggest that the study and classification of target functions across species is the next big step needed in order to enhance the predictive power of precision toxicology frameworks (Priority research question 1 - Table 2). For example, such functional information could be used to guide the development of robust, quantitative AOPs and AOP networks (Margiotta-Casaluci *et al*., 2016; Spinu *et al*., 2020) that, in turn, may inform the development of 3Rs-driven testing strategies to identify biological biomarkers that could be used for eco-pharmacovigilance. One important challenge to this vision is that the mapping of drug target functions across species would require significant resources, and in practical terms, a full mapping may be unachievable. However, a step-by-step strategy may be used to overcome this challenge. Firstly, a large volume of knowledge has already been generated over decades of comparative physiological research. We suggest that future research efforts should be focused on the extraction and classification of such information (e.g. with the help of text-mining tools supported by Artificial Intelligence) within informatic databases designed to support evidence synthesis (Lee *et al*, 2020; Brooks *et al*., 2021) (Priority research question 2 - Table 2). This ambitious work has already been initiated by ongoing international activities, such as the MONARCH Initiative (https://monarchinitiative.org/), which is an integrative data and analytic platform that extracts and synthesises all available evidence to connect phenotypes to genotypes across species (Shefchek *et al*., 2020). In addition to this data mining approach, modern genetic modification techniques coupled with advanced imaging approaches offer an exciting opportunity to shed light on the functional role of drug targets in fish and other species of environmental relevance. This approach can be applied to cells or directly *in vivo*, for example using non-protected zebrafish larvae and is already being applied for assessing drug safety and efficacy for human health purposes. For example, we have used a combination of *in vivo* imaging and CRISPR/Cas9-gene editing in larval zebrafish to assess the impact of risk gene knockout on cardiovascular function (Winter *et al*., 2022). Using a pan neuronal Ca^{2+} sensing transgenic zebrafish and light sheet microscopy we have also profiled the effect of 57 CNS active drugs/chemicals on brain activity in non-protected larval zebrafish (Winter *et al*., 2021). Using these types of approaches, gene-phenotype relationships

can be established for virtually any desired organs/system, and, as the second study shows, the number of compounds that can be realistically assessed is typically much greater than that using traditional ecotoxicological testing paradigms. Higher throughput and higher content approaches such as this also offer advantages in terms of low compound requirements, higher statistical power, along with equal suitability for method standardisation. Most importantly, in certain contexts such imaging-based approaches provide greater opportunities for the identification of specific mode-of-action relevant effects that can help to establish a quantitative relationship between target modulation and adverse phenotype (Priority research question 3 – Table 2). For example, the ability to link alterations in drug-induced neuronal activity with specific brain regions known to be target-rich (Winter *et al*., 2021) suggests that a large-scale application of functional assessment and gene editing approaches may allow highly granular mapping of gene function in fish, using 3Rs-friendly methods.

Higher throughput ecotoxicological testing is also being aided by the availability of automation systems which were previously the preserve of pharmaceutical company *in vitro* drug screening facilities. These include wider application of high content imaging systems (Green *et al*., 2019; Westhoff *et al*., 2020; Lempereur *et al*., 2022), microfluidics for animal manipulation and exposure (Pulak 2016), and advanced image analysis scripts for processing the large amounts of data generated by such platforms (e.g. Caicedo *et al*., 2017; Otterstrom *et al*., 2022). The data generated using these approaches could then be used to build and train models to predict the potential impact of functional conservation, without the need for costly animal testing at all. Of course, caution still needs to be applied with regards to the interpretation of such data in the context of its potential relevance for the ERA process. Specifically, it would be essential to consider the quantitative relationship between novel functional data (e.g. cardiovascular parameters) and the apical functions typically considered by the ERA framework (i.e. development, growth, reproduction), which is centred on the protection of population dynamics rather than on the protection of individuals (as in the case of human risk assessment) (Priority research question 3 - Table 2). Whilst the use of these techniques can help us to reveal the potential for drug-target interactions in environmentally relevant species, understanding the wider ecological consequences of such effects is crucial in order to use these data appropriately in the refinement of ERAs. This is perhaps where the more research into the integration of approaches such as

functional brain imaging, alongside more directly ecologically relevant holistic markers of effect (e.g. behaviour) may prove especially fruitful (Bertram *et al*., 2022).

Large-scale species extrapolation of experimental data using modelling approaches

One of the major sources of uncertainty during the cross-species extrapolation process is the limited availability of species-specific ADME parameters that would allow the accurate prediction of drug concentrations within target tissues (e.g., via PBPK modelling) and, in turn, the assessment of hazard and risk associated with organ-specific target engagement dynamics. This translational challenge is exacerbated by the fact that drug exposure and uptake routes for wildlife (e.g., uptake via gills and skin, ingestion via food, etc.) are generally different from the administration routes typically used in most pre-clinical and clinical studies (e.g., oral administration of a capsule/tablet, or injection). As discussed above, recent research has led to the development of novel *in vitro* models to characterise drug uptake and ADME in a small number of fish species, for example rainbow trout and zebrafish. Refining, validating, and scaling up those models should be considered a research priority. For example, high-throughput human *in vitro* ADME (HT-ADME) screening has become an important step in drug discovery, and the large volume of data generated with these approaches is guiding the development of AI-powered *in silico* tools for the prediction of ADME profiles (Davies *et al*., 2020; Goller *et al*., 2020). In line with these advancements, we foresee that medium or HT-ADME screenings could become a reality for fish species too, eliminating the need to rely on animal tests for this purpose. Furthermore, mass-spectrometry (MS)-imaging and high-sensitivity-MS applied to both whole zebrafish larvae or adult fish organs would allow the quantification, with high precision, of drug concentrations and distribution within specific tissues/organs. This, in turn, would shed light on fish-specific *in vivo* ADME that may be difficult to predict via *in silico* or *in vitro* methods alone (Assian *et al*., 2021). Such MS-based approaches could also be used to detect changes in protein expression and more precisely reveal the distribution of a given drug target (Lombard-Banek *et al*., 2016; Brunner *et al*., 2022). These approaches have already started to be applied to ecotoxicologically relevant species groups such as daphnids and zebrafish (Schirmer *et al*., 2022; Perez *et al*., 2017) achieving relatively high levels of spatial resolution. Integrating these techniques with those discussed for the investigation of target function (e.g. *in vivo* imaging and gene editing) could prove incredibly powerful in identifying tissue specific drug concentrations needed to trigger phenotypically observable target-

mediated effects. This, in turn, could be used to inform the development of more predictive computational models. However, with more than 34,000 teleost species identified to date (of which about 50% live in freshwater; Froese and Pauly, 2019), achieving satisfactory taxonomic coverage using experimental tools is likely unrealistic. This challenge becomes even more problematic if we consider the millions of invertebrates and terrestrial species that could also be exposed to pharmaceuticals in the environment.

In parallel with the experimental approach focused on a small number of species, modelling methods should, therefore, play a major role in the extrapolation of ADME profiles across species (Davies *et al*., 2020) (Priority research question 4 - Table 2). For example, Bayesian-PBPK modelling (Krauss and Schuppert, 2016), multitask deep neural network models (Wenzel *et al*., 2019), and population-based ADME simulators (Wedagedera *et al*., 2022) are just a few examples of advanced modelling methods currently used to predict human PK profiles and estimate their variability within human populations. Here we foresee that comparable modelling concepts could be applied to simulate the variability of uptake and ADME data across species for ecotoxicological purposes, either from a few fish species to many, or from humans to non-humans. A major obstacle to this predictive approach, however, is the limited knowledge of the key mechanistic and functional processes that determine uptake and ADME in wildlife species. Hence, any future research in this field cannot ultimately advance without an investment in fundamental biological research. This mechanistic-comparative effort should leverage the rapid advancements achieved in genome sequencing, with genome assemblies available for 3,278 species distributed across 24 different phyla and 64 classes (of which 684 ray-finned fish species) (Hotaling *et al*., 2021). This vast amount of data could be used to drive the assessment of evolutionary and functional conservation of key molecular drivers of ADME processes across species. In turn, this knowledge could be integrated in existing databases (e.g., ECODrug) to guide the cross-species extrapolation of both PD and PK.

From a PD perspective, chemical-induced -omics signatures generated in a standardised manner using cell lines from a wide array of species may support comparative toxicology evaluations, including the assessment of a drug's modes of action. In the biomedical field, large scale transcriptomic and proteomic signatures generated for thousands of genetic and chemical perturbagens (including pharmaceuticals) across many different human cell lines have unlocked novel

opportunities to expand our understanding of the mode of action of marketed pharmaceuticals, supporting, for example, drug repurposing efforts (Subramanian et al., 2017). It possible to envisage that the expansion of such approaches to wildlife-derived cell lines may enable mode of action concordance studies and hazard assessment across a wide set of more relevant species groups. Recent initiatives have started to explore this avenue. For example, the EcoToxChip project is aiming at generating PCR arrays for wildlife (including fish, amphibians, and birds) designed to assess the effects of chemicals on 384 genes of ecotoxicological relevance (Basu et al., 2019). It is important to highlight that experimental reproducibility, inter-cell type response variability, biological coverage, phenotypic and *in vivo* relevance, cell metabolic competence, and data interpretation within a regulatory framework remain important challenges to overcome for the robust application of -omics signatures for chemical safety assessment within a regulatory decision-making context. Nonetheless, embedding NAMs-based -omics approaches within a wider weight of evidence framework may provide a valuable resource to address the challenge of predicting drug-induced effects across species.

Overcoming barriers for regulatory acceptance

Governments have evolved different chemical regulation frameworks over many years. Essentially, inherent hazard is balanced against risk to reach a point of protection for human health and the environment. Risk assessors use a wide range of information on chemicals in their evaluations, ranging from the knowledge of molecular structure to the results of complex *in vivo* studies. The risk assessors then inform the risk owners (e.g. governments), and chemicals are either permitted (with or without additional restrictions), or declined. This demarcation between risk assessor and risk owner is critical as it is at the heart of the barrier for regulatory acceptance. Risk assessors are scientific experts and have the appropriate skill sets to reach a conclusion on the assessment, while the risk owner is unlikely to be an individual, but rather public and governmental authorities acting on behalf of an elected government that accept the assessor's judgement on behalf of society. In this context, key barriers to the acceptance of novel hazard and risk assessment methods (e.g. New Approach Methodologies, NAMs) concern the confidence, transparency, and domain of applicability of such methods, so that the risk assessor can assure the risk owners of the safety of a chemical. That threshold for assurance is THE barrier to regulatory acceptance. If an

assessor has limited confidence in the data presented, then they cannot recommend acceptance to the risk owner.

In this context, cross-species extrapolation frameworks should be seen as a valuable strategy to support the overall evidence synthesis and weight of evidence exercise underlying the safety assessment of chemicals as well as the development of ecotoxicology-relevant NAMs with high potential for regulatory acceptance. For example, comparative pharmacology and toxicology knowledge could be used to identify human *in vitro* assays/endpoints that can be readily extrapolated to fish without the need to develop fish-specific assays or, on the other hand, identify toxicity endpoints for which the development of fish-specific NAMs is essential (Priority research question 5 - Table 2). It is important to note that the considerations discussed in the present article are specifically referred to pharmaceuticals; however, they can be adapted and applied to any chemical class, including pesticides and personal care products.

A critical action needed to bring this vision to life is the expansion and integration of databases explicitly designed to facilitate the synthesis of comparative biology data relevant for chemical risk assessment (Priority research question 2 - Table 2). Novel AI-powered text mining tools could play a major role for the practical implementation of this concept. For example, the MONARCH Initiative Database (Shefchek *et al*., 2020) has developed a tailored data analytics pipeline to extract, annotate, and integrate a wide range of gene-to-phenotype data across species, which are presented using a user-friendly interface. Other databases have also been established with an explicit comparative vision (e.g. Comparative Toxicogenomics Database; ECOTOX Knowledgebase) (Davis *et al*., 2022; Olker et al., 2022), while others are expanding very rapidly and effectively the integration of multiple relevant cross-species data types (e.g., ChEMBL, US EPA CompToxChemical Dashboard). Although some expert judgement is still needed to evaluate the toxicological relevance of inter-species data and its practical implications, these examples highlight that a tremendous volume of comparative biology knowledge is already available to any user willing to implement cross-species extrapolation as part of their ERA of pharmaceuticals. Nonetheless, future research should certainly focus on increasing the effectiveness and integration of available databases with the explicit aim of supporting future chemical ERA. Another prerequisite for progress is the training of the next generation of experts with the right skill set needed to lead comparative pharmacology and toxicology research and its translation

into decision making and/or regulatory applications. Hence, here we call for an active and synergistic involvement of academia, governmental agencies, regulators, and industry to support and strengthen comparative biology and toxicology education at global level.

Conclusions

One of the most important observations that can be made concerning the last 10 years of research in the cross-species extrapolation field – applied to the ecotoxicology of pharmaceuticals – is that the tremendous advancements achieved to date have emerged thanks to the synergistic partnership between industry, government agencies, and academia. Here we propose that this synergy should be expanded further if we want to achieve our ambitious global protection goals for all species, and not just a few. In line with our position, LaLone *et al*. (2021) have called for the formation of a global cross-sector collaborative consortium aimed to advance the development and implementation of cross-species extrapolation methods in regulatory toxicology. The authors have led the formation and launch of a new "International Consortium to Advance Cross-Species Extrapolation in Regulation (ICACSER; https://www.setac.org/page/scixspecies)". Here we welcome and support this initiative, and we encourage any interested reader to join the ICACSER consortium. Cross-species extrapolation is also at the heart of a new large research project, funded by the European Commission, called Precision Toxicology. This project, started in 2021, will explore the concept of "PhyloToxicology", which aims at replacing traditional animal testing with an "evolutionarily diverse model suite of organisms from multiple branches of the tree of life". Although many research questions remain open, the progress obtained in the last 10 or so years, and the rejuvenated global interest and initiatives in comparative toxicology suggest that cross-species extrapolation research will remain at the centre of future ecotoxicological research. Implementing this vision in regulatory toxicology would enable us to achieve the true global protection of human and environmental health.

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Graphical Abstract. Enhancing chemical safety assessment with high-precision ecotoxicology predictions.

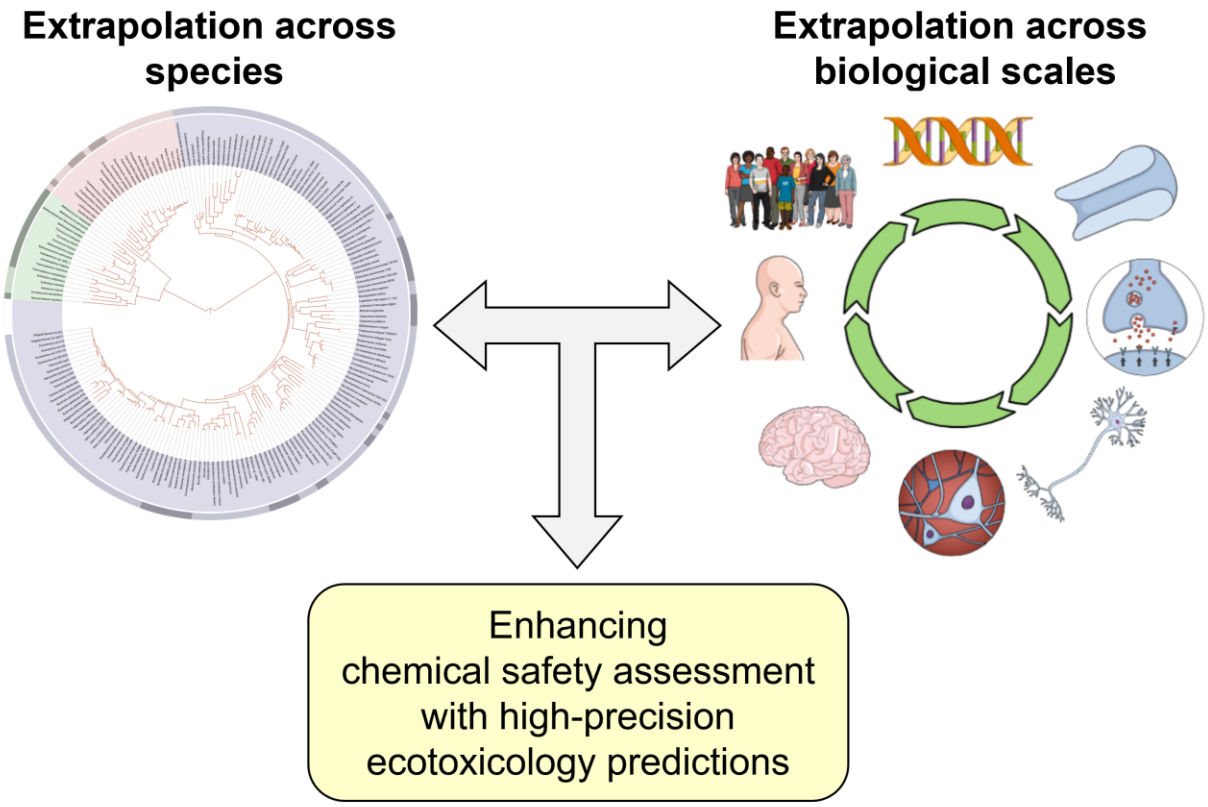

***Table 1**. Examples of experimental studies carried out using (wild type) fish models highlighting the potential wider translational value of mammalian data generated during the drug discovery and development process.*

Target system	Study	Species	Drug	MoA-relevant endpoints
Central Nervous system	Valenti *et al*. (2012)	Fathead minnow (*Pimephales promelas*)	Sertraline	• Plasma drug concentration • SERT binding • Behaviour
Central Nervous system	Margiotta-Casaluci *et al*. (2014)	Fathead minnow (*Pimephales promelas*)	Fluoxetine	• Plasma drug concentration • Drug metabolism • Behaviour
Central Nervous system	Huerta *et al*. (2016)	Fathead minnow (*Pimephales promelas*)	Oxazepam	• Drug concentration in plasma, brain, liver, muscle • Behaviour
Central Nervous system	Tanoue *et al*. (2017) Tanoue *et al*. (2019)	Fathead minnow (*Pimephales promelas*)	Tramadol	• Drug concentration in plasma & brain • Drug metabolism • Behaviour
Central Nervous system	Winter *et al*. (2008a)	Zebrafish *(Danio rerio)*	25 compounds	• Seizure-like behaviour • Maximum tolerated concentration
Central Nervous system	Winter *et al*. (2021)	Zebrafish *(Danio rerio)*	57 compounds	• Whole body drug concentration • Behaviour • Brain activity
Cardiovascular system	Milan *et al*. (2003)	Zebrafish *(Danio rerio)*	100 compounds	• Heart rate
Cardiovascular system	Parker *et al*. (2014)	Zebrafish *(Danio rerio)*	• Adrenaline • Cisapride • Haloperidol • Terfenadine • Theophylline • Verapamil	• Whole body drug concentration • Maximum tolerated concentration • Heart rate • Atrium:ventriculum beat ratio • Stroke volume • Blood flow • Blood vessel diameter
Cardiovascular system	Margiotta-Casaluci *et al*. (2019)	Zebrafish *(Danio rerio)*	• Captopril • Losartan • Propranolol	• Maximum tolerated concentration • Heart rate • Atrium:ventriculum beat ratio • Stroke volume • Blood flow • Blood vessel diameter
Reproductive system	Runnalls *et al*., 2013	Fathead minnow (*Pimephales promelas*)	• Desogestrel, • Drospirenone, • Gestodene • Levonorgestrel	• Reproductive activity • Egg production • Breeding frequency • SSCs

Reproductive system	Margiotta-Casaluci *et al.*, 2013	Fathead minnow (*Pimephales promelas*)	Dutasteride	• Reproductive activity • Egg production • Breeding frequency • SSCs • Plasma E2, T, 11-KT • Gonad histopathology • Sperm quality
Reproductive system	Runnalls *et al.*, 2015	Fathead minnow (*Pimephales promelas*)	• Ethinylestradiol • Levonorgestrel	• Plasma drug concentration • Reproductive activity • Egg production • Breeding frequency • Plasma E2 and 11-KT • SSCs
Examples of experimental studies carried out using (wild type) fish models highlighting the potential wider translational value of mammalian data generated during the drug discovery and development process.				
Reproductive system Respiratory system Development	LaLone *et al.* (2011)	Fathead minnow (*Pimephales promelas*)	• Dexamethasone	• Growth • Reproductive activity • Egg production • Breeding frequency • F1 hatching rate • *Ex vivo* gonad T and E2 production • SSCs • Gill histopathology • Expression of MoA-relevant genes
Immune system Metabolism	Kugathas & Sumpter (2011)	Fathead minnow (*Pimephales promelas*) Rainbow trout *(Oncorhynchus mykiss)*	• 10 synthetic GCs (*in vitro*) • Beclomethasone dipropionate • Prednisolone	• Target transactivation • Plasma glucose • Blood leukocyte count
Immune system Metabolism HPI axis	Kugathas *et al.*, 2013	Fathead minnow (*Pimephales promelas*)	• Becloemthasone dipropionate	• Plasma cortisol • Plasma glucose • PEPCK and GR gene expression • Blood leukocyte & thrombocyte count • SSCs
Immune system Metabolism Secondary pharmacology	Margiotta-Casaluci *et al.* (2016)	Fathead minnow (*Pimephales promelas*)	• Becloemthasone dipropionate	• Drug metabolism & plasma drug concentration • PEPCK and GR gene expression • Blood glucose • Leukocyte sub-populations response • AR gene expression • SSCs
Immune system Renal system GI system	Mehinto *et al.* (2010)	Rainbow trout *(Oncorhynchus mykiss)*	Diclofenac	• Drug metabolism & bile drug concentration • ptgs1 and ptgs2 gene expression • Kidney, liver, intestine histopathology

Immune system	Patel *et al*. (2016)	Fathead minnow (*Pimephales promelas*)	Ibuprofen	• Plasma drug concentration • Prostaglandin E • ptgs1 and ptgs2 gene expression

***Abbreviations**: E2 = estradiol; 11-KT = 11 keto-testosterone; MoA = Mode of Action; SERT = Serotonin Transporter; SSCs = secondary sexual characteristics; T = testosterone; GI = gastrointestinal; GR = glucocorticoid receptor; HPI = hypothalamic-pituitary-interrenal; PEPCK = phosphoenolpyruvate carboxykinase; ptgs = prostaglandin-endoperoxide synthase.*

***Table 2**. Priority research questions to advance the application of comparative pharmacology and toxicology methods to guide the environmental safety assessment of pharmaceuticals.*

Question 1 In the last 10 years, we have successfully characterised the evolutionary conservation of the genes coding almost all drug targets in many non-target species. Can we generate a similar knowledge map for the functional conservation of drug targets?
Question 2 Comparative biology knowledge is currently scattered across many different sources. What is the best way to extract that knowledge and make it usable for environmental toxicology applications?
Question 3 Cross-species extrapolation approaches can generate highly granular predictions at sub-apical level. On the other hand, regulatory decision making is only interested in apical adverse effects. What is the quantitative relationship between sub-apical adverse endpoints and apical effects across species?
Question 4 Can we use modelling approaches to overcome the experimental (and resource) limitations of using model laboratory organisms?
Question 5 Can we develop a scalable battery of NAMs for the characterisation of uptake, PK, and PD of chemicals in fish species?

***Abbreviations**: NAMs = New Approach Methodologies; PK = pharmacokinetics; PD = pharmacodynamics.*