Development of the fish invitrome for animal-free environmental risk assessment of chemicals

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Funding statement: This work was supported by the National Research Programme "Advancing 3R – Animals, Research and Society" (NRP 79) of the Swiss National Science Foundation (SNSF), project number 407940_206439; the Lead Agency Project Grant jointly funded by the Slovenian Research Agency (ARIS) and the Swiss National Science Foundation (SNSF) (project numbers J4-3097 and 205321_197327, respectively); and the CRACK IT Challenge "SAFE" (project number NC/C021203/01).

Conflict of interest: The authors declare no conflict of interest.

Author Contribution Statement:
Marion Revel: Data curation; Writing–original draft; Writing–review & editing.

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Sven Lukas Mosimann: Writing-review & editing

Colette vom Berg: Conceptualization; Writing–review & editing; Funding Acquisition.
Anze Zupanic: Conceptualization; Writing–review & editing; Funding Acquisition.
Bernhard Truffer: Conceptualization; Writing–review & editing; Funding Acquisition.
Kristin Schirmer: Conceptualization; Data curation; Writing–review & editing; Funding Acquisition

Data availability: No additional data was used apart from data presented in the article.

Revel et al.

Environmental Toxicology

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Abstract: Given the need to reduce animal testing for environmental risk assessment, we aim to develop a fish invitrome, an alternative fish modular framework capable of predicting chemical toxicity in fish without the use of animals. The central module of the framework is the validated RTgill-W1 cell line assay that predicts fish acute toxicity of chemicals (Organization for Economic Cooperation and Development Test Guideline (OECD TG) 249). Expanding towards prediction of chronic toxicity, the fish invitrome includes two other well-advanced modules for chemical bioaccumulation/biotransformation and inhibition of fish growth. This framework is expected to continuously evolve with the development of modules that predict, for instance, neurotoxicity and reproductive toxicity. We envisage the fish invitrome framework to become

part of the broader academic field of New Approach Methodologies (NAMs), where it will remain flexible and open to integration of new developments from research groups around the world. To accelerate the development and uptake of this framework, we strive for transdisciplinarity, integrating both natural and social sciences, along with broader stakeholder interactions. A stepwise socio-technical approach has been chosen, where mainstreaming the fish invitrome involves progressive adoption across various ecotoxicological contexts. The framework will be co-designed with stakeholders from academia, industry, and regulatory bodies. Rather than aiming for immediate regulatory acceptance, this approach aims to build trust and familiarity with fish cell line-based testing among stakeholders. By doing so, it encourages broader use of the framework in practical applications while gradually overcoming institutional, cultural, and technical barriers. Additionally, establishing a clear roadmap for mainstreaming the fish invitrome will help identify and address challenges to its uptake, ensuring a smoother transition to non-organismal testing methodologies.

Keywords: new approach methodologies (NAMs), rainbow trout cell lines, toxicity testing, socio-technical approach, co-design with stakeholders

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1 Introduction

Modern society heavily depends on chemical industry, with more than 350,000 chemicals registered on the global market (Wang et al., 2020). Aquatic environments often become a sink for chemicals (Bertram et al., 2022), which may cause significant impact on these ecosystems and the biodiversity within them (Groh et al., 2022). In this context, it is crucial to elaborate effective risk assessment procedures to prevent unwanted chemical effects. Fish play a vital role in aquatic ecosystems and are an important food source for humans, linking environmental and

human health. Therefore, assessing the risk of chemicals to the aquatic environment involves evaluating effects on fish population health, using, e.g., survival, growth, and reproduction as proxies. However, traditional testing methods use large numbers of fish each year and are very resource demanding, raising ethical, practical and economic concerns.

Against this background, the past decades have seen varied efforts to develop alternatives to traditional animal-based testing, lately referred to as New Approach Methodologies (NAMs; ECHA-17/R/24/EN), including NAMs to replace testing with fish. These entail quantitative structure activity relationships (QSARs) (Khan et al., 2019; Toropov et al., 2020), machine learning models (Gasser et al., 2024) as well as bioassay-based alternatives, namely the fish embryo acute toxicity test (FET; Organization for Economic Cooperation and Development Test Guideline (OECD TG) 236; OECD (2013b)) and the fish cell line acute toxicity test using the rainbow trout (*Oncorhynchus mykiss*) gill cell line, RTgill-W1 (OECD TG249; OECD (2021)). To strengthen confidence in the predictions provided by these NAMs, there are proposals to combine them in a defined approach (DA), to provide an objective output, or in an integrated approach to testing and assessment (IATA), which may also rely on expert judgment.

The advances described above provide proof that alternatives to animal testing with fish can indeed be established. While the focus thus far has largely been on acute fish toxicity, it is now time to lay out strategies that might also allow for the prediction of long-term, i.e., chronic effects of chemicals on fish. In this context, we here propose the framework of the fish invitrome for environmental risk assessment of chemicals. As defined by Bols et al. (2017), an invitrome is a group of cell lines, derived from a specific organism or linked by a common theme, such as their tissue of origin, specific functions or application cases – here environmental risk assessment. This article, therefore, aims to review the origin of the fish invitrome concept,

outline the information that the proposed framework should provide to support current risk assessment schemes and discuss the current research that seeks to establish this framework. We will close by emphasizing the need for stakeholders in academia, industry and regulation to co-design this framework to facilitate its uptake.

2 Established conventional and alternative tests for fish toxicity

To study chemical effects on fish, a wide range of *in vivo* bioassays has been developed and regularly used, following established guidelines, such as OECD TG. They aim to capture impacts on fish that are of relevance to fish population health, such as bioaccumulation, survival, growth and reproduction. Alternative methods should strive to provide a comparable information value with regard to chemical toxicity, e.g., to inform about the extent of bioaccumulation or effective concentrations causing reduced survival or growth.

2.1. Conventional tests

Chemical as well as water sample testing with *in vivo* bioassays leads to the sacrifice of many animals, which, in the case of fish, was estimated to be around three million juvenile and adult fish per year accounting for reports from Europe, the United States and Canada (Scholz et al., 2013). However, animal numbers used for chemical testing are often recorded as total numbers, without further information on their specific applications. Burden et al. (2020) published the number of OECD TG studies performed in 15 contract research organizations between 2014 and 2017 (Figure 1). This analysis highlighted the three most frequently performed tests on fish: the fish acute toxicity test (OECD TG 203; OECD (2019)), which is used for chemical testing but also water quality assessment (Paparella, 2020); the Fish Early-life Stage Toxicity Test (FELS-Test; OECD TG 210; OECD (2013a)), which assesses survival but

also hatching, behavior and growth; and Bioaccumulation in Fish (OECD TG 305; OECD (2012)) to provide information on the chemical's behavior and effects in the organism.

Though the three assays mentioned above dominate the testing landscape, a much wider range of OECD TG is available to study chemical toxicity to fish. The difference between these tests lies in the exposure time, the age of the organism, and the combination of endpoints measured. Table 1 provides a summary of the endpoints assessed by each OECD TG for fish in the context of chemical risk assessment, based on information available in OECD documents. Mortality (respectively survival) is recorded in all tests while growth is considered in five out of eight chronic toxicity assays. Appearance and behavioral abnormalities are commonly used to better interpret mortality data or to gain further information on the mode of action of the chemicals. Several endpoints are measured to study the reproduction of fish (e.g. secondary sexual characteristics, biomarkers such as vitellogenin and androgenic activity, and reproductive fitness). These endpoints give further information on the mode of action of the chemical, by revealing its impact on endocrine and reproductive functions. Table 1 also reveals some redundancy between the tests. For instance, while OECD TG 210 and OECD TG 212 (OECD, 1998) differ in duration and the life stage of the fish assessed, they both measure similar outcomes of chemical exposure. Overall, these *in vivo* fish tests are highly resource-demanding (infrastructure, time, personnel), ethically concerning, and scientifically limited, as they view fish as 'black boxes' without clarifying the molecular pathways and mechanisms underlying the apical endpoints measured (Lillicrap et al., 2016; Scholz et al., 2013).

2.2. Alternative biological tests

A total of four OECD validated NAMs exist currently: the already mentioned acute toxicity tests using fish embryos (OECD TG 236) or the RTgill-W1 fish cell line (OECD TG

249); and the fish (cryopreserved) hepatocyte (OECD TG 319a; OECD (2018a)) or liver S9 fraction (OECD TG 319b; OECD (2018b)) to analyze chemical depletion for calculation of bioconcentration factors. While all of them can be considered NAMs, the RTgill-W1 cell line assay is the only one no longer requiring fish sacrifice at any stage. This is the motivation for using fish cell lines and the basis for proposing the fish invitrome framework for environmental risk assessment of chemicals.

3 Developing the first module of the fish invitrome

We define the fish invitrome as a modular framework that employs fish cell lines as alternatives to animal testing for chemical risk assessment. The initial module predicts chemical acute toxicity to fish using the RTgill-W1 cell line.

3.1. Fish cell lines as models

A cell line is a population of cells from a multicellular organism that can be propagated outside the organism (*in vitro*) through a serial transfer of cells from one culture vessel to another (Schaffer & Terminology Committee Chair Tissue Culture, 1990). With cells being the smallest units of life, one can expect that changes which start in cells can result in noticeable effects in tissues and entire organisms (Schirmer, 2006). Therefore, cellular responses can be considered as proxies for toxicological manifestations that might be extrapolatable to *in vivo* outcomes.

Mammalian cells have long been used in biomedical and environmental research (Verma et al., 2020). However, for fish-related research, fish cell lines are preferable because each cell line has unique characteristics that make them better suited to study species-specific physiology and responses to chemicals. Many fish cell lines immortalize spontaneously, meaning that they can multiply indefinitely without becoming cancerous or needing genetic manipulation, in

contrast to mammalian cells that often undergo senescence or come from a cancer background (Bols et al., 2005; Schirmer, 2006).

The use of fish cell lines as models to study fish physiology, nutrition or toxicology is continuously increasing (Goswami et al., 2022; Nguyen et al., 2024). This growing interest is reflected in the extensive database maintained by Cellosaurus, a Switzerland-based knowledge resource on cell lines, which now includes over 940 fish cell lines (Bairoch, 2018) from approximately 210 species (Cellosaurus v.50, https://www.cellosaurus.org/). These cell lines were isolated not only from different species, but also from various organs (e.g. gill, liver, gut, brain), allowing for the development of diverse fish-specific cell line models that reflect the different target sites of chemicals.

3.2. The RTgill-W1 cell line assay as a starting point for fish invitrome

Tremendous progress toward the aim of predicting fish toxicity with cell lines has already been made over the past decades with a focus on substituting specific regulatory toxicity tests. The most advanced of these developments is the RTgill-W1 cell line assay for acute fish toxicity. The RTgill-W1 cell line was derived from gill lamellae of rainbow trout (*Oncorhynchus mykiss*) (Bols et al., 1994), the primary site of waterborne contaminant uptake in fish. The assay measures the impact of chemicals on cell viability through indicator dyes revealing cytoplasmic membrane damage, metabolic activity impairment, and lysosomal integrity. The assay conditions were optimized by using a simple buffer, L-15/ex, where protein components were eliminated to improve bioavailability of test chemicals. Dosing methods were also optimized and procedures to measure actual exposure concentrations were introduced. These developments allowed the RTgill-W1 assay to show a very good correlation between *in vitro* and *in vivo* acute toxicity data for ~70 organic chemicals with diverse toxicity profiles (Fischer et al., 2019; Natsch et al., 2018;

Tanneberger et al., 2013). By rational design and rigorous validation, including an international round-robin study (Fischer et al., 2019; Natsch et al., 2018; Tanneberger et al., 2013), the RTgill-W1 assay has reached global adoption as an International Organization for Standardization (ISO) standard (ISO21115; ISO (2019)) with a focus on water sample testing, and as the first fish cell line-based OECD TG in ecotoxicology (OECD TG 249) for chemical toxicity testing. Considering that almost all fish-based OECD TG include the assessment of survival/mortality (Table 1), we view this assay as the first module of the fish invitrome framework (Figure 2).

4 Expanding the options: Other advanced modules

The successful demonstration that the RTgill-W1 assay could predict chemical acute toxicity in fish paved the way to the expansion of the fish invitrome framework. Below we discuss additional modules that were developed using various fish cell lines and methods to assess a broader range of chemical effects on fish.

4.1. Acute toxicity in other tissues

While the OECD TG 249 effectively predicts acute toxicity under waterborne conditions, application to other exposure scenarios, such as for dietary exposure, calls for using other cell lines. Indeed, the assay procedure can be adapted to include alternative cell lines, such as RTgutGC, derived from rainbow trout intestine (Kawano et al., 2011; Schug et al., 2020). The intestine encounters more hydrophobic chemicals via food uptake, making it a critical site for biotransformation and potential toxicity of such compounds. In exposing this cell line to similar chemical groups and in the same exposure medium (L-15/ex), the acute toxicity was demonstrated to align closely with the one observed with the RTgill-W1 cell line assay (Schug et al., 2020). These bioassays also allow for the determination of lowest-observed effects or of non-toxic concentrations (Stadnicka-Michalak, Knöbel, et al., 2018) and thus can serve as a range

finder for "downstream" studies focusing on bioconcentration/biotransformation or on sublethal, chronic toxicity.

4.2. Bioaccumulation/biotransformation

Bioaccumulation is a critical endpoint commonly measured for risk assessment, typically expressed as the bioconcentration factor (BCF) or the biomagnification factor (BMF) for dietary uptake. Computational models (e.g. QSAR) are often employed to predict these factors, reducing the need for *in vivo* bioassays, such as the OECD TG 305. However, these models tend to overestimate bioaccumulation compared to *in vivo* data as they do not account for biotransformation processes (Laue et al., 2020).

Significant progress has been made in developing *in vitro* assays to predict biotransformation in fish using the OECD validated methods for freshly isolated and cryopreserved fish hepatocytes or the liver S9 fraction from fish to measure biotransformation rates (OECD TG 319a/b). Through applying *In Vitro-In Vivo* Extrapolations (IVIVE), these biotransformation rates can then be used to provide more accurate estimates of bioaccumulation in fish. Although these *in vitro* methods still require the sacrifice of fish, they offer a significant reduction in animal use compared to *in vivo* test methods. Once isolated, S9 fractions and freshly isolated hepatocytes offer fast, high-throughput analysis of biotransformation kinetics within few hours. In contrast, permanent fish cell lines, such as those derived from liver cells (e.g., RTL-W1), can be used to study bioconcentration without animal use and for extended exposure durations, which can be helpful for, e.g., slowly transformed chemicals (Balk et al., 2023). The possibility to estimate BCF in fish cell lines has been demonstrated: here, cellular concentrations were measured and IVIVE procedures applied to estimate a cell-based BCF. To date, the bioaccumulation potential of chemicals in fish has been predicted using three different

permanent cell lines: RTgillW1 and RTgutGC, representing primary sites for environment-epithelial barrier interactions, and RTL-W1 derived from liver, a major biotransformation site (Lee et al., 1993). *In Vitro-In Vivo* Extrapolations has been applied in two different ways – by (i) calculating a BCF based on the ratio of cell-internal concentration to exposure medium concentration (Balk et al., 2023; Balk et al., 2024) or by (ii) inputting the *in vitro*-derived depletion rate constants into a (physiology-based) toxicokinetic model (Stadnicka-Michalak et al., 2014; Stadnicka-Michalak, Weiss, et al., 2018). The latter study specifically showed that the combination of cell lines led to better prediction of the *in vivo*-based BCF for the applied test chemical compared to using only a single cell line, highlighting the importance of considering different fish organs when studying bioaccumulation.

4.3. Effects on growth - cell proliferation assay

Chronic toxicity testing, which involves prolonged exposure, is more complex than acute toxicity testing. As well, it requires an even larger number of fish, highlighting the necessity for alternative non-animal testing methods.

Growth is an important endpoint commonly measured when evaluating chronic toxicity (Table 1). Stadnicka-Michalak et al. (2015) offers an alternative method to predict that endpoint by combining an RTgill-W1 cell population growth assay with mechanistic models for IVIVE. Inhibition of cell population growth is being measured under chemical stress and is directly linked to animal weight using the von Bertalanffy growth model. This study quantitatively predicted reduced fish growth based on inhibited cell population growth for two fish species—rainbow trout and fathead minnow—and two non-volatile fungicides—cyproconazole and propiconazole (Stadnicka-Michalak et al., 2015). In addition, two currently unpublished studies

expand this concept to a wider range of structurally and functionally diverse chemicals in a higher throughput set-up.

4.4. Important role of computational models, such as PBTK models, to link modules

Computational models, such as physiology-based toxicokinetic (PBTK) models, can link modules of the fish invitrome (Stadnicka-Michalak & Schirmer, 2019). By considering the chemical distribution across different organs, PBTK models are applicable for an IVIVE procedure in order to predict chemical toxicity and bioaccumulation in fish. For instance, reverse PBTK can convert the internal concentration calculated from RTgill-W1 cell acute toxicity assays into an *in vivo* lethal concentration (LC_{50}). By combining *in vitro*-derived biotransformation rates with the PBTK model, chemical BCF can be predicted in fish. Furthermore, linking *in vitro* growth assay data with the PBTK model enables the prediction of chemical impacts on fish growth, integrating cellular responses into organism-level effects.

5 Molecular mechanisms of toxicity: a pathway to develop further modules

Cell-based assays reviewed above were developed to specifically target selected apical outcomes relevant to current risk assessment procedures, specifically survival, bioconcentration and growth. This research direction allowed for the efficient establishment of useful alternative methods that can now be further developed and applied in product development and risk assessment practices. However, in these assays, similarly to conventional studies with live fish, cells are essentially treated as a black box. Shedding light on the molecular mechanisms underlying the effects of chemicals on cells would aid in the understanding of complex biological phenomena, such as those leading to reduced growth or altered reproduction. This, in turn, could strengthen the practical application of these assays, for example, by providing a

scientific basis for read-across to other chemicals or cell models representing different tissues. It could also increase their predictive power, particularly for the assessment of complex chronic toxicity outcomes with relatively simple cell-based models, where reliance on mechanistic toxicity information and molecular predictors of higher-level outcomes may become a necessity (Rehberger et al., 2018). Here, the adverse outcome pathways (AOP) concept (Ankley et al., 2010; Groh et al., 2015) has put forward a useful mechanistic framework allowing to collect and organize knowledge about the linkages between regulatory-relevant adverse outcomes occurring *in vivo* and the molecular/cellular alterations that precede them (some of which could also be observed *in vitro*). Through establishing these connections, AOPs can help to (i) identify key events leading to chronic toxicity (Groh et al., 2015), (ii) demonstrate their association to respective NAMs that can be used to generate relevant assessment data (Knapen et al., 2020), and (iii) extrapolate from the effects observed *in vitro* to whole-organism contexts (Villeneuve et al., 2021).

Omics methods —such as (epi)genomics, transcriptomics, proteomics, or metabolomics— can deliver large-scale overviews of toxicant-induced molecular changes. Collectively known as toxicogenomics, these technologies are great tools for enhancing mechanistic understanding, and there have been calls for a broad integration of omics into chemical risk assessment schemes, particularly for transcriptomics (Brockmeier et al., 2017; Buesen et al., 2017; Schirmer et al., 2010; Vinken, 2019). In fish cells, molecular analyses have also proved beneficial. For example, immune system-relevant responses to pathogen surrogates were revealed in RTgutGC cells (Schug et al., 2020; Wang et al., 2019). Another approach is high content imaging referred to as cell painting (Nyffeler et al., 2023; Willis et al., 2020).

Such large-scale approaches to chemical risk assessment require advanced concentration-response modelling to derive combined effective concentrations such as Benchmark or Points of Departure Concentrations. When integrated with, e.g., PBTK models, these values can support IVIVE. Further, computational models and approaches, including but not limited to causal networks, Bayesian inference and quantitative AOPs (qAOPs), can be applied to advance analysis of omics data in order to reveal complex molecular pathways involved in specific toxicity outcomes in fish (Li et al., 2020; Li et al., 2021; Mittal et al., 2022; Perkins et al., 2022; Ramšak et al., 2022; Zhang et al., 2018).

Thus, we consider mechanistic toxicity studies to be a great opportunity to extend the fish invitrome framework by either enhancing the existing modules (e.g., growth) or developing completely new modules, e.g., for reproductive toxicity or neurotoxicity (Figure 2). Indeed, fish growth could be better understood by looking at the molecular pathways leading to reduced fish cell population growth after chemical exposure. This approach could potentially allow to discover shared molecular mechanisms involved in growth reduction induced by different chemical groups. Similarly, molecular approaches could enable prediction of the effects of chemicals on fish reproduction. Fish cell lines as such cannot replicate apical endpoints like reproductive fitness and sexual characteristics, but molecular endpoints could be used as proxies for certain reproductive functions or relevant effects. The success of this approach depends on understanding specific pathways critical to growth (e.g. MAPK, mTOR, JAK-STAT) or reproduction (e.g. estrogen, testosterone, or thyroid signaling), along with identifying biomarkers that can serve as early indicators of pathway disruption, which can also be supported by respective AOPs (Brockmeier et al., 2017; Brooks et al., 2024; Song & Villeneuve, 2021; Vinken, 2019).

Similarly, fish cell lines can shed light on the toxicity mechanisms of neurotoxic compounds. Although environmental contaminants are frequently observed to cause neurotoxicity (Busch et al., 2016), development of neurotoxicity-related NAMs for ecotoxicology are much less advanced compared to neurotoxicity NAMs developed in the human-health context (Fritsche et al., 2018; Masjosthusmann et al., 2020), especially what purely cell-based assays are concerned (Fitzgerald et al., 2021; Legradi et al., 2018; Lillicrap et al., 2016). The use of rainbow trout gill cells for the evaluation of chemicals with neurotoxic mechanisms of action is limited because they do not express nervous system-specific targets (Tanneberger et al., 2013). However, permanent fish brain cell lines have been established from several marine and freshwater species (Le et al., 2017; Li et al., 2016; Long et al., 2021), and some have been proposed for use in ecotoxicological assessment of neuroactive substances (Avalos-Soriano et al., 2021; Morcillo et al., 2017). The RTbrain cell line of rainbow trout, introduced by Steinmoeller et al. (2009), has the potential to be developed as the fish invitrome module for neurotoxicity assessment in cold freshwater fish. This would require a thorough characterization of this cell line, including a molecular analysis to define their identity and check for the presence of neurotoxicity-relevant molecular targets and pathways, as well as a structural and functional analysis to assess the presence of synaptic connections and neuronal activity (Fitzgerald et al., 2021).

So far, molecular mechanisms of toxicity *in vitro* have been mainly explored by looking at the mRNA abundance. mRNA is used as a proxy for the corresponding protein abundance because it is the proteins that are directly involved in many cellular functions that ultimately define the phenotype of an organism (Liang et al., 2020). However, mRNA and protein abundance do not always correlate, due to additional factors influencing protein expression

levels, such as post-transcriptional modifications, translation efficiency, and post-translational modifications (Liu et al., 2016). Therefore, relying only on mRNA expression changes, which are often transient, may be insufficient when studying the progression of toxicity over time. In contrast, studying protein changes could offer more substantiated insights into different cellular responses and their consequences, as proteins are more closely linked to phenotype (Groh & Suter, 2014). Recent advances in proteomics technology attained in the biomedical field have enabled the performance of efficient proteomics analyses in an ecotoxicological context as well (Faugere et al., 2020; Liang et al., 2020; Pollard et al., 2024), including in the fish cell lines (Degeratu et al., 2024; Tierbach et al., 2020).

Ultimately, omics analyses are moving in the direction of multi-omics, as it has been demonstrated that integration of several different omic data streams holds the potential to enhance the depth and relevance of the resulting mechanistic insights (Bakker et al., 2023; Legler et al., 2020; Song et al., 2023).

6 Mainstreaming the fish invitrome approach

Bringing a new, non-organismal-based method, such as the RTgill-W1 acute toxicity assay, all the way from conception to international regulatory acceptance has been a tedious process. It took about ten years to achieve endorsement by ISO and OECD after the proof-of-concept had been published (ISO, 2019; OECD, 2021; Tanneberger et al., 2013). Achieving this status therefore took about as long as bringing a new pharmaceutical onto the market. If we project this experience to estimate the time it would take to establish the whole fish invitrome approach, several decades will pass before a full substitute to fish-based tests will be available. Different approaches for developing, diffusing, and implementing test procedures are thus needed. Regulatory acceptance might not be the first achievable goal on this journey, due to the

highly institutionalized process of method acceptance and their uptake and use in regulatory frameworks. Instead, we suggest a stepwise and indirect approach for mainstreaming the fish invitrome to make it widely understood, trusted and actually used in everyday ecotoxicological applications.

The successful application of the fish invitrome does not only depend on the scientific validity of the core ecotoxicology modules but also needs to take into account the organizational contexts where companies, non-governmental organizations (NGOs) and ultimately regulators apply these tests. The fish invitrome framework therefore must be understood as a "socio-technical" object, respecting toxicological coherence alongside various practice-based requirements and organizational and institutional conditions of its application contexts. Earlier studies explaining the limited uptake of NAMs pointed at vicious cycles of lacking legitimacy, perceptions of immaturity of cell line-based assessments, cultural and legal barriers, and a lack of familiarity, trust and confidence among toxicology practitioners (Čavoški et al., 2023; Mathisen et al., 2024; Rehberger et al., 2018; Schiffelers et al., 2012). Mainstreaming of the fish invitrome requires overcoming these multiple barriers in a stepwise manner, with different segments of practice adopting the modules in stages. As experience is gained in individual segments, they create the conditions for other users to build on the pioneers' experiences. Regulatory acceptance and full substitution will then be potential endpoints of such a trajectory rather than its imminent target.

An important element of this roadmap is to devise and gain legitimacy for the use of modular frameworks, such as the recently recommended cell-based *in vitro* testing strategy for human developmental neurotoxicity (Masjosthusmann et al., 2020; OECD, 2023) or the fish invitrome framework as proposed here. To enable a rapid accumulation of critical experiences

with cell-based testing strategies in modular frameworks, we need to better understand how they are already being applied across a wide range of application contexts. Additionally, it is important to identify which application contexts have the potential for implementation with minimal additional investment soon. Experiences gathered in these application fields would then enable to gradually diffuse the method to other contexts. This process would be guided by an evolving understanding of data and information requirements and the necessary toxicological endpoints for the framework. This evolving knowledge will help to refine the framework and support its broader application across various organizational and institutional contexts. Full substitution of *in vivo* tests may not always be the primary goal in the different application contexts. More complex assessment situations may still require complementing cell-based methods with *in vivo* assays, such as first *in vitro* determining appropriate exposure concentrations for subsequent *in vivo* tests. Cell line-based approaches may also enable a deeper understanding of toxicological pathways than *in vivo* assays alone. Key for successful application is that the modular cell-based testing strategies should be understood as methods that align with practice-based requirements in heterogeneous organizational and institutional risk assessment contexts.

To better understand ecotoxicological risk assessment practices by different regulatory agencies, industries, NGOs and researchers, we are developing a typology of risk assessment contexts that considers the needs of actors to achieve their goals with the tests, which might include alignment with specific regulatory requirements. For this, we will map out various user segments in which cell line-based assays are reliably applied first as well as by potential subsequent adopters. For a selected sample of assessment situations, we will elaborate in-depth case studies to specify how scaling and mainstreaming processes could look like. Based on

insights from these case studies, we may then sketch out specific roadmaps for their mainstreaming and identify barriers that would need to be overcome. This represents a systematic design strategy for the fish invitrome framework and suggests critical steppingstones for developing the individual assessment modules.

An important aspect of this process is the co-design with stakeholders from different risk assessment contexts. This co-design will lead to critical reflection on the practical viability of using the framework. It also will create a community of professionals across various user segments. By following such a stepwise and inclusive socio-technical approach we are confident to break out of the "vicious cycle" (Čavoški et al., 2023) of mutual reluctance between industry and regulators, thus allowing for a faster acceptance and broader practical application of the fish invitrome framework.

Funding statement: This work was supported by the National Research Programme "Advancing 3R – Animals, Research and Society" (NRP 79) of the Swiss National Science Foundation (SNSF), project number 407940_206439; the Lead Agency Project Grant jointly funded by the Slovenian Research Agency (ARIS) and the Swiss National Science Foundation (SNSF) (project numbers J4-3097 and 205321_197327, respectively); and the CRACK IT Challenge "SAFE" (project number NC/C021203/01).

Conflict of interest: The authors declare no conflict of interest.

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Data availability: No additional data was used apart from data presented in the article.

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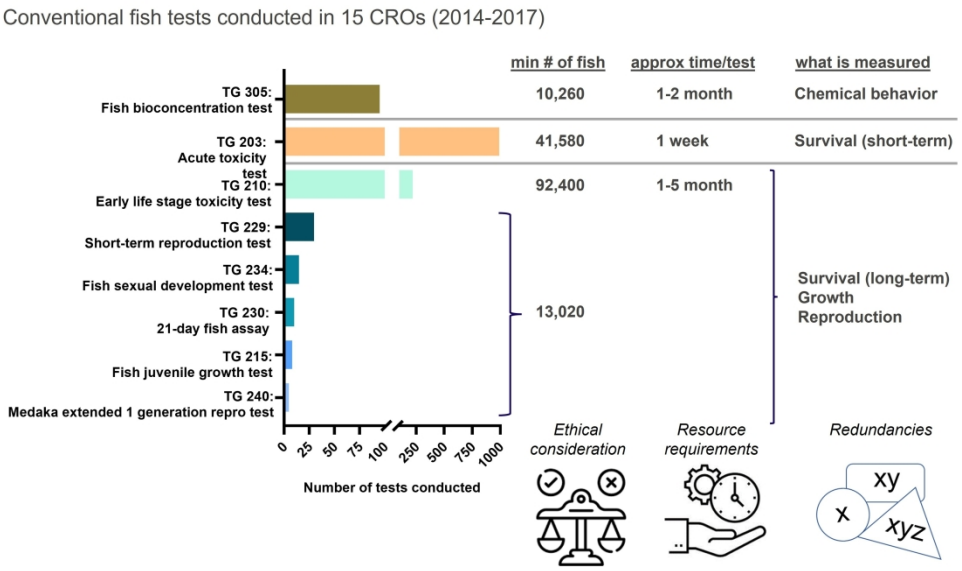
Figure 1. Types and frequencies of use of Organization for Economic Co-operation and Development Test Guidelines (OECD TG) using fish for environmental risk assessment, conducted by 15 Contract Research Organizations (CROs) between 2014 and 2017, as reported in Burden et al., 2020. The figure includes how often these tests were performed, along with an estimated number of fish sacrificed (ethical dimension), time required per actual test (resource dimension) and what is measured in general terms (recognizing redundancies, see also Table 1).

Figure 2. Representation of the different modules of the fish invitrome framework. RTgill-W1 cell line assay is presented as the foundational model for other cell-based assays in this framework. Dashed lines represent the modules that are under development.

Table 1. List of endpoints measured by Organization for Economic Co-operation and Development (OECD) Test Guidelines (TG) and Guidance Documents (GD) using fish for environmental risk assessment. An "X" indicates that a specific endpoint is assessed in the corresponding TG.

TG	Title	Survival	Bioconcentration factor (BCF)	Embryonic stage	Hatching	Morphological abnormalities	Behavioral abnormalities	Growth		Reproduction					
								Length	Weight	Secondary sexual characteristics	Gonadal histopathology	Reproductive fitness	Genetic sex determination	Biomarkers: vitellogenin	Biomarkers: androgenic activity
203	Fish, acute toxicity test	X													
305	Bioconcentration: Flow-through fish test		X												
236	Fish embryo toxicity test	X		X	X	X									
215	Fish, Juvenile Growth test	X				X	X	X	X						
212	Fish, short-term toxicity tests on embryo and sac-fry stages	X		X	X	X	X	X	X						
210	Fish, Early-life Stage Toxicity Test	X		X	X	X	X	X	X						
229	Fish short-term reproduction assay	X				X	X			X	X	X		X	X
230	21-day fish assay	X				X	X			X				X	X
234	Fish sexual development test	X		X	X	X	X	X	X	X			X	X	
GD 148	Androgenised Female Stickleback Screen (AFSS)	X				X	X								X
240	Medaka extended one generation reproduction test (MEOGRT)	X		X	X			X	X	X	X	X		X	



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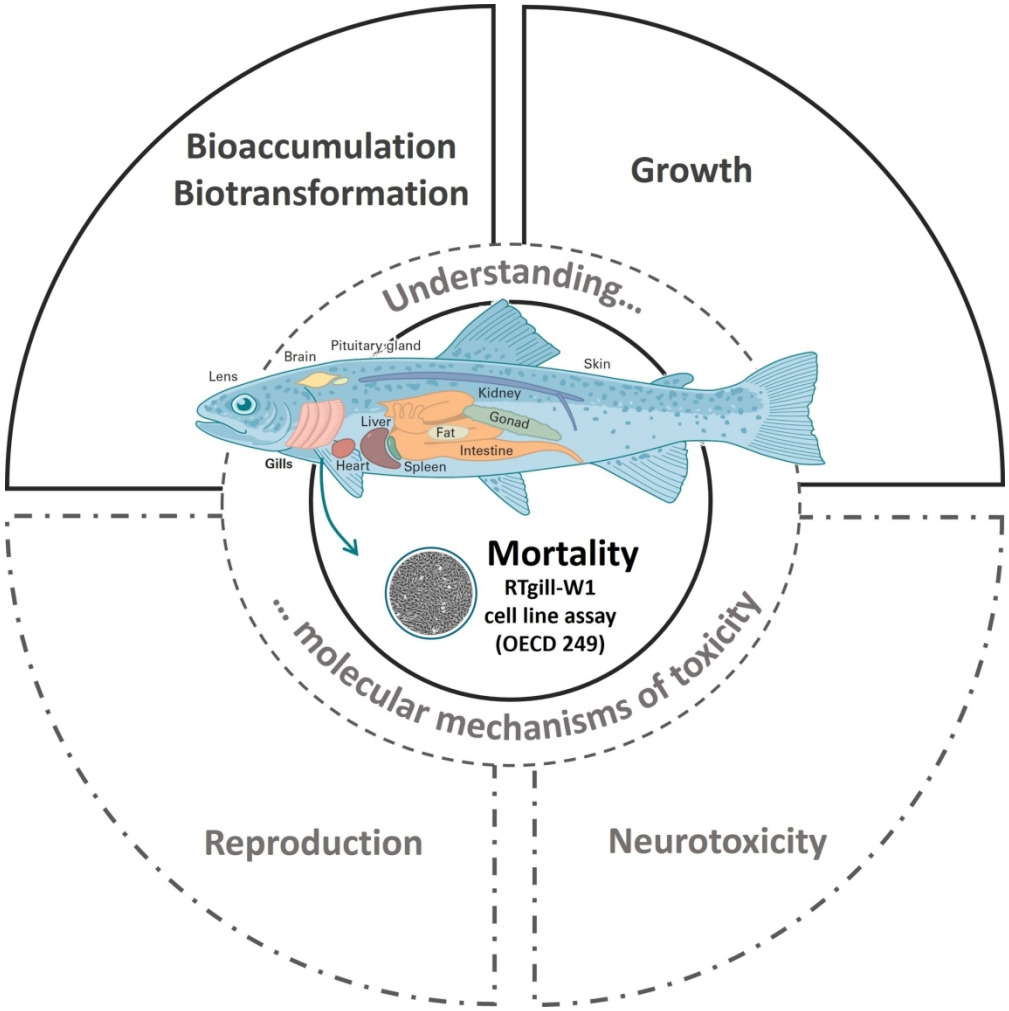

Figure 2. Representation of the different modules of the fish invitrome framework. RTgill-W1 cell line assay is presented as the foundational model for other cell-based assays in this framework. Dashed lines represent the modules that are under development.

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