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Commentary:

Promoting the 3Rs to enhance the OECD Fish Toxicity Testing Framework.

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Fish testing has been used to understand chemical and effluent toxicity since the 1860's and continues to play an important role towards defining safe levels of chemical contaminants in lakes, rivers and coastal waters (Sprague 1971; Hunn 1989). Historically, many severe chemical pollution problems led to fish kills giving rise to a focus on acute lethality testing of chemicals and effluents. More recently, the focus of concern is on long term effects of chemicals directly on fish and also indirectly via impacts on invertebrate prey species and other taxa. Consequently, fish toxicity testing is embedded in most regulatory programmes for prospective and retrospective assessment of individual chemical substances and effluents. Current regulations implementing environment protection (e.g. REACh and Plant Protection Products legislation) increasingly incorporate the wider societal view that vertebrate animal use should be Replaced, Reduced and Refined (the 3Rs) where possible. Such a paradigm shift also supports scientific and business needs to consider the 3Rs. The OECD Fish Toxicity Testing Framework (OECD 2012) provides a useful structure with which to simultaneously address the needs of high levels of environmental protection whilst implementing the 3Rs. This commentary aims to encourage awareness of this activity and promote the implementation of the recommendations of the OECD Fish Toxicity Testing Framework.

The historic need to be identify and prevent chemical impacts on fisheries and water quality led in 1981 to the Organisation for Economic Co-operation and Development (OECD) adopting the Fish Acute Toxicity Test Guideline 203 (updated in 1984 and 1992). Subsequently the Fish Early-Life Stage Toxicity Test Guideline 210 was adopted in 1988 (since updated in 2013). As scientific knowledge of the environmental risks posed by diverse chemicals has grown, the OECD has adopted a growing number of fish test guidelines to address bioconcentration, development and reproduction in fish. Today's OECD 'toolbox' of

test guidelines plays a central role in supporting an internationally consistent approach to the environmental safety assessment of chemicals. We define the environmental safety assessment of chemicals as the evaluation of the predicted environmental exposure of a chemical with the predicted *in vivo* biological effect of the chemical, supported by mechanistic *in silico* and *in vitro* data describing the intrinsic (eco)toxicological properties or mode-of-action (MOA) of the chemical. In contrast, the environmental risk assessment of chemicals typically focusses solely on comparing the predicted exposure concentration (PEC) to the predicted no effect concentration (PNEC) derived from *in vivo* experiments for relevant taxonomic groups (fish, aquatic invertebrates and algae) in the absence of MOA data. Though perhaps a subtle distinction, the safety assessment approach offers scope for a more comprehensive use of all *in silico*, *in vitro* and *in vivo* information at multiple levels of biological organisation. Balancing the need for high standards of environmental protection with the demands and desire to implement the 3Rs is one of the key challenges for environmental safety assessment today. As part of addressing this challenge, the OECD (2012) developed the Fish Toxicity Testing Framework in order to provide guidance on how best to deploy the various fish toxicity and bioconcentration test guidelines, including consideration of the 3Rs. In our view, the OECD's Fish Toxicity Testing Framework provides a logical and transparent approach to this complex aspect of environmental safety assessment. A simplified version of the OECD Fish Toxicity Testing Framework is shown in Figure 1 (see OECD (2012) for full details). The OECD (2012) also considered a number of important outstanding questions of scientific and regulatory concerns, including for example, what are the options for reducing animal numbers in fish toxicity tests and how can less severe endpoints be given priority in decision making?

More broadly, we believe there is reason to be optimistic that the 3Rs can be successfully applied to the OECD Fish Toxicity Testing Framework to support environmental safety assessment. Firstly, in terms of replacement, Figure 1 summarises some key opportunities and gives priority to the replacement of *in vivo* fish testing, where feasible, through the use of validated *in silico* and *in vitro* tools. However, these can only be applied with confidence within the chemical domains of the data used for their validation. Replacement of fish acute tests by the Fish Embryo Acute Toxicity Test Guideline 236 (adopted July 2013) may be possible under some regulations. Replacement of fish toxicity testing with suitable invertebrates may also be useful. Replacement may take the form of establishing targeted threshold test levels for fish (determined by full invertebrate tests) or complete replacement but this needs to be justified scientifically by an understanding of the exposure relevant MOA of a chemical in order to derive robust environmental safety assessments. For example, a the this mechanistic approach could offer a positive way forward to address the replacement of fish with arthropod toxicity tests where there is a shared MOA (e.g. ion channel mediated neurotoxicity of pyrethroids) or other *a priori* knowledge of a particularly more sensitive taxonomic group of invertebrates. This would be in contrast to a very different mode-of-action specific to vertebrates (e.g. receptor–mediated feminization of fish by steroidal oestrogens) (ECETOC 2007). The development of adverse outcome pathways, as strongly supported by the OECD, could in the future help to identify where cross-species extrapolation is appropriate based upon a common Molecular Initiating Event (Ankley et al., 2010; Burden et al. 2015a; OECD (2015)). Invertebrates may also provide an environmentally relevant alternative bioconcentration test guideline given the potential for chemical uptake into lipid rich invertebrates (ECETOC 2007).

Secondly refinement of the severity of the experimental endpoints (i.e. degree of suffering induced) is another aspect of the OECD Fish Toxicity Testing Framework that warrants attention. For instance, minimising the assessment of lethality in fish and optimising the experimental design to focus on sublethal endpoints via the Maximum Tolerated Concentration (MTC) approach (Hutchinson *et al*., 2009) is one aspect of refinement considered by the OECD (2012). The OECD (2012) also recommended introducing the term 'moribund' in the fish acute toxicity Test Guideline 203, which would represent a significant refinement. Discussions are currently ongoing regarding this guideline revision. Test Guideline 204 (Fish, Prolonged Toxicity Test: 14-Day Study) was deleted after it was deemed as 'ethically indefensible' and concerns have also been raised about the lack of feeding in the Test Guideline 212 (Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages).

Thirdly, further effort is needed to reduce the numbers of fish toxicity tests through a variety of approaches. As summarized in OECD (2012), these approaches include moving away from automatic 'tick box' testing to more efficient tiered testing frameworks and 'intelligent' or 'integrated testing strategies' which make better use of *in silico*, *in vitro* and *in vivo* information. However, operating such a flexible approach will undoubtedly result in greater regulatory complexity. Further, work to explore the application of test guideline validity criteria was also recommended. This could determine which deviation(s) (or magnitude of the deviation) from criteria fundamentally undermines study outcomes and overall test performance (hence necessitating repeat studies), and conversely which do not impact on the scientific quality of studies (thus negating the need for their repetition).

121 The OECD framework is not comprehensive of all opportunities to address the 3Rs as
122 described elsewhere. However, it offers tangible opportunities to address the issues with what
123 constitutes the building blocks of the current regulatory data requirements (i.e. test guideline
124 studies mandated by the various chemical legislations). As such it fits with the current legal
125 frameworks and so offers an ability to improve 3Rs application in the short and medium
126 terms whilst fundamentally different approaches are developed and mature sufficiently for
127 regulatory implementation (Burden et al. 2015b). A number of the OECD Fish Toxicity
128 Testing Framework recommendations have already developed as projects and made it on to
129 the OECD's work plan (see Table 1).
130
131 In conclusion, the OECD (2012) generic framework provides a highly valuable opportunity
132 to improve fish toxicity and bioconcentration testing. The OECD framework highlights key
133 3Rs opportunities that are consistent with the scientific and ethical principles increasingly
134 required by regulations, industry and society for chemical safety assessment. We encourage
135 international stakeholders to take up the recommendations from the OECD framework in
136 order to further promote the reduction, replacement and refinement of fish toxicity testing
137 within the environmental safety assessment context.
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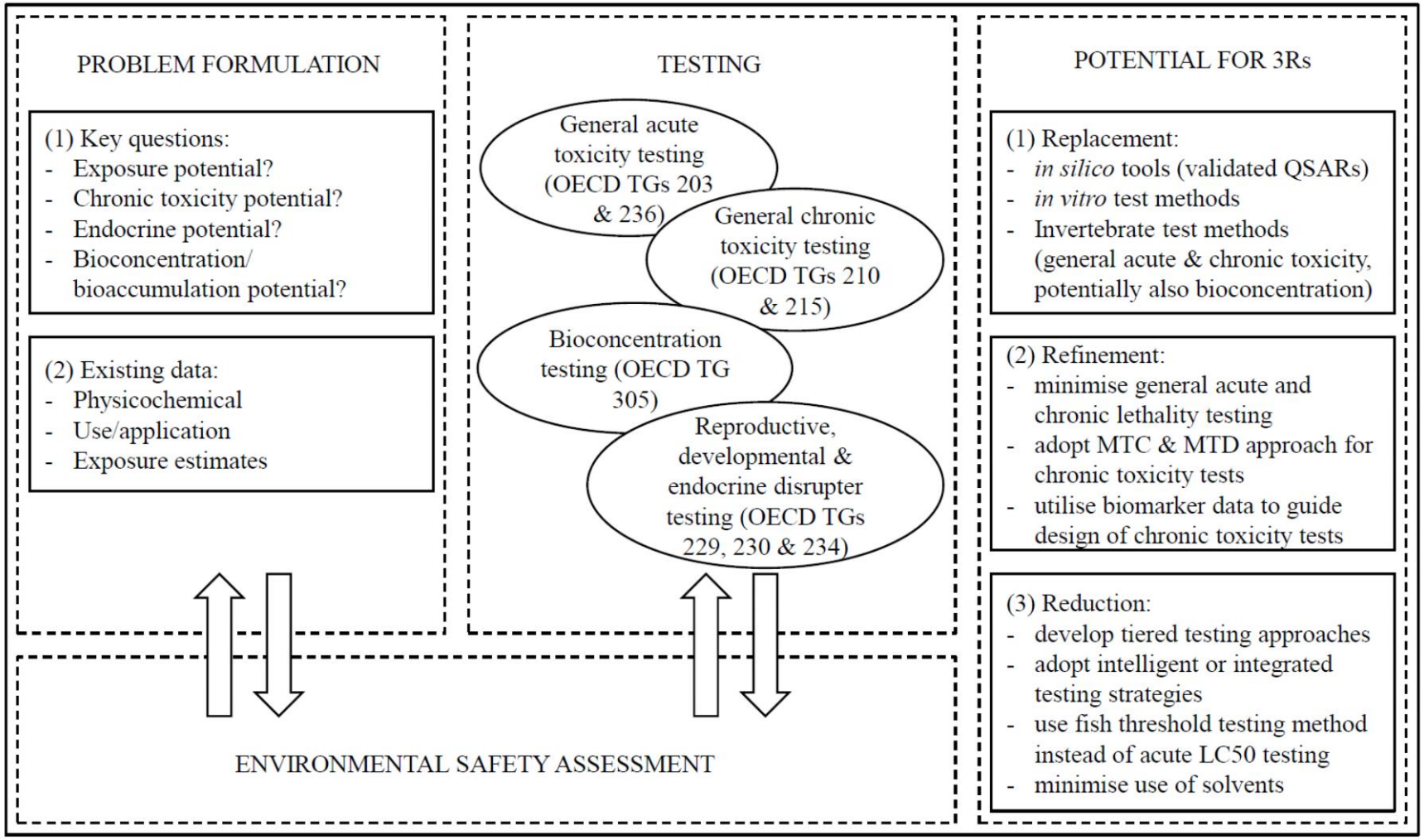

Figure 1. Simplified OECD generic testing strategy for fish toxicity and bioconcentration assessment with priorities for promoting the 3Rs (see OECD (2012) for the detailed flow-chart of the testing strategy).

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187 Table 1. Summary of OECD projects on (or proposed) the work plan associated with the 3Rs
188 in guideline ecotoxicity tests.

Project Number	**Title**	**Date included**	**Lead country**	**Issue**
2.50	Revision of TG 203 Fish Acute Toxicity Test	2014	Switzerland/ United Kingdom	Definition and implementation of moribund to allow early of termination of individuals to prevent suffering (reliable prediction of death)
2.54	Guidance Document on IATA for Fish Acute Toxicity Testing	2015	Austria	Integrated Approaches to Testing and Assessment for acute fish toxicity testing
2.55	Use and analysis of control fish in toxicity studies	2015	European Commission	Review and update of poorly soluble substance guidance. Detailed Review Paper of use of controls in ecotoxicity tests
Proposed	Critical assessment of deviations from OECD Vertebrate Ecotoxicology Test Guidelines	2016	United Kingdom	Review of test guideline validity criteria. Update of test guidelines and guidance on interpretation to avoid unnecessary repeats.

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