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TITLE

Advancing the 3Rs in Regulatory Ecotoxicology: A Pragmatic Cross-Sector Approach

RUNNING HEAD

Advancing the 3Rs in Regulatory Ecotoxicology

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ABSTRACT

The ecotoxicity testing of chemicals for prospective environmental safety assessment is an area where a high number of vertebrates are used across a variety of industry sectors. Refining, reducing and replacing the use of animals such as fish, birds and amphibians for this purpose addresses the ethical concerns and the increasing legislative requirements to consider alternative test methods. Members of the UK-based National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) Ecotoxicology Working Group, consisting of representatives from academia, government organisations and industry, have worked together over the past six years to provide evidence bases to support and advance the application of the 3Rs in regulatory ecotoxicity testing. The group recently held a workshop to identify the areas of testing, demands and drivers that will impact on the future of animal use in regulatory ecotoxicology. As a result of these discussions we have developed a pragmatic approach to prioritise and realistically address key opportunity areas, to enable progress towards the vision of a reduced reliance on the use of animals in this area of testing. This paper summarises the findings of this exercise and proposes a pragmatic strategy towards our key long-term goals – the incorporation of reliable alternatives to whole organism testing into regulations and guidance, and a culture shift towards the reduced reliance on vertebrate toxicity testing in routine environmental safety assessment.

KEY WORDS

3Rs; fish; chemicals; ecotoxicology; regulatory

INTRODUCTION

Many organisations across various sectors have an aspirational goal that scientific and technological advances may eventually enable the replacement of animal toxicity testing in the evaluation of product safety. Approaches which support the 3Rs principles (Reduction, Refinement and Reduction of animal testing; Box 1) are already practiced in some sectors; in particular, replacements are being sought to traditional animal tests through the use of cell-based (*in vitro*) and computational *(in silico*) methods within the cosmetics and personal care products industry, due to restriction or exclusion of the testing of such products in animals. However, until such approaches are more widely developed, validated and accepted, regulatory safety standards and industries' duty of care to humans and the environment means that today some animal tests remain integral to the assessment of the health, safety and potential environmental impact of chemical, agrochemical and pharmaceutical products, intermediates and raw materials. There are opportunities however to challenge existing practices and determine how they can be improved, while at the same time exploring and developing the use of alternatives to *in vivo* testing.

Ecotoxicity testing is widely undertaken to prospectively assess the impact of chemicals on wildlife populations and ecosystems and is one area which represents a significant source of vertebrate animal use across a number of industry sectors. Fish, followed by birds and amphibians, are the most commonly used vertebrate species in such regulatory environmental safety assessments. According to the most recent statistics from the European Union (EU), almost 200,000 animals from these groups were used in 2011 for toxicological or other safety evaluations, with over half of those tests carried out to meet regulatory requirements (EC 2013). However this area has historically received much less attention in terms of the 3Rs principles than the mammalian toxicity testing undertaken to assess human safety.

Advancing the 3Rs in ecotoxicology will not only address ethical concerns but also the legislative demands to find alternative non-animal methods, and share data wherever possible. In Europe, for example, this is relevant to Article 62 of the Plant Protection Products Regulation (PPPR; EC 2009), Article 25 of the Registration, Evaluation, Authorisation & restriction of CHemicals (REACH) Regulation (EC 2006), and Article 62 of the Biocidal Products Regulation (BPR; EU 2012). Furthermore, it is no longer an option for the cosmetics industry to utilise animal tests in safety evaluations of their products in many parts of the world, for example in Europe (EC 2009), India (Indian Ministry of Health and Family Welfare 2014) and possibly with Australia and the US following suit (www.alp.org.au/cosmeticstesting; www.congress.gov/bill/113th-congress/house-bill/4148). The benefits of applying 3Rs approaches in ecotoxicology are potentially far reaching, particularly considering the large numbers of substances requiring approval to sell worldwide (Muir and Howard 2006).

The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) is a UK-based independent scientific organisation set up by Government in 2004, to lead the discovery and application of new technologies and approaches which minimise the use of animals in research and improve animal welfare. The organisation's in-house programmes aim to provide scientific evidence bases which support the application of the 3Rs in a range of research areas. This is achieved through the creation of a proactive, collaborative environment which engages key stakeholders such as authorities, regulators, academics and industry practitioners, and is strengthened by the forging of international links to stimulate change at the global level. In 2008 the NC3Rs began a dedicated programme of work in the area of ecotoxicology, supported by a working group of experts in the field across academia, government agencies (including regulators), contract research organisations and industry (principally agrochemicals, personal care products and petrochemicals). The projects

initiated within the NC3Rs Ecotoxicology Working Group over the past six years have largely focussed on reducing the use of fish in the safety assessment of industrial chemicals and agrochemicals (Burden et al. 2014; Creton et al. 2014; Creton et al. 2010; Creton et al. 2013), and build on the earlier 3Rs-related work of some members of the group (Douglas et al. 1986; Hutchinson et al. 2003; Jeram et al. 2005).

This paper summarises discussions held at a recent workshop of the NC3Rs Ecotoxicology Working Group, which considered the future of animal use in the ecotoxicity testing required for product or substance registration. The group has used this sharing of knowledge and experience to develop a pragmatic approach to enable progress towards the vision of a reduced reliance on the use of animals in this area.

TRENDS IMPACTING CURRENT AND FUTURE ANIMAL USE IN ECOTOXICOLOGY

There have been considerable advances made in recent years in the science and methodology of alternative test methods in ecotoxicology, particularly in the area of acute fish toxicity (for example, the development of the zebrafish embryo test - Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 236 (OECD 2013)), and there is evidence emerging from regulatory authorities that companies are increasingly making use of alternative approaches for regulatory purposes (e.g., see ECHA 2014). Efforts have also been made to refine and reduce the animal numbers used in *in vivo* tests, as demonstrated by the development of OECD TG 223 (avian acute oral toxicity test, OECD 2010a) and the provision of guidance for the threshold approach for acute fish toxicity (OECD Guidance Document (GD) 126; OECD 2010b). There is nevertheless potential for further headway to be made towards both the refinement of animal tests and the application

of non-animal alternatives, for aquatic and terrestrial ecotoxicity testing. The detailed assessment carried out by the Working Group identified the areas of testing where high numbers of vertebrates are currently being used or a significant increase is anticipated, as well as the opportunities which exist to decrease or refine animal use. This review was used to pinpoint key focus areas for the group's future activities, and is summarised in Supplementary Table 1. Four key areas of potential current and future high vertebrate usage were determined as: 1) the identification of endocrine disruptors; 2) the assessment of bioaccumulation; 3) acute and 4) chronic toxicity. Within some of these areas there is potential for a high degree of suffering to be experienced by the test animals (higher "severity" tests).

Some universal drivers, which may contribute to changes in animal use across all these areas of testing, were highlighted by the group. One of these is the growth in complexity and breadth of risk assessments to address the needs of the legislation and the concurrent increase in uncertainty regarding some of the exposure estimates used. Many of these estimates are based on theoretical assumptions, rather than real exposure measurements, and often aim to cover a wide range of possible exposure scenarios. Typically 'worst case' exposure assessments are used, although refinement is possible to provide more 'realistic' values. However, refinements can be difficult especially for diffuse exposure scenarios,, Therefore, refinement may be pursued by undertaking additional toxicity studies as a means to reduce uncertainty in the effects assessment endpoint, and hence the overall risk assessment. For example, as outlined in European Chemicals Agency (ECHA) Guidance Documents (e.g. ECHA 2011) the sparser the available data, the higher the assessment/uncertainty factor. Hence, having more acute and chronic toxicity data for different trophic levels (including fish) can reduce the assessment/uncertainty factor used to determine the predicted no effect concentration (PNEC), thus reducing the "apparent" risk

posed by chemicals. More realistic exposure modelling may go some way to alleviate the issue, so that the use of data from vertebrate test subjects to refine effects assessments is required less often. Further, more spatially or temporally explicit techniques could be employed to better represent the chemical contribution to overall risk (Hope 2004). The increased breadth of the legislation has contributed to an increase in the scope of animal testing; under the PPPR for example, chronic fish testing is now a core requirement, as well as the inclusion of an assessment of endocrine disruption. The combination of these factors potentially leads to an increase in vertebrate testing. It is worth noting however that where informed exposure-led assessments are carried out, this can help to better design testing strategies and thus could contribute to a decrease in numbers of animals used; consideration of internal exposure concentration can also help to avoid the dosing of excessively high concentrations in later studies thus reducing the likelihood of inducing unnecessary animal suffering (Creton et al. 2012).

Secondly, there is evidence that animal studies can be duplicated in closely related species from the same taxon (e.g. freshwater cyprinid fish (Oris et al. 2012)) to address different regulatory needs across the globe, and it is possible that higher severity/more animal intensive methods are being used to meet different national requirements to ensure the global registrations. Despite the obvious ethical concerns this raises, registrants generally incorporate the most extensive testing necessary to ensure global acceptability and associated freedom to market and sell their products. This is largely due to a lack of comprehensive harmonisation of global regulations, which occurs due to regional preferences and disagreement on standard test species or approaches. Furthermore, although test data generated in any OECD member country in accordance with OECD Test Guidelines and Principles of Good Laboratory Practice (GLP) can be accepted in other member countries for assessment purposes, requirements can exist for toxicity tests to be carried out locally,

particularly in the non-OECD member countries, despite data already being available from GLP OECD studies carried out elsewhere. The Globally Harmonized System of Classification and Labelling of Chemicals (GHS) is a rare example where fish toxicity tests carried out in any species under the relevant OECD Test Guideline are globally accepted.

Finally, legislative demands in certain chemical sectors in some regions are contributing significantly towards increases in *in vivo* ecotoxicity testing, despite stipulations within various frameworks that animal testing should only be carried out as a last resort. This is in part a result of the reluctance of some national regulators to accept modelled information (derived from *in silico* or *in vitro* data), category and read across approaches, and a lack of recognition that regulatory endpoints may not automatically require a vertebrate test to fulfil them. Data packages containing non-animal data which are acceptable within the EU are not always globally accepted, and therefore additional animal testing may be undertaken to meet the demands of other regulatory authorities. It is worth noting that there are scientific barriers that still need to be overcome before non-animal methods can be more widely utilised. In the case of computational models for instance, there is not always sufficient toxicity data available on certain molecular structures to be able to make confident predictions. Furthermore, information on toxicokinetics and thus relevant internal concentrations can be difficult to capture using cell-based assays. There is also recognition that alternative methods cannot realistically replace the whole animal tests on a like-for-like basis. This may be addressed in the long term by the development of better extrapolation models and integrated approaches to testing and assessment. Consideration of integrated approaches also highlights the importance of, and need for, the scientific aims of the risk assessment *a priori* – this will also help to avoid "box-ticking" exercises and ensure that vertebrate tests are only carried out when they add genuine value.

A PRAGMATIC APPROACH TOWARDS ADVANCING THE 3Rs

This assessment of the current and future landscape was intended to not only highlight the potential to apply the 3Rs in ecotoxicology, but to help build a strategy whereby organisations such as the NC3Rs can apply their experience to address some of the issues, and reach key long-term goals. This must undoubtedly be approached in a practical and realistic way, through concurrent investment in both short- and medium-term endeavours, towards the key long term aims, as detailed in Figure 1. Adopting a complementary approach ensures that immediate impacts regarding a reduction in animal use can be achieved, whilst working towards those more time and resource-intensive aims. The undertaking of short-term projects will help to facilitate the medium-term approaches, and will also have value in addressing some of the drivers impacting on animal usage over the next three to five years (such as the EU REACH 2018 registration deadline, and the identification of Substance of Very High Concern (SVHCs) by 2020).

When determining individual focus areas in which to invest and ensure maximum impact, the prioritisation of efforts and resource can be achieved through a cost/benefit analysis which considers a number of factors, including:

- 3Rs benefit – how many of the Rs will be addressed? What will the impact be in terms of decreasing the severity of tests/numbers of animals used?
- Expected outcomes and impacts – how likely are the recommendations to be taken up across the scientific and regulatory communities and will they apply across sectors nationally and/or globally?
- New areas compared with previous work/programmes of work undertaken within other scientific organisations, to ensure minimal duplication and add maximum value.

- Potential for collaboration across sectors, and with other scientific organisations where common interests and goals have been identified.

Based on these factors, the Working Group assessed the potential 3Rs impact associated with investing resource into the opportunities to reduce, refine or replace animal use (as detailed in Supplementary Table 1) for each of the key focus areas of ecotoxicity testing, and prioritised them in the following order:

1) Assessment of acute toxicity (fish and bird) – scope for a large reduction in both animal usage and testing severity.
2) Assessment of bioaccumulation (fish) – scope for a large reduction in animal numbers used; although physico-chemical properties are already extensively used to predict bioaccumulation, *in vivo* testing is still often triggered as the metabolisn processes can often be a driving factor that is not well predicted *in silico.*
3) Identification of endocrine disruptors (fish, amphibians and birds) – scope to further explore the necessity for high animal usage in reproductive/developmental screening assays carried out to meet regulatory protection goals (though note that test severity tends to be relatively low in the relevant *in vivo* tests).
4) Assessment of chronic toxicity (fish) – scope to reduce animal usage, particularly where similar tests are conducted on multiple species (e.g. for global registrations).

Furthermore, there are two key overarching themes where progress is essential to advance the global recognition and applicability of the 3Rs, with potential to impact on all four of the identified focus areas, and beyond: i) improvements in study design across all areas of ecotoxicity testing in order to provide adequate statistical power within studies, which

could lead to either the use of fewer animals and/or the generation of better quality data from *in vivo* tests (which can be achieved in the short-term); and ii) the harmonisation of global data packages (a longer-term aim). In order to achieve global harmonisation, dedicated assessments will need to be carried out in the first instance, to determine appropriate recommendations based on the areas where there is scope to align testing and data requirements. This will need to be followed by co-ordinated large-scale international efforts and collaboration, to ensure that the recommendations are heeded. It is also of note that the recent interest and increasing investment into the development of adverse outcome pathways (AOPs; pathways which link a molecular initiating event for a chemical to an apical endpoint and subsequent organism/population effects, through a scientifically proven causal chain of events). Application of this concept in the long term has potential to transform the practice of (eco)toxicity testing, and provide tangible 3Rs benefits, for example by increasing confidence in cross-species extrapolations and read-across approaches, as well as perpetuating the development of newnon-animal methods for use in safety assessment (Burden et al. 2015). This concept is being underpinned by a global initiative currently underway at the OECD, which provides an explicit, consensus-led framework under which AOPs are developed and brings together the relevant communities; thus, establishment of robust AOPs for ecotoxicology endpoints is also considered a key long-term goal (Figure 1). The AOP framework provides a valuable opportunity for ecotoxicologists to share data on a large scale; initiatives such as this which facilitate data sharing, between both the regulatory and scientific communities, have potential to not only improve the science of ecotoxicology, but to accelerate the development and validation of non-traditional methods and approaches.

Activities such as those related to improvements in study design and reporting will apply broader concepts to the field of ecotoxicology that are also relevant to academic

research. Many areas of basic research have started to apply the ARRIVE (Animal Research: Reporting of *in Vivo* Experiments) guidelines. These guidelines are a 20-point checklist of the essential information that should be included in publications reporting animal research, to improve standards of reporting and ensure that the data from animal experiments can be fully evaluated and utilised (Kilkenny et al. 2010). In this way consistency in the reporting of, for example, exposure routes and exposure concentrations can be ensured to enable comparisons between test methods on a like-for like basis. This is a key factor considering that alternative test methods are often 'validated' against existing *in vivo* data. If such an approach were globally endorsed, replication of experiments could be avoided and higher confidence attributed to methodology and results, thus increasing the robustness of open literature data. This is particularly significant because such data must now be included in dossiers submitted under various chemicals regulations. Better reporting and study design of basic research could therefore help to avoid the triggering of new animal tests, as reliable open literature data will be available more frequently. It is also paramount that detailed ecotoxicity data submitted to regulatory agencies is made available wherever possible, and is easily accessible. Promotion and uptake of standard reporting guidelines within ecotoxicology is a good example of how a short-term approach has scope to feed into longer-term 3Rs benefit.

LOOKING TO THE FUTURE

The next steps involve continuing to apply and progress the proposed strategy, through the continuation of efforts to work internationally, with the aim of addressing the key areas of concern. Mapping of priority areas to the existing opportunities to decrease or refine animal use, and the development of projects to facilitate novel advances within these areas, is

now underway. The commitment of industry to working alongside organisations such as the NC3Rs, who can take on the role of a neutral scientific forum to enable data sharing activities and involve regulators at the early stages of projects, stands the community in good stead to progress the 3Rs and improve animal welfare in this ever-evolving field. Through the continuation of committed collaboration and international coordination, the achievement of these aims will be increasingly realised.

DISCLAIMER

The views and statements expressed in this paper are those of the authors alone. The views or statements expressed in this publication do not necessarily represent the views of the organisations to which the authors are affiliated, and those organisations cannot accept any responsibility for such views or statements.

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Box 1. Definition of the 3Rs.

REPLACEMENT
Methods which avoid or replace the use of animals

REDUCTION
Methods which minimise the number of animals used per experiment

REFINEMENT
Methods which minimise suffering and improve animal welfare

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