

Towards alternatives to animal testing of industrial chemicals in Australia: A scoping report

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EXECUTIVE SUMMARY

This scoping report (the report) was prepared for the Commonwealth Department of Health and Aged Care to provide an overview of progress, in Australia and overseas, towards reducing reliance on animal testing of cosmetics and other industrial chemicals for human health and environmental impacts. The report presents best practices, innovative alternative approaches and opportunities for Australia to benefit from increased domestic and international collaboration.

The report includes a targeted review of the scientific literature on alternatives to animal testing. This is not an exhaustive, systematic review of every test method and approach, but rather an overview of the science, challenges and trends in the development and use of non-animal ‘new approach methodologies’ (NAMs) for chemical toxicological assessment. It is supported by a scan of how NAMs are being accommodated and encouraged by chemical regulatory frameworks in Australia and internationally. The report considered advice from a stakeholder reference group that included Australian regulators and Commonwealth agencies, peak bodies and non-governmental organisations.

An industrial chemical is one that has an industrial use but is not an agricultural or veterinary (agvet) chemical product, therapeutic good, nor is it used in human or animal food. Cosmetics in Australia have the unique classification (for a Western jurisdiction) of being industrial chemicals. The scope of this report is confined to industrial chemicals and does not extend to pharmaceuticals or medical testing. Many methods are applied across multiple domains and are referred in the report because of the high degree of overlap with industrial chemical testing.

An overview of the key findings of the report is provided below.

Key finding: Animal models for human chemical toxicity are often ineffective. NAMs for toxicity assessment promise significant benefits in both animal welfare and performance.

The use of animals in research and training traditionally has been pivotal to science. This is founded on the belief that animals are effective models for humans, such that toxicity to animals reflects likely toxicity to humans. The use of animals in research is increasingly scrutinised and more stringent rules and ethical guidelines are applied. Application of the 3Rs is increasingly important: ‘replacement’ (methods that permit a given purpose of an activity or project to be achieved without the use of animals), ‘reduction’ (methods for obtaining comparable levels of information from the use of fewer animals in scientific procedures or for obtaining more information from the same number of animals) and ‘refinement’ (methods that alleviate or minimise potential pain and distress, and enhance animal wellbeing). This approach has improved animal welfare outcomes.

Animal models may not be as effective as first thought. Most new drugs fail phase III clinical studies. It is challenging to extrapolate ecological toxicity from small, controlled studies of chemical exposure in a target species. This, and knowledge gaps in the mode of action of a (toxic) chemical and/or differing modes of action between species, can lead to errors in concluding human toxicity from animal test results. Alternatives to animal models may be beneficial on both ethical and performance grounds.

Key finding: NAMs are under rapid development. These include organ-, cell-, omic-, chemical-, and silico-level models of aspects of body function and/or toxicity.

Animal model replacements fall into several categories: *in vivo* (‘within the living’), *ex vivo* (‘outside of a living body’), *in vitro* (studies of living cells ‘within glass’), *in chemico* (studies of chemical effects on bioactive organic macromolecules) and *in silico* (using computer models). Human volunteers to test chemical safety are occasionally valid alternatives. There are advantages and deficiencies of each model

for replacing animal tests. Integrated systems for evaluating biological effect typically combine several methods, and these are increasingly used to take advantage of the strengths of each model.

In vivo models do not always replace animals but rather shift welfare impact onto species or life stages considered to be of lesser sentience (e.g. chicken embryos). *Ex vivo* methods involve the use of living tissues or organs taken from an animal, or human donor, so are not complete animal alternatives in the strictest sense. *In vitro* methods include cell cultures, 'organs-on-chips' and artificial membranes. Live cell/organ-based methods can provide direct models for toxicity by using cell extracts from the parent species, but they can be difficult to construct into standardised, repeatable, reliable, and acceptable models. They are hard to maintain in differentiated cellular form, so often are poorly suited to long-term exposure studies. An emerging field is 'omics', the common collective describing several related areas of study, notably genomics, transcriptomics, proteomics and metabolomics. This 'omics' cascade maps the sequence from gene to effect on cell function and is receiving considerable research attention in the European Union (EU) and United States (US) for exploring adverse outcome pathways. *In chemico* methods examine how a change in concentration or activity of the bioactive organic macromolecule impacts organism function.

Key finding: NAMs can replace animal models for some toxicity end points. This generally requires the mode of action of toxicity to be known.

Whilst animal models monitor the whole organism for the adverse effect of a chemical exposure, models that consider only a subset of biological pathways in the organism (such as *in chemico*, *in vitro* or 'omics') demand the relationship between a (measurable) response at chemical/cellular level and the (toxic) effect at organism level to be known. This means the toxic end point must map to the measured end point. The mode of action of many chemicals is unknown, especially for chronic exposures. Exposures that produce reproductive or developmental pathology, mutagenicity or carcinogenicity are often hard to study for this reason. Many NAMs are qualitative and so are useful to identify hazards, but less able to quantify dose-related risk. Chemical toxicity risk assessments consider both innate chemical toxicity and exposure risk. Quantifying the biological response is one component of evaluating risk. Animal studies can quantify toxicity responses even when the mode of action is unknown by measuring animal responses to exposure. This is not possible with NAMs. Points-of-departure on dose-response curves are difficult to identify/interpret in NAM models as often (intermediate) measurement variables do not mirror responses of whole organism.

Key finding: Chemical toxicity can rarely be assessed using a single NAM; often a suite of NAMs is required to cover the gamut of potential toxicity. Systems and processes are required to combine information from a suite of NAMs into an overall evaluation of toxicity. This need is driving a weight-of-evidence and battery-of-tests approach.

Chemicals can have multiple toxic effects, so determining chemical safety often requires assessment across a toxicity spectrum including cell survival, cell function/physiology, development, reproduction, carcinogenesis and mutagenesis. Typically, several NAMs are needed across the toxicity spectrum, as most NAMs test a single specific toxicity. A battery-of-tests approach, whereby a suite of NAMs is used, is often required. The challenge is in identifying sets of NAMs that comprise a complete suite. 'Read-across' methods are often needed when there are gaps for specific NAMs. This relies upon natural chemical groupings (with structural, mechanistic or biological similarities) such that a result from a NAM for a related chemical may substitute an unavailable NAM for the target chemical. Few chemicals have a complete suite of NAMs and this has placed increased reliance on read-across techniques. Good Read-Across Practice guidelines are in development, which may increase registration dossier acceptance, because many fail due to inappropriate/inadequate use of read-across methods.

The *in silico* methods such as quantitative structure-activity relationship (QSAR) modelling can explore a wide gamut of toxicity effects, but are dependent upon the quality and completeness of the underpinning knowledge base, and the effectiveness of data-mining algorithms. It is not possible to code the full complexity of living organisms and all the interactions between living cells and toxins. Comparative studies of *in silico* models, such as QSAR, have shown poor agreement between models at predicting toxicity. Predictive performance increases as extra information (such as mode of action and pharmacokinetics) is added to the knowledge base used by QSAR models. These extra data sources are likely to enhance *in silico* model performance.

There is increasing use of a 'weight-of-evidence' approach in chemical toxicity assessment, which is an integrated science-based approach to assessing toxicity. It combines information on mode of toxicity action with expert scientific judgement to assess, review and integrate all available information to form a conclusion on toxicity. This way any gaps in science are considered using hypothesis-driven thinking. Information on mode of action, study data and design, and relationships between responses and assessment endpoints are considered when assessing potential impacts of any missing information.

Key finding: Once developed, NAMs must gain regulator acceptance. This requires the methods to be made available and fully described to ensure repeatable, reliable and accurate deployment by external users. International organisations are collaborating to address market failures.

There is significant international activity and collaboration to develop NAM-based approaches to chemical testing. The Organisation for Economic Cooperation and Development (OECD) is especially prominent, driving focus onto addressing testing gaps, integrating information from various approaches, sharing data and resources, recognising new techniques and developing new paradigms for determining toxicity. Collaboration is vital because multi-disciplinary methods and expertise are essential to evaluate toxicity. Regulators are not prescriptive and do recognise the weight-of-evidence approach. The challenge is to ensure that techniques and methods developed at one site can be reliably and repeatedly applied at other facilities. Development of standards and good practice systems are vital.

Non-animal tests bring benefits to society. Many already provide effective screening of chemicals for toxicity. Advances in this field have reduced the number of animals used in toxicity testing or confirmatory studies. However, the process of bringing a NAM to market is laborious and lengthy. Five stages are described: (1) development, where the NAM is created, optimised and partially tested; (2) validation, where NAM accuracy and reliability are independently assessed; (3) formal test method, describing the NAM in a highly-specified, standard document; (4) regulatory acceptance, where regulators decide if they accept the NAM; and (5) deletion of the animal test, where an existing animal-based test is formally disallowed.

The NAM development, use and acceptance process is challenging. NAMs often do not progress to commercialisation if developed in academic environments or enter the public domain if developed in private enterprise. Validation can be challenging and sometimes exposes deficiencies in the prevailing animal tests the NAM seeks to replace. Greater understanding of the requirements of the regulator to accept a NAM and on the formal process for replacing (deleting) the animal test is needed.

Barriers to implementation of NAMs include the inertia of the current scientific paradigm, legislation, bureaucracy, funding, entrenched interests, lack of enforcement, threat of litigation and a lack of clarity in how to bring a NAM to market. Enablers include legislation, regulation, guidelines/standards, education and training, leadership, governance, improved reporting of experiments in publications, databases and information sharing, ethics committee processes, industry involvement and collaboration, research support, policies and frameworks, and workforce roles.

Key finding: In Australia, chemical registration often relies upon data generated in other countries. Australia has some unique requirements (including ecological toxicity) suggesting that greater involvement in international activities to develop, validate and adopt NAMs would be valuable for Australia.

In Australia, the *Industrial Chemicals Act 2019* (the Act) established the Australian Industrial Chemicals Introduction Scheme (AICIS) as the body regulating introductions of industrial chemicals. AICIS is administered by the Office of Chemical Safety, which replaced the National Industrial Chemicals Notification and Assessment Scheme (NICNAS) in 2020. A party seeking to introduce an industrial chemical, or products containing industrial chemicals, must register with AICIS and must categorise each chemical, including those in products into one of five introduction categories. Medium- to high-risk introductions are assessed for human safety, and/or environmental safety according to AICIS-specified (typically OECD) guidelines or equivalents.

The Act prohibits the use of data obtained from animals on or after 1 July 2020 to support introduction of new ingredients with an end-use only in cosmetics and chemicals with multiple end uses (including in cosmetics), with some exceptions. Australia also has more general protections for the welfare of animals used in science, notably the *Australian code for the care and use of animals for scientific purposes* (the NHMRC Code), which is endorsed by the major research funding bodies and research providers. The NHMRC Code is adopted under state and territory legislation, providing a framework for ethical and responsible care and use of animals for scientific purposes in Australia.

It appears that most chemical introductions originate and have safety data generated overseas. Registration data may be produced in-house or by a contract research organisation (CRO). The CRO and consultancy sector for industrial chemical registration in Australia is underdeveloped. Industrial chemicals may not offer the profit margins of specialised pharmaceutical or pesticides, limiting the incentive to develop NAMs for these chemicals. Often overseas-generated submissions do not meet Australian standards because of deficiencies in human safety data specifically required by the Australian regulator. They may also fail to address unique Australian requirements, for example, for toxicological data relevant to ecosystems found only in Australia.

There is a complex web of legislation, governance mechanisms and participants in the global 3R and NAM domain. The European Union, United Kingdom, and the United States are the most active, but other countries also have initiatives. The main elements of the jurisdictional responses are:

- # Regulations to restrict animal testing, although these are generally confined to cosmetic testing.
- # Centres to facilitate the advancement of the 3Rs, such as the National Centre for the 3Rs (NC3Rs) in the UK. These centres work with academia and industry to support research into NAMs and the development of skills and change in institutions to support their adoption.
- # Bodies to validate and publish on NAMs, such as the EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and the US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). These bodies cooperate internationally.
- # Strategic documents to guide advancement of the 3Rs, such as the US 'roadmap'.

Australia has world-class research groups developing test methodologies. However, often the path to commercialisation of research is not clear, exacerbated by the under-developed CRO network. Australia also lacks the coordinating, cross-disciplinary mechanisms, present in other countries, to promote the development, validation and application of 3Rs and NAMs. Australian individuals and organisations contribute to global activities (e.g. OECD), but there is little formal coordination within Australia.

Key finding: Support for stakeholders may be needed to provide NAM solutions to ensure that specific domestic needs are met for the Australian market. A roadmap for development and leadership is desirable.

Australia could enhance its relationships with jurisdictions that have larger chemical sectors and more advanced institutional responses to encourage progression to NAMs in toxicity testing by:

- # Promoting funding for 3Rs research, and specifically NAM development and commercialisation.
- # Promoting activities to advance 3Rs in Australia, in all fields that use animals, such as coordinating information exchange, supporting researchers, facilitating access to resources and providing training. The effectiveness and sustainability of efforts to advance the 3Rs will require fully engaged public and private stakeholders.
- # Promoting adoption of internationally-validated NAMs/alternative test methods, with a specific focus on unique Australian needs. This would help Australian companies access globally-accepted NAMs needed for overseas registration. Participation in the International Cooperation on Alternative Test Methods would ensure an Australian representation in global decision-making on NAMs.

Australia needs a strategy or 'roadmap' that articulates its needs and ambitions in relation to the 3Rs and identifies the means to achieve them. The strategy should consider what data is available or required to set performance targets. This requires national leadership and facilitation. The US roadmap provides a national model for consideration.

Australia stands to benefit from advancement in alternatives to animal testing. It is a relatively small player in industrial chemicals and NAM development by international standards and may not be able to replicate all of the mechanisms available in larger jurisdictions. Coordination is key for Australia and could stimulate research in NAM development and commercialisation. Economic benefits for Australian chemical companies would flow for those working in global markets. A national strategy would help Australia address its unique (e.g. ecological) needs for chemical assessment and regulation, which is particularly important for Australia as a 'data taker'. Last, and above all, animal welfare outcomes are likely to be improved more rapidly as the 3Rs gain a greater focus.

ACRONYMS AND ABBREVIATIONS

ACROYMNS	DESCRIPTIONS
Agvet	Agricultural and veterinary
AICIS	Australian Industrial Chemicals Introduction Scheme
ANZCCART	Australian & New Zealand Council for the Care of Animals in Research and Teaching
AOP	Adverse outcome pathway
APCRA	Accelerating the Pace of Chemical Risk Assessment
APVMA	Australian Pesticides and Veterinary Medicines Authority
CADD	Computer-assisted drug design
CAAT	Center for Alternatives to Animal Testing
CaCVAM	Canadian Centre for the Validation of Alternative Methods
CAS	Chemical Abstracts Service
CCAAM	Canadian Centre for Alternatives to Animal Methods
CRO	Contract research organisation
DNA	Deoxyribonucleic acid
EAGMST	Extended Advisory Group on Molecular Screening and Toxicogenomics (of the OECD)
ECETOC	European Centre for Toxicology and Ecotoxicology of Chemicals
ECHA	European Chemicals Agency
EPA	Environmental Protection Agency (of the US)
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
EU	European Union
FDA	Food and Drug Administration (of the US)
FSANZ	Food Standards Australia and New Zealand
GLP	Good Laboratory Practice

GRAP	Good Read-Across Practice
HREC	Human Research Ethics Committee
HRIPT	Human repeat insult patch test
HTTK	High-throughput toxicokinetic
IATA	Integrated Approaches to Testing and Assessment
ICATM	International Cooperation on Alternative Test Methods
ICCR	International Cooperation on Cosmetics Regulation
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ILSI-HESI	International Life Sciences Institute – Health and Environmental Science Institute
INCI	International Nomenclature Cosmetic Ingredient
JaCVAM	Japanese Center for the Validation of Alternative Methods
KoCVAM	Korean Center for the Validation of Alternative Methods
MAD	Mutual Acceptance of Data
MoA	Mode of action
NAM	New approach method / methodology
NC3Rs	National Centre for the Replacement, Refinement & Reduction of Animals in Research
NGRA	Next Generation Risk Assessment
NHMRC	National Health and Medical Research Council
NGO	Non-governmental organisation
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NTP	National Toxicology Program
NZ	New Zealand
OCS	Office of Chemical Safety
OECD	Organisation for Economic Cooperation and Development

PBTK	Physiology-based toxicokinetic
PoD	Point of departure
QSAR	Quantitative structure-activity relationship
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
RNA	Ribonucleic acid
SEARCH	Sharing Experimental Animal Resources, Coordinating Holding
SEURAT	Safety Evaluation Ultimately Replacing Animal Testing
SRG	Stakeholder reference group
TG	Test Guideline (of the OECD)
TGA	Therapeutic Goods Administration
TSCA	Toxic Substances Control Act
US	United States
USDA	United States Department of Agriculture
WNT	Working Group of National Co-ordinators of the TGs programme

1. PURPOSE OF THE REPORT

This report is prepared for the Commonwealth Department of Health and Aged Care and addresses a commitment by the Australian Government during the passage of the *Industrial Chemicals Act 2019*. This commitment was to investigate the development and uptake of new methods to replace animal use in regulatory testing of industrial chemicals.

The purpose of the project is to develop a report that:

- ⊕ examines existing practical examples, as well as emerging techniques to reduce the reliance on animal testing across relevant sectors (including cosmetics and other industrial chemicals) and identifies best practices and innovative approaches already being undertaken
- ⊕ analyses domestic and international evidence around non-animal testing methods
- ⊕ identifies opportunities for collaboration domestically and internationally, and the potential for Australia to leverage activities and research occurring both nationally and overseas
- ⊕ investigates data accessibility and develops advice on practical steps to support innovation and information sharing (shared information/data/publish statistics).

The report has been confined to industrial chemicals and does not encompass pharmaceuticals or medical testing.

2. METHODOLOGY

The project methodology comprised the following steps:

1. Stakeholder reference group. A stakeholder reference group (SRG) was established, comprising individuals representing Australian regulators and Commonwealth agencies, peak bodies and non-governmental organisations (NGOs) identified in consultation with the Department of Health and Aged Care and the National Health and Medical Research Council (NHMRC). The organisations participating in the SRG are listed in [Appendix 1](#).

The SRG met by videoconference in August 2021 to share its perspectives on the major gaps in progress towards non-animal testing and to provide guidance to the consultancy team on the best sources of information for the project.

2. Targeted literature review and environmental scan. The consultancy team undertook desktop research into the industrial chemical registration environment in Australia and internationally, with a more specific focus on the development and uptake of alternatives to animal testing. Members of the SRG provided documents and pointers to information that were very useful to the project.

The consultancy team also conducted a targeted review of the scientific literature on the development of alternatives to animal testing. It is important to note that this scoping report is not an exhaustive, systematic review of every test method and approach, but rather an overview of the domain with information on current progress, barriers, international collaborations and regulatory trends in this evolving field.

3. Consultations. The team held one-on-one discussions by videoconference with a range of individuals from within and outside the SRG, including international experts. Additional information was provided by email by some experts. The consultation process was focused on collecting information on international research and the status of Australia's progress towards non-animal testing. A uniform and systematic consultation process could not be applied to identify scientific information and resources. The team also considered advice from the SRG to finalise the report.

3. SCOPE AND DIMENSIONS

This report is confined to the testing of industrial chemicals. It does not specifically encompass related domains such as pharmaceutical development, although such fields are tangentially referred to in parts of the report because of the high degree of overlap with industrial chemical testing.

The Australian Industrial Chemicals Introduction Scheme (AICIS) defines industrial chemicals as those with an industrial use. Industrial use is defined by exclusion, that is, it is any use that is not:

- ⊕ an agricultural chemical product – as defined by the AgVet Code
- ⊕ a veterinary chemical product - as defined by the AgVet Code
- ⊕ use as a substance or mixture of substances prepared by a pharmacist or veterinary surgeon, or in the preparation of these – as defined by paragraph 5(4)(a) of the AgVet Code
- ⊕ a therapeutic good – as defined by the *Therapeutic Goods Act 1989* or
- ⊕ use as food for humans or animals, or in the preparation of it (What is an industrial chemical? n.d.)

Industrial chemicals include:

- ⊕ any chemical element with an industrial use

- # compounds or complexes of chemical elements, where the compounds or complexes have an industrial use
- # UVCBs [unknown or variable composition or biological substances] with an industrial use
- # chemicals released by an article, where the article has an industrial use
- # naturally-occurring chemicals with an industrial use.

Industrial chemicals are used in such applications as cosmetics (including skincare, make-up and hair products, soaps, deodorants, perfumes, toothpastes), plastics, paint, glue, ink and cleaning products. Industrial chemicals often have multiple uses.

It is notable that Australia is unique (at least among major Western jurisdictions) in regulating cosmetics as 'industrial chemicals', which are not regulated as medicines.¹ Other jurisdictions have specific regulations for cosmetics or treat them similarly to drugs. For example, cosmetics are regulated by the Federal Food, Drug and Cosmetic Act (Food and Drug Administration 2022a) in the United States (US), and by the 'cosmetics regulation' in the European Union (EU). This is further discussed in section 7 of the report.

In relation to animal testing, two important concepts are introduced here:

1. The '3Rs'. The concept of the 3Rs was developed over 50 years ago. The *Australian Code for the Care and Use of Animals for Scientific Purposes* (the 'NHMRC Code') defines alternatives under each of the 3Rs as:

- # Replacement: Methods that permit a given purpose of an activity or project to be achieved without the use of animals.
- # Reduction: Methods for obtaining comparable levels of information from the use of fewer animals in scientific procedures or for obtaining more information from the same number of animals.
- # Refinement: Methods that alleviate or minimise potential pain and distress and enhance animal wellbeing (National Health and Medical Research Council n.d.).

The 3Rs provide a valuable framework for understanding how the humaneness of research can be progressively improved. They acknowledge that, whilst 'replacement' of animals in research should be the ultimate goal, important advances can be made in 'reduction' and 'refinement'. The 3Rs is well accepted internationally and explored in greater detail throughout this report.

It should be acknowledged that the 3Rs have been challenged as a policy tool to improve the welfare of animals in medical and scientific research. This view argues that the 3Rs have been at once a notable success and a failure. They have succeeded, when measured by their widespread acceptance and recognition. On the other hand, the 3Rs have failed, because they have neither reduced the number of animals used in experiments nor ameliorated their suffering. The principles enshrined in the 3Rs are often watered down in practice by phrases such as 'wherever practical'. Blattner argues that the 3Rs should be retained, but that three fundamental reforms are needed (Blattner 2019). First, the hierarchy of the 3Rs should be reversed to put 'replacement' first² and give it absolute preference; second, the balance of interests between human and animal in justifying an

¹ C. Brock, pers comm

² Note that this reversed sequence is the one used in this report

animal test should be completely reframed; and third, legal frameworks should establish explicit, intrinsic rights of animals.

2. Definition of 'animal'. The *Industrial Chemicals Act 2019* defines animal test data as that obtained from any live vertebrate animal other than a human being, or 'any animal of a kind prescribed by the rules'. The *Industrial Chemicals (General) Rules 2019* prescribe the inclusion of data from cephalopods (such as octopus or squid) in this definition.

There is considerable debate as to the extent to which sentience, '*the ability of animals to feel and experience emotions such as joy, pleasure, pain and fear*' exists in lower-order animals (Proctor et al. 2013). Even cephalopods, which are widely regarded as highly developed and 'intelligent' among the invertebrates, may not possess '*a capacity for conscious emotion and for suffering after noxious experience*' (Walters 2018). Alternatively, cephalopods and even less-developed species may in fact possess this attribute, but it may never be possible for humans to tell. For example, zebrafish may be considered a less sentient organism than rodent species. Zebrafish are also easier to house and therefore become less stressed than larger animals, improving the outcome of experiments (*Five reasons why zebrafish make excellent research models* | NC3Rs n.d.).

This report does not seek to make any contribution to this particular debate. The issue is raised here because the use of alternative species is often considered part of a valid 3Rs approach. Notably, the UK is set to amend its new animal welfare legislation to recognise the sentience of decapods and cephalopods ('Eating a lobster this Christmas? It may once have had feelings' 15 December 2021). It is also notable that the NHMRC Code applies to 'the care and use of all live non-human vertebrates and cephalopods' (National Health and Medical Research Council n.d.).

4. THE SCIENCE OF ANIMAL TESTING: LITERATURE SUMMARY

This section provides a summary of the scientific literature on animal testing and the development of alternative, non-animal test methods and other 3Rs approaches. This section is an overview of the domain and not an exhaustive, in-depth review of every new test method, as the domain is enormous in scope and continuously expanding. The objective is to provide an overview of status, progress, challenges and opportunities for replacement of animal tests in chemical toxicity assessment. This section is not confined to the testing of industrial chemicals. It would not have been useful to have confined the review to industrial chemicals, as many of the new testing technologies discussed are used in related fields such as disease research and drug discovery. The material presented has been written with educated readers rather than scientific experts in mind.

4.1 HISTORY OF ANIMAL TESTING

The use of animals in research and training has been regarded as pivotal to the advancement of science for many centuries (Cheluvappa et al. 2017). This reflected the thinking that animals make effective models for humans and as such can be used (substituted for human subjects) to study a range of fields including anatomy, physiology, biochemistry, pathology, and toxicology.

Over time, the use of animals in research and teaching has become subject to increasingly strict rules and ethical guidelines. The '3Rs' approach to the use of animals in research was first developed in the 1950s (Kirk 2018). As noted in section 3, the 3Rs are replacement, reduction and refinement. The research community was initially slow to adopt these principles. Medical historians have attributed this lag

to ambiguity in the original 3Rs publication and to a different emphasis held at the time between scientific and human values. Over time, human values have increasingly come into conflict with the drivers underpinning scientific advancement. This widening gap appears to have been rooted in the (artificial) division of society into two cultures, the humanities and the sciences, that was prevalent within British-based universities of the 1950s and 1960s. Scientists held firmly to the belief that the practice of (good) science, with its openness to truth, was a model for good citizenship. The humanists believed that the 'institutionalisation of the sciences' eroded human values and thinking in favour of a focus on 'scientific proofs'. Over time, a middle view gained predominance that society should position itself somewhere between total abolition of animal testing and scientific libertarianism (Balls 1994).

The increasing understanding that the experimental animal's world is (for the most part) a human construct has made the engagement of both the humanities and the sciences accepted by all, and essential for determining the most suitable role for animals in research. Animal ethics is not a set of stringent rules mandating researcher conduct but is more a guideline designed to express the human moral obligation towards the animals used in research.

Publications recommending humane treatment of research animals have been produced from the nineteenth century onwards. The first statute protecting animals was the *Cruelty to Animals Act 1876* in the UK, and this was matched by similar laws in other jurisdictions in the following years. The 1986 Council of Europe Convention produced a directive specifically addressing obligations of member states on the ethical use of animals for research (amongst other animal-welfare based directives) that reflected and extended the 3Rs approach to recognise that animals have capacity for 'suffering and memory'.

Of the 3Rs, replacement is the method that historically was the least used for minimising animal use in experiments (Gruber and Hartung 2004). However, the development of more replacement technologies (such as organs-on-chips) has allowed scientists to perform experiments without animals that would not have otherwise been possible without using traditional animal models (MacArthur Clark 2018). Despite this, the number of animals used in experiments and testing in Europe continues to increase.

A review of trends in non-medical toxicity/safety testing in Great Britain comparing the number of procedures (of all tests) undertaken between 1987 and 1992 found an 84.8 per cent reduction in tests of cosmetics and toiletries and an 84.8 per cent reduction in tests of environmental pollutants. However, the same review found only a 0.6 per cent reduction in tests of agricultural chemicals and a 30.6 per cent increase in both animal and non-animal tests of industrial chemicals (Balls 1994). A more recent publication (2014) confirmed reduced animal use between 2006 and 2010 (53 per cent) arising from the application of 3R principles for pharmaceutical toxicity testing (Törnqvist et al. 2014). However, an analysis of overall trends in animal use in toxicology, with a European focus, found variation between countries (Allen and Waters 2013). Data for the twenty-first century from France, Germany, the United Kingdom, Italy and Belgium was analysed using 1999 as the reference year (each included country used 200,000 animals or more for toxicity testing in 1999). The study found an overall modest decline of 13 per cent in animal use in 2008 compared to 1999, but with important country-specific variations. Italy's decline was substantial, Belgium bucked the trend and increased its use of animals, Germany's use pattern was steady, France's numbers increased then declined rapidly before 2008 and the UK declined consistently in the early 2000s but experienced a dramatic increase from 2005 to 2008. These findings suggests that use of animals for toxicity testing in non-medical and non-therapeutic chemicals remain an important challenge for reducing animal use.

Gowans reported in 1974 that the Hansard from the House of Commons for 11 May 1973, when discussing the *Cruelty to Animals Act 1876*, stated '*People will not search for alternatives as long as they have animals to use*' (Gowans 1974). It seems this is still relevant today. The key driver of change to non-

animal-based testing will be limiting the right to use animals for certain research or testing. Concrete legislation mandating the ethical use of animals in research is now considered essential for effective implementation of best practice use of animals in research.

4.2 ADVANTAGES AND DISADVANTAGES OF ANIMAL MODELS

Animals have traditionally been used in research because of (modified from Gallup and Suarez 1985):

- # **Complexity:** animals are functional organisms that best represent the human targets of study. This complexity allows trust in results being extrapolated to the equally complex human targets.
- # **Control:** animals can be placed into controlled environments and managed to minimise variation, except for the variable in question. This is more difficult to do with free-living human subjects.
- # **Interventions:** in general, a wider range of interventions can be studied using animals than are available to researchers using human subjects or non-animal models.
- # **Objectivity:** it is easier for researchers to objectively assess responses of animals using a system of measurements and observations. A larger number of detailed objective measurements are also possible in animal studies, for example organ weights and histopathology of a wide range of tissues. Maintaining researcher objectivity can be difficult when working with (articulate) human subjects.
- # **Genetics:** selective breeding can be used to provide suitable animal study subjects (like nude mice³). This is not possible for human studies.
- # **Practicality:** in general, live animal studies are more convenient and accessible than alternatives.

Notwithstanding these perceived benefits, animal models may not be as effective as first thought. It is becoming clearer that there has been an over-reliance on a limited number of model organisms, many of which have been subsequently shown to have diverse (i.e., different) molecular initiation events and endocrine-disrupting and -modulating chemical sensitivities or pathways (Gaw et al. 2019).

Ecological toxicity of a chemical can also be difficult to estimate from limited studies of a single chemical exposure in a target species.

Only around 11 per cent of chemicals (i.e. candidate drugs) trialled in human subjects reach the application step for registration, with 25 per cent of application dossiers subsequently failing registration. Over half of all new chemical entities fail in phase III clinical studies (large-group efficacy studies) (Holmes et al. 2009). Most candidates progress towards registration using data from animal studies. The high human-use failure rate suggests that for many situations an animal is not a suitable substitute (or model) for the human target.

Thus, alternatives to animal models may be required on both ethical and performance grounds to fully understand new chemicals, diseases or treatments.

4.3 DEVELOPMENTS IN THE 3RS

As noted above, an enormous research effort has been made to reduce the impact on animals of chemical testing and related fields such as medical research. This section aims to provide a cross-section

³ A 'nude mouse' is a laboratory mouse from a strain with a genetic mutation that causes a deteriorated or absent thymus, resulting in an inhibited immune system due to a greatly reduced number of T cells

of these 3Rs approaches and salient examples. It is intended only as a high-level survey and does not provide an exhaustive listing of every published model.

IN VIVO MODELS

In vivo models do not always replace animals but rather shift the welfare impact to species or life stages considered to be of lesser sentience such as chicken embryos or invertebrates such as insects and worms. *In vivo* models are generally considered to be the least preferred replacements to existing animal models because they may only shift the burden of harm between species.

Incubated chicken eggs can be used to study ocular and mucosal toxicity, angiogenesis, heavy metal toxicity, tumour biology, drug impact etc. Incubated chicken eggs have been used to replace nude mice and results correlate well with cell culture studies and *in vivo* animal studies. The chorioallantois is a vascularised membrane surface that has excellent characteristics for many studies, including chemotherapy efficacy studies, in which cancer cells are grafted onto the chorioallantois before exposure to the chemotherapy agent. Chicken eggs have been used to produce monoclonal antibodies, but this approach has since been superseded by *in vitro* (phage-based) methods. The embryo is insensitive in the first seven days of incubation, so egg testing meets the 3Rs requirements if done within this period (Rashidi and Sottile 2009). However, there are jurisdictional differences in the maximum allowable age of chicken embryos for use in experiments.

Terrestrial invertebrates are used in many disciplines of research (U.S. Congress, Office of Technology Assessment 1986). *Caenorhabditis elegans*, for example, has been investigated by the US Food and Drug Administration (FDA) as a model to detect developmental neurotoxins (Hunt et al. 2018). Marine invertebrates remain an untapped alternative. Invertebrates offer some advantages as specimens for biological research including their ability to reproduce rapidly, and experiments can be conducted quickly because large numbers of invertebrates are readily available. They are also inexpensive to keep and maintain and are not prone to spreading disease within their colonies.

Zebrafish (*Danio rerio*) have been used to study human cancers, especially spontaneously developed cancers. This is due to the similarity between fish and humans in gene sequence and expression (*Five reasons why zebrafish make excellent research models* | NC3Rs n.d.). Zebrafish are also recognised to be an effective species for development biology and for nuclear hormone receptor studies. Hydra are a genus of small fresh-water invertebrates with unique properties (such as an apparent lack of aging) and they have been used in studies of regeneration and environmental toxin exposure (Penza et al. 2009). Jellyfish, nematodes, earthworms, leeches, and fruit flies are other species commonly used in research that may have wider application to testing and chemical impact studies.

Whilst hydra are non-sentient, there is increasing evidence that fish are sentient and can experience and remember pain, suggesting that fish may not meet the 'replacement' criterion of the 3Rs under modern thinking. Fish are recognised as sentient in most Australian jurisdictions and in the NHMRC Code.

Plants and fungi have also been used as test models. Plants provide an advantage over animals in research in that they lack anything resembling an animal nervous system, so presumably they do not feel pain (U.S. Congress, Office of Technology Assessment 1986). A major disadvantage of plants, especially in toxicological/exposure studies, is that they possess a rigid cell wall, which can limit the transfer of chemicals across the wall into the cell. Plants are therefore often poor analogies to humans for toxin exposure studies.

Yeasts are eukaryotic, single-celled members of the fungus kingdom. As such they do not possess a rigid cell wall, and this makes them useful for chemical exposure/toxicology studies. They also possess active

steroid hormone systems and so can be used to study endocrinology. Brewing yeast has been used regularly to study cell death processes (Pasupuleti et al. 2016).

Whilst transgenic animals are still animals, they have been successfully used in circumstances that allowed researchers to avoid using higher-order species such as non-human primates, as well as rats and mice. Transgenic animals (with specific, targeted characteristics) also support smaller studies than may occur if animals were selected from the wider general population. The downside is that often large numbers of animals are required to produce a transgenic animal population and transgenic animals may be at greater risk of developing diseases such as cancers, infections, and diabetes.

EX VIVO MODELS

'Ex vivo' means 'outside of a living body' and refers to the use of tissues or organs taken from a living animal. Suitable animal organs can often be obtained from abattoirs. These can be maintained in isolated organ perfusion chambers and subjected to a range of experiments. For example, pig hearts have been used to study the effect of cardiotropic drugs on the electrical activity of the heart (Gruber and Hartung 2004). Abattoir-harvested pig brains have been used to study blood-brain barrier disease (Patabendige 2012). Blood-brain barrier research has increased exponentially since the 1970s because of the increasing prevalence of neurodegenerative disease in the aging human population. An effective non-animal alternative model with potential to provide human-relevant data will have a large effect on the use of animals in that area of research.

Two examples of *ex vivo* models that can be used for the testing of industrial chemicals in Australia are the Organisation for Economic Cooperation and Development (OECD Test Guideline (TG) 437 (bovine corneal opacity) and TG 438 (isolated chicken eye) (*Appendix - acceptable test guidelines for categorisation* n.d.).

Isolated organs are particularly suited to toxicity studies where proven diagnostic tests for organ function can be applied to organs exposed to high levels of a chemical (higher than may be possible in a live animal study operating under an animal ethics committee approval). Similarly, drug dosage studies can be effectively undertaken in perfused organs. Human tissues and organs can be used similarly to animal equivalents. The question is how human organs are obtained, stored, and dispensed for use in research. Patients (donors) must agree to the use of their tissues in research in some jurisdictions yet must actively refuse their use in research in other jurisdictions. The creation of a managed network of research tissue banks has been suggested to allow reasonable access by researchers to tissues and organs. This is important to ensure consistency in supply and to control variability in tissues and organs, thereby providing confidence in results obtained using these methods. A repository of fresh gestational tissue (PLACENTA; PathLink Acquired gEstatioNal Tissue bAnk) was recently piloted in the US to allow for molecular, cellular and proteomic research (Arck 2019). The use of human tissues has been supported by the development of systems for maintaining tissue health and functionality in research. The ethics and challenges of using human tissue for *in vitro* research have been extensively reviewed (Thasler et al. 2006). There are legal, ethical, and commercial considerations in the use of human tissue in research and testing. This is discussed further below.

IN VITRO MODELS

In vitro models are studies of living cells 'within glass'. The definition has expanded to include organised structures of cells and cell lines. A high-level summary of the advantages and disadvantages of *in vivo* and *in vitro* studies is presented in Table 1.

Table 1: Summary of advantages and disadvantages of in vivo and in vitro test methods

Source: Adapted from Gruber and Hartung 2004

MODEL TYPE	ADVANTAGES	DISADVANTAGES
<i>In vivo</i> models	<ul style="list-style-type: none"> ⊕ Homeostatic environments can be more easily maintained and replicated ⊕ Completeness – absorption, distribution, metabolism and excretion of chemicals and toxins can be studied ⊕ Systemic responses can be studied ⊕ Low tech system or basic research – just need animals 	<ul style="list-style-type: none"> ⊕ Ethics approval can be challenging to obtain ⊕ Species differences – animal model findings may not extend directly to humans ⊕ Cost – can be prohibitive ⊕ Statistical power often low – it can be challenging to include sufficient replicates ⊕ Difficult to replicate or reproduce
<i>In vitro</i> models	<ul style="list-style-type: none"> ⊕ Cost – often low to run (can be expensive to establish the facility and resources to begin with but comparatively less expensive than animal experiments) ⊕ Throughput – often much easier and faster to get required numbers (study power) ⊕ Replicates – easier to get sufficient power with additional (low marginal cost) replicates ⊕ Simplicity – a specific response of a cell line can be studied in isolation from the rest of the organism ⊕ Accessibility – it is usually easier to measure and monitor cell lines than living animals ⊕ Less variability – this can control variance and increase study power ⊕ Potential to use (waste) human materials – such as skin removed in surgery, blood etc (often called <i>ex vivo</i> models) 	<ul style="list-style-type: none"> ⊕ Difficult to control all artefacts – such as those introduced by media or reagents ⊕ Low variability in cell lines – may not reflect the population at large, making extrapolation difficult ⊕ De-differentiation – cell lines may receive signals in vitro that makes them lose differentiation (i.e., regress away from the target tissue), making results less applicable to the target tissue ⊕ Long-term exposure and maintenance can be challenging ⊕ Cell density issues – <i>in vitro</i> cell lines are typically orders of magnitude less densely packed than target tissues, not reflecting the cellular interaction in the organism and this may impact results ⊕ Oxygen supply – this limits size of <i>in vitro</i> replicates; cells can only access the oxygen dissolved in the media so cultures cannot be too large

Several of the advantages and disadvantages listed in Table 1 are arguable. For example, it is not clear that it is more difficult to control artefacts in an *in vitro* than an *in vivo* model.

CELL CULTURES

Cell cultures are the mainstay of *in vitro* methods. Whilst great gains have been made in cell culture, there is still an incomplete understanding of how to maintain cells and organs indefinitely *in vitro*, and how to adapt them to substitute animals in research effectively. For example, hepatocyte cultures have not been able to maintain liver-like function for prolonged periods of time. This limits their use in long-term exposure studies. Studies that hold hepatocytes in a liver-like structure (with collagen) and include recruitment of hepatic stem cells to control the generation and maintenance of the hepatocyte line and the co-habitation with macrophages, to maintain the cellular environment and to allow effective hepatic inflammation studies, are showing promise. Such lines are providing *in vitro* data that has a strong correlation to *in vivo* study data.

Human brain cell cultures may provide the opportunity to study cell electrical responses to exposure to chemicals. These so-called 'mini-brains' can amplify the response of brain cells to foreign substances. Neural imaging provides increased sensitivity to detecting neuron responses to stimulation (including toxins). Brain-cell and neural imaging combinations appear to be very effective for studying neurotoxicity (Walum et al. 2005a, 2004).

TISSUE MODELS

Tissue models are constructs that are, essentially, *in vitro* cells maintained within a biochemical, physiological, and architectural environment that mimics the tissue as it would be within the human homeostatic environment.

Several human skin equivalent models exist (including the pioneer EpiDerm®). These have been developed to suit a range of uses and pathophysiologies, many to study specific diseases such as malignancies (Cheluvappa et al. 2017).

Multi-cell-type tissue models have been developed. The gut-liver axis model contains liver parenchymal cells and immune system cells in an appropriate architecture to allow meaningful study of sepsis and sepsis risk. Similarly, stem cells have been used to develop myocardial and neuronal cells for examining impacts of chemical exposures on cardiac health.

Tissue models are further classified into two-dimensional (2D) or three-dimensional (3D) forms.

2D models are classical monolayer cultures of cells. These are often effective for studying the impact of certain chemicals and inflammatory mediators on cell function (Loewa et al. 2018a). They can be challenging to use in studies of inflammatory cell responses, and for this reason co-cultures with immune cells have since been developed. However, because combinatorial cultures do not have the appropriate tissue architecture, they are less suitable for studying the interplay between cells and cell barriers, such as the epidermis.

3D models are representations of the cells *in situ*. They contain relevant cells on an artificial three-dimensional solid scaffold. They are more suitable for studies of organs and membranes than 2D models. They have been used to successfully study skin, liver, lung, heart and the central nervous system (Loewa et al. 2018a). A collagen gel on a silicon mould has been used to build a microvessel 3D structure suitable for studying many aspects of blood vessels (Rosania 2013). Three-dimensional culture techniques continue to improve allowing study of membranes and organelles within tissues. For example, 3D epidermal models have been used to study the effects of cytokine cocktails on keratocyte response, and to examine genetic modifications on the formation of the epidermal layer. The capacity of a 3D model

to include migrating immune cells provides greater capacity for *in vitro* studies of immune responses and diseases in tissues that invoke an immune response.

A high-level comparison of 2D and 3D models by Loewa *et al.* is provided in Table 2.

Table 2: Advantages and disadvantages of *in vitro* 2D and 3D models

Source: Loewa *et al.* 2018a

	2D MODELS	3D TISSUE MODELS
Structure	Simple monolayer or co-cultures, no stratification	Epidermal differentiation and maturation, stratification
Application	Initial studies on cell reactivity and cell-cell interactions	Investigation of more complex tissue-specific effects in normal and diseased states
Air-liquid interface	No	Yes, crucial for stratification
Dynamic cultivation	No	In principle possible, but improvements are required
Preparation and cultivation time	Short; a few days	Up to several weeks
Cost	Low	High

Tissue models also offer advantages over basic cell culture models and animal models in study design (e.g. Loewa *et al.* 2018b). They can be designed and constructed to minimise (natural) variation between study units, and this can reduce the number of replicates required to provide sufficient power for studies. They also can partly overcome some of the issues of lack of extrapolatable results from animal or human cell culture-based toxicological studies (Ripoli *et al.* 2019).

The challenge for tissue models is to effectively incorporate multiple (i.e., more than two) cell types to mimic an organ structure, and then to define suitable disease or toxicity models for study using these constructs. Tissue models are also hampered by a lack of vasculature that mostly arises from their static cultivations. This limits the size of the tissue model, and the resultant physical characteristics of the constructed tissue may differ in important ways from the real tissue. Dynamic perfusion chamber models may help address some of these limitations.

Despite the sophistication of 3D models, they still cannot mirror the integrated complexity of the animal model. This means systemic responses can be difficult to study in 3D (and 2D) models. This is emphasised by the absence of the full suite of immune cells. Finally, there can be a lack of comparability between models. Specific models, built with dedicated cell lines and held in different media, may not provide comparable results when subject to the same study. Interpreting results can be a challenge.

There is movement towards building (natural) complexity into 3D models by incorporating techniques from multiple disciplines. This integrated approach is called tissue engineering and it is an interdisciplinary field with inputs by scientists, chemists, bioengineers, biologists, physiologists, and clinicians. Tissue engineering is contributing to treatment (through therapeutic implants) as well as research in areas such as skin corrosivity and irritancy, and epithelial surfaces such as oral, corneal, urogenital, digestive, and pulmonary linings (Holmes *et al.* 2009).

Notably, tests based on 3D tissue models have been validated for skin and eye irritation. For example, the reconstructed human epidermis (RhE) model, which is comprised of living human keratinocytes cultured to form a highly differentiated epidermis, is used for skin irritation testing under OECD TG 439. The reconstructed human cornea-like epithelium (RhCE) model is used for eye irritation testing under OECD TG 492 (Barthe et al. 2021). Both of these tests are accepted for use for testing of industrial chemicals in Australia (*Appendix - acceptable test guidelines for categorisation n.d.*)

The challenge in using tissue engineering to build non-animal research models is in defining the essential components of the microenvironment. Developments in incorporating simple vascular networks into 3D tissue models are progressing, but they do not currently (and perhaps may never) match the complexity of whole organs (or organisms). The vision of engineering replacement organs for implantation into human subjects is driving interest and activity in tissue engineering, and successful implants have been developed (and implanted).

Tissue engineering and subsequently developed tissue models may not fully represent the target tissue, making it challenging to fully rely upon them for drug registration (for example). However, they are at least mature enough to provide an effective screening role and thereby eliminate toxic candidates without requiring animal testing.

ORGANS-ON-CHIPS

Organs-on-chips are micro-engineered systems that use computer chip manufacturing technology to produce microfluidic channels lined by living human cells in an architecture that mimics the structure and functionality of a target organ (Cheluvappa et al. 2017). These systems can be used to study organ-level pathophysiology. A lung-on-chip has proven successful at mimicking pulmonary oedema (Rosania 2013). Multi-organ-system chips are being developed to replace animals in pre-clinical testing of drugs and for toxicology studies of cosmetics and chemicals as well as pathophysiological conditions such as type 2 diabetes (Ripoli et al. 2019). Mass production of organ-on-chip systems has been trialled by Sony. A placenta-on-a-chip device has been developed to assist in study of reproductive development (Arck 2019).

OTHER APPROACHES

Animal **stem cells** can be directed into any of the germ layers. They can be controlled to express or suppress individual genes, and this allows for *in vitro* loss-of-function, gain-of-function, teratogenesis, pharmacological (cellular) and growth studies. Stem cell models are subject to the other challenges of *in vitro* cell cultures discussed above.

Immortalised cell lines often have reduced metabolic activity in certain areas. **Genetic modification** of these cells to make them more suitable to specific studies allows immortal cell lines to provide suitable cells for *in vitro* studies indefinitely, that is, without requiring future cell harvest from animals.

Artificial membranes are useful to study the transfer of chemicals across membranes, a fundamental area of research. Many chemicals, drugs, toxins, and hormones must transfer across a membrane to gain biological activity. Systems to study membranes are essential for research. Models of the blood-brain barrier developed from co-cultures of capillary endothelium cells and astrocytes have been developed to allow studies into neuropathological syndromes and treatments. The increasing prevalence of degenerative neurological conditions in man requires more detailed study of the potential neurotoxicity of agents administered to or arising from exposure in humans. These artificial membranes offer great promise for rapid and extensive scale up of these studies without requiring animals or human volunteers.

Many areas of research require specific **antibodies**. Monoclonal antibodies can now be derived from bacterial culture (sometime modified by bacteriophages), thereby avoiding the need to harvest antibodies from antigenically-stimulated animals. Antibodies can also be obtained by inoculating chicken eggs and extracting the antibodies from the egg yolk. If done before the seventh day of incubation there is no functioning nervous system within the chicken embryo and depending on the local jurisdictional requirements regarding embryo age, it may be used for experiments.

Bacteriophages (also informally called phages) are viruses that replicate within bacteria and other single-celled organisms. Phages have been used to cleave human antibodies into segments suitable for use in recombinant antibody studies and as vehicles for presenting antibodies or antibody fragments. Traditionally, animals were required to generate the antibody fragments, but this technique effectively replaces the use of animals. Around half a million animals are used every year to generate antibodies for use and research. Often, monoclonal antibodies made in laboratories are superior to polyclonal antibodies extracted from animals as they have greater specificity than polyclonally-derived alternatives. Monoclonal antibodies and antibody fragments built using these so-called phage models appear to be superior to animal-derived antibodies in diagnostic tests, therapeutics and non-therapeutic applications.

Human blood derivative models provide a working blood model replete with cellular and biochemical components. They are used to estimate the inflammatory, immune, and cell-based responses of the blood to challenge. They have been used to develop and are included within the monocyte activation test and cytokine-release immunotoxicity test and could potentially replace the limulus amoebocyte lysate test (that currently requires horseshoe crab blood). These models may be adapted to study the effects of toxins. An advantage of human-cell derived models is the removal of the inter-species difference effect on results that can sometimes be apparent within animal models.

Systems to grow **parasites** outside of the animal using media and exposing them to chemicals and/or antibodies have reduced the need for animal studies in this domain.

‘OMICS’

‘Omics’ is the common collective term for several related areas of biology study, the most common being genomics, transcriptomics, proteomics and metabolomics. This ‘omics’ cascade maps the sequence from gene to effect on cell function.

Genomics is the study of the structure, function, evolution, mapping and editing of genomes (a genome is the complete set of an organism's DNA). Genomics studies the interplay between all the genes and how this influences organ function. Most genes code for proteins, so genomics explores how changes to the genome alter the pattern of production of proteins and the interactions between proteins. This extends to cell and organ structure and function. Genomics is possible because of ability to identify the function of individual genes through DNA sequencing and bioinformatics.

Transcriptomics is the study of all RNA transcripts within an individual or cell. The transcriptome describes the subset of genes that are transcribed into RNA, and that eventually go onto produce proteins and intracellular signals. Not all RNA encodes a protein.

Proteomics is the large-scale study of proteins. The proteome is the entire set of proteins produced by an organism. This is coded by the genome (specifically, the transcriptome). Proteomics enables the identification of an increasing number of proteins and their activities. It is the next step in the study of biological systems. It is increasingly more complex as, whilst the genome is constant, the products of the genome (the proteins) are not; they are a function of which genes are active at the time and differ depending on the cell or tissue type and on the external stimuli received. Proteomics is used to identify

new chemicals that may have biological activity, proteins that interact with other proteins, and biomarkers that indicate a biological process is occurring, and to undertake structural analysis of proteins (to determine the architecture of binding sites for example).

Metabolomics is the scientific study of chemicals and intermediaries in chemical processes (called metabolites), or the 'chemical fingerprint', of a cell or organism. Metabolomics is the next stage of biological understanding after proteomics in that it examines the markers of the biochemistry and physiology of the cells, that is, the activity between the proteins and the chemicals in the cell. Metabolomes are often used to explore the toxicity profile of chemicals and other insults to living systems. For example, liver damage may be associated with a particular combination of chemicals and the identification of this footprint confirms hepatic toxicity following chemical exposure. Tools such as mass spectroscopy and nuclear magnetic resonance spectrometry allow the impact of a chemical or exposure on the functioning of a cell or organism to be examined by rapidly measuring the suite of chemicals within the substrate.

For the most part, 'omics' technology is applied as a tool in toxicology. The various 'omics' may be used together. For example, the hazardous effects of a chemical may be initially identified via metabolomics, and subsequently traced back to the proteins within the organism that are affected by exposure (proteomics), the RNA that encodes the manufacture of these proteins (transcriptomics) and finally the genes that code the proteins themselves (genomics). This sequence of known (or at least assumed) events from molecular initiation to adverse toxic effect is known as the 'adverse outcome pathway' (AOP). Once established, an AOP is a valuable inclusion into toxicity databases and this knowledge can assist in the identification of other toxic chemicals that use the same or a similar pathway.

There are gaps in this AOP reasoning, mostly related to the problem that many key events, whilst necessary, are often not sufficient on their own to continue the sequence (i.e., many may be shown to demonstrate a potential for toxicity rather than be proven to have a direct toxicity). Often these other (necessary) effects relate to exposure dynamics (i.e., pharmacokinetics) and the environment of exposure (e.g., other concurrent exposures). A 'mode of action' (MoA) pathway approach finds plausible substance-specific key events that follow the interactions between a chemical (toxin) and the metabolites of an organism which subsequently result in an adverse (toxic) outcome. A MoA may not fully define the pathway but identifies the association between exposure and toxicity and as such, MoAs are often incomplete. Practically, this means a chemical that is shown to be safe for a particular metabolic pathway may subsequently be found to be toxic to another (yet untested) metabolic pathway.

The OECD has taken a close interest in the development of 'omics' and its application in animal-free research (Organisation for Economic Co-operation and Development 2009). The OECD has formed working groups and has organised workshops to explore knowledge gaps in the use of the 'omics' in toxicological research and chemical registration. Toxicology is a one-to-many search; the question it seeks to answer is whether a particular chemical adversely affects *any* biochemical pathways. This wider-reaching toxicological application is currently beyond the capability and knowledge base underpinning 'omics' technology and precludes the use of 'omics' as a stand-alone suite of tools for determining toxicity (Boverhof and Zacharewski 2006). A more recent review found that despite significant progress in 'omics' science, barriers to routine use of 'omics' data by regulators include low transparency of the data processing methods used to convert raw 'omics' data into information, and non-standardised reporting of 'omics' data, metadata and methodologies to generate results. The OECD Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST) has established a project to develop guidelines for reporting of 'omics' data aimed at fostering regulatory approval (Harrill et al. 2021).

These MoA gaps in the link between cause (i.e. genes, RNA, protein etc.) and effect (metabolite) limit confidence in ‘omics’ output that show or clear chemical toxic responses (Buesen et al. 2017). Regulators such as the US Federal Department of Agriculture (USDA) are examining how to best use toxicogenomic data within their approval process. Currently, this is not obvious nor the pathway clear.

The requirements for effective ‘omics’-based toxicological assessment have been summarised (Boverhof and Zacharewski 2006), as shown in in Table 3.⁴ Many of these requirements have been addressed since this original publication. However, the regulatory environment demands reliable, standardised and repeatable approaches to be used. Regulatory dossiers must demonstrate and describe a consistency in conduct, analysis, reporting and inference from ‘omics’ data at a level that is typically beyond that required for most scientific publications. A formal, modular reporting framework developed by the EAGMST to support use of ‘omics’ data by regulators is presented in Table 4 (Harrill et al. 2021).

Table 3: Toxicogenomic challenges

Source: Modified from Boverhof and Zacharewski 2006

APPLICATION	NEED
Prioritisation of chemical lists	Establishment of a knowledge base
Deciphering mechanisms of action	Accessible and complete databases
Identifying biomarkers of exposure	Conserved metabolite, protein, mRNA and genetic fingerprints
Identifying biomarkers of toxicity	As above, with consistent analytical approach
Cross-species extrapolation	Using agreed logic and to the required quality standard
Identifying species sensitivities	Application of recognised risk-assessment methods
Analysis of mixtures toxicity	Cross-platform, cross-technology, multi-species, data aggregation methods

Table 4: Modules of an ‘omics’ data reporting framework

Source: Modified from Harrill et al. 2021

MODULE	DESCRIPTION
Study summary reporting module (SSRM)	Describes the minimum reporting set of elements required to provide a high-level overview of a regulatory toxicology ‘omics’ study

⁴ Boverhof and Zacharewski use the term ‘toxicogenomics’ to describe the integration of ‘omic’ technologies, bioinformatics and toxicology

MODULE	DESCRIPTION
Toxicology experiment reporting module (TERM)	Captures and reports key descriptors of <i>in vivo</i> or <i>in vitro</i> toxicology studies from which samples/data is used for 'omics' analysis
Data acquisition and processing reporting modules (DAPRMs)	Capture and report descriptions of 'omics' assays, data capture and processing prior to statistical analysis
Data analysis reporting modules (DARMs)	Capture and report statistical analysis method descriptions and analytical objectives and outputs used for 'omics' data

For the reasons described above, 'omics' has most application, at its present stage of development, as a screening tool for toxicity. Toxicogenomic databases are currently not at the required level of completeness to allow (solely) 'omics'-based registrations so other supporting data is required. Example databases include the International Life Sciences Institute – Health and Environmental Science Institute (ILSI-HESI), US National Center for Toxicogenomics (NCT), GeneLogic, ToxExpress and CuraGen. A collaboration between the ILSI-HESI Genomics Committee and European Bioinformatics Institute is developing a data format and standard for toxicogenomic data storage and exchange (Chan and Theilade 2005). The Biomedical Information Science and Technology Initiative (BISTI) is also examining the information system and risk assessment framework – how to infer and quantify toxicity risk from 'omics' data.

IN CHEMICO MODELS

An *in chemico* model assesses the interaction between a test chemical and a biological macromolecule. Several organic covalent interactions (between a chemical and a biological macromolecule) are known to result in alteration of the chemical's biological function (and toxicity profile in the host organism). Often the consequence of these interactions can be interpreted through assessment of the mechanistic organic chemistry reaction following mixing of the two. The stoichiometry of the resultant chemical reaction provides a quantitative measure of change in the chemical-organic molecule combination.

In chemico modelling requires knowledge of the MoA of the biological macromolecule and of the (altered) form of the macromolecule following interaction with the chemical. If the MoA is known, the effect of exposure to the chemical can be extrapolated from the measured change to the biological macromolecule. The challenge in toxicology is to define the relationship between the change in the concentration of the chemical-organic molecule and the assessment endpoint. This information mostly originates from animal studies. Where the relationship can be mapped, the resultant *in chemico* test presents as a quantitative toxicology method.

IN SILICO MODELS

Computer modelling can be used to study some pathophysiological phenomena, such as exposure to a toxin. It relies upon the ability to effectively code key aspects of the interaction between the chemical and the body, including pharmacokinetic parameters such as rates of gut / tissue absorption, tissue distribution, protein binding, metabolism and excretion, and pharmacodynamic activities of the molecule. Advanced techniques such as quantitative structure-activity relationship (QSAR) modelling can produce sophisticated estimates of a chemical's hazard-inducing capacity. These techniques have been successfully used to study pesticides, chemical carcinogens, and aromatic amines amongst others.

The receptor model of cell stimulation (lock and key) can be partly mimicked using pseudoreceptor modelling. This elucidates the three-dimensional shape of the receptor from knowledge of the shape of bioregulators (modulators of various cellular processes) and other ligands that bind to the receptor to varying degrees. This is the basis of QSAR technology. QSAR models are cheap, fast, and efficient, and as the training datasets improve and increase in size and breadth of chemicals within their library, they will become increasingly more adaptable. It is predicted QSAR methods alone will reduce the number of animals used in research by 30 per cent (Gruber and Hartung 2004). A study of sensitivity of pre-clinical data to detect drug-induced liver injury compared *in vivo*, *in vitro*, and QSAR analysis found that the QSAR-based method was more sensitive than *in vitro* approaches which were in turn more sensitive than *in vivo* studies at identifying hepatic toxicity risk (Dirven et al. 2021).

Computer-assisted drug design (CADD) is a recognised and mature approach to identifying new pharmaceutical compounds (Gruber and Hartung 2004). Many companies and research organisations use CADD to identify and to screen candidate molecules. CADD uses a similar approach to QSAR modelling. A database of chemicals and chemical structures is linked to a biological effect database and artificial intelligence is used to identify the key chemical moieties and structures that may have favourable biological activity. This suggests that QSAR models for replacing animals in research will develop to a similar degree, although perhaps the incentives to develop them are not as strong as for CADD.

A notable worldwide research initiative is the ‘virtual physiological human’ that aims, ultimately, to create an individualised ‘virtual twin’ that would permit personalised diagnosis and therapy of patients (*The Virtual Physiological Human – your ‘digital twin’* | Royal Society n.d.).

An emerging area of interest is nuclear hormone receptors. These are proteins found within cells that bind to hormones such as the sex hormones and thyroxine that subsequently attach to cellular DNA to modify gene expression. These are difficult to study because chosen animal models may not contain the same nuclear hormone receptor pathways, cell lines used in *in vitro* studies may also not mirror the parent tissue, and *in vitro* models are unable to be maintained for the periods required to study long-term exposure risk amongst other challenges (Penza et al. 2009). Whilst this may be an area for *in silico* studies, the complexity of the cascades that follow hormone activation / deactivation may be beyond current QSAR model capacity. Researchers in this area rely on proven animal models (such as zebrafish) and have developed molecular imaging techniques (using bioluminescence, for example) to identify changes following exposure.

However, computer modelling is limited in its reach. Whilst an existing computer model can be used to replace use of animals in research, all models are first built by codifying the exposure and biological activity and responses of animals; a new model requires this baseline data for it to be constructed. Also, as complexity is added to existing models, new relationships between model variables may have to be defined from animal studies. There is also a limit to the capacity to fully ‘code’ an animal and its response to a chemical. Whilst processor speed and computing resources continue to advance, the technique will be held back more by the inability to fully code every (essential) part of the working animal. This will likely limit the range of studies that can be undertaken and may limit the capacity of the model to identify all responses of the animal to exposure. The reasonably regular identification of adverse reactions to registered pharmaceuticals (post phase III trials) implies that not all causal pathways are known. For this reason, *in silico* models may have more of a role in screening testing rather than as a (complete) replacement model for adverse response assessment to chemical exposure (Walum et al. 2005b). QSAR models are ideal for high-throughput screening (HTS), allowing rapid assessments of a multitude of chemicals and metabolites.

Validation of a technique is a formal system of assessing appropriateness and usefulness of a tool for its intended purpose (Griesinger et al. 2016). Validation processes for non-animal model toxicity testing methods (including *in silico* models) require description of methodology (software for *in silico*), data sources, decision systems, supporting data and method documentation. The underlying methodologies and systems must be able to be independently assessed for completeness and fitness for purpose by independent parties.

Stanford University has developed a software simulation of an entire, single-cell organism and its life cycle. The model includes every gene and all of their known functions contained within 28 algorithms that code each of the major cell functions (Rosania 2013). The work is seen as the first step towards 'computerised laboratories' that could conceivably carry out a myriad of experiments and screen for new chemicals that may provide favourable biological activity.

Large databases of toxic chemicals that include information on the chemical structure and the toxicological responses in organisms can be used to develop specific artificial intelligence that predicts the toxicity response of novel chemical based on similarities to other chemicals (or chemical sub-structures), toxicity observations and biochemical mechanisms (see Ball et al. 2016 as an example). This is analogous to QSAR and CADD systems. This offers potential to screen new chemicals *in silico* for potential toxicity.

Most QSAR models are also used in drug discovery. These may be of limited use in toxicology because the questions asked by toxicologists have different emphasis, and the underpinning database of knowledge is also subtly different to that used in drug discovery. Many QSAR models used in drug discovery are proprietary and this limits accessibility. The OECD has developed a (Q)SAR Toolbox that is freely available and transparent to foster effective use of *in silico* tools in chemical toxicity profiling (*The OECD QSAR Toolbox - OECD n.d.*). Performance of QSAR models would likely be improved if basic biokinetic information (for example, net flux across bio-membranes and basic pharmacokinetic/dynamic parameters) could be included in databases. The CompTox Chemicals Dashboard, managed by the US Environmental Protection Agency (US EPA) provides much of this ancillary information for over 900,000 chemicals from 300 chemical groupings based on structure or category (US EPA 2016).

Other *in silico* tools dedicated to chemical toxicity include TopKat®, ToxTree® and Derek® Nexus. A 2016 study evaluated the performance of each *in silico* model for predicting ocular irritation of chemicals listed within the European Centre for Toxicology and Ecotoxicology of Chemicals (ECETOC) (Bhatarai et al. 2016). The models agreed on toxicity prediction for less than 65% of chemicals in ECETOC. Importantly, predictive performance was improved by additional chemical data including physicochemical property and electrophilic behaviour. This suggests that more than just association between chemical moiety (rule-based or statistical) and adverse response is needed for models to perform to the required standard. The addition of MoA and physicochemical property information improved classification performance by *in silico* models. This implies that toxicological assessments for chemicals without MoA information solely undertaken using *in silico* analysis are insufficient.

STUDIES WITH HUMAN VOLUNTEERS

Human volunteers offer some potential for replacing animals in research. An appropriately-informed human volunteer freely decides to participate or not for any study – whereas animal subjects do not have access to this choice. The use of human volunteers in cosmetic testing was examined by the European Commission Expert Panel on Effective Ways of Investing in Health (*Opinion - Guidelines on the use of human volunteers in the testing of potentially cutaneous irritant cosmetic ingredients or mixtures of ingredients adopted by the plenary session of the SCCNFP of 25 November 1998 | Scientific Committees*

n.d.). The committee considered the human testing of cosmetic ingredients should not be preferred to animal testing and summarised the testing of cosmetic ingredients on humans as follows:

- # Often there is limited (validated) animal-test data such that the predictive value of these tests is limited. This makes confirmatory (skin irritancy) testing in humans necessary on scientific and ethical grounds;
- # Confirmatory testing of ingredients or formulations in humans must be limited to situations where no irreversible damage to the subject is likely, and the study goal is reasonable, limited and of small scale; and
- # Recruitment of human volunteers should conform to the World Medical Association Declaration of Helsinki ('WMA - The World Medical Association-Declaration of Helsinki' n.d.) and the Good Clinical Practice for Trials on Medicinal Products in the European Union ('Good Clinical Practice for Trials on Medicinal Products in the European Community: CPMP Working Party on Efficacy of Medicinal Products' 1990).

Whilst the Expert Panel statements imply that human volunteers cannot replace all animal testing for cosmetic registration, the use of human volunteers can reduce the number of animal tests required to register some products.

Human volunteers fully represent the target species (humans); they are complete, functioning organisms and they can articulate their feelings and assess their own response (in addition to measured variables).

Human-volunteer studies appear suitable for drug microdose evaluation. Microdose studies are primarily aimed at determining the pharmacokinetics of a test chemical and are carried out using sub-therapeutic (theoretically safe) doses of a test drug on human subjects. Around 80 per cent of microdose studies estimated key pharmacokinetic parameters within a suitable margin of error to actual therapeutic doses when used in a clinical setting. However, microdosing studies by their very nature are not suitable for studying toxicity effects and cannot by themselves fully replace the use of animals in drug studies.

Biomarkers are parameters that can be measured objectively that can indicate the performance of a biological process. They can be used to measure normal biological or pathological processes and response to therapy or chemical exposure. Monitoring biomarkers in volunteer studies can provide insights into likely benefits of risk of wider-scale or larger exposures (Langley et al. 2005).

A test that is often used to generate data for fragrances and cosmetics is the human repeat insult patch test (HRIPT). The HRIPT is used to predict the risk of allergic contact dermatitis, a Type IV delayed hypersensitivity that may be induced by fragrances, preservatives, metals, and other substances. The HRIPT involve the application of a series of 'induction' patches followed by a 'challenge patch'. A recent review found wide variation in HRIPT protocols. There are marked differences in parameters such as type and size of patch, duration of patch application, test substance concentration, and number of participants per study. The authors of the review propose a framework for standardisation of HRIPT data collection and interpretation (Bormann and Maibach 2021).

Human volunteers in pain research provide more insight into the condition and its management than animal models because the human subject is able to articulate their perception of pain to the researcher, something an animal cannot do (Langley et al. 2008). Surrogate measures for human pain, such as chemical and electrical responses, can be substituted for human assessments of pain itself. Neuroimaging provides great insight into brain mechanisms and studies that use functional imaging often require fewer subjects than non-imaging-based methods. This especially has application in neuropharmacological studies, where microdoses can be studied for the effects on the brain through analysis of brain images.

There are some important constraints and caveats to the use of human volunteers. Ethics approval must still be obtained, even though a human participant can provide informed consent to participate. Human Research Ethics Committees (HRECs) are used in Australia when there are human participants in research. Studies that use humans or animals in research must meet respective requirements and satisfy relevant ethics committees before commencing. The remit of these committees most likely will extend to include review of studies in which humans serve as ‘guinea pigs’. There are also increased legal considerations; the responsibility for the well-being of the human subjects falls onto the researchers and research institute. This increases their requirement to design and justify their choice of experimental method. It is pertinent to note that all research involving the administration of drugs, chemical agents or vaccines to humans or devices in humans must be considered by an HREC to assess the appropriateness of their use. If such research is part of an Australian-based clinical trial, then it may need to be disclosed to or approved by the Therapeutic Goods Administration (TGA), which administers the Clinical Trials Notification/Approval schemes. Human volunteers are also more likely to agree to a study of a new therapeutic whereas fewer would submit to a toxicity study of an industrial (i.e., non-therapeutic) chemical, even within a microdose study design. As such there is likely to be only a limited role for human volunteers in chemical toxicity testing. NHMRC provides details on the ethical conduct of human research (*National Statement on Ethical Conduct in Human Research (2007) - Updated 2018* | NHMRC n.d.). All human research funded by the Australian Government must follow these guidelines and meet HREC approval.

4.4 AVAILABILITY OF NON-ANIMAL RESOURCES

TISSUE BANKS

Often animal model experiments have surplus tissue or a bank of potentially valuable material remaining at the end of the study. Some of this material may be useful later or to other scientists. A framework for collating and sharing these resources has been proposed (Morrissey et al. 2017). The Sharing Experimental Animal Resources, Coordinating Holding (SEARCH) builds upon findings that 70 per cent of surveyed researchers used animals in their research but 70 per cent of those who did not use animals said their work would benefit from an *in vivo* component. Of researchers who used animals, 70 per cent said they retained surplus material and 95 per cent would be willing to share their surplus material with other researchers. The SEARCH framework would provide a mechanism for researchers to donate surplus material, a database for researchers to interrogate knowledge bases to find suitable (surplus) tissues where available, and a collaborative network encouraging exchange of material and ideas and the techniques for reuse of surplus materials.

The Australian & New Zealand Laboratory Animal Association and the Australian & New Zealand Council for the Care of Animals in Research and Teaching (ANZCCART) provide information and resources for sharing of animal tissues between researchers within and between institutions in Australia (*Australian and New Zealand Laboratory Animal Association - Home* n.d.; *Home* n.d.).

Human tissue banks are challenging to establish and operate (Thasler et al. 2006). Whilst use of surgical waste human tissue can replace the need for using animals there are legal and ethical considerations and public concern that such tissues may be used for commercial profit. It is important to have a clear set of procedures for tissue donation, collection, supply, and allocation. Informed consent by donors is critical. The tissue supply must also be controlled in a transparent and independently managed way. A key question is how the use and distribution of a patient’s donation is managed; that is, who are the brokers of patient donations? What protects the personal (genetic) information contained within the donated tissues? What limitations apply to the researcher when using the donated tissue?

In Australia, the Organ and Tissue Authority has many of these procedures either in place or under review (Department of Health 2018). The Organ and Tissue Authority works with states and territories, clinicians and the community sector to improve organ and tissue donation and transplantation outcomes. It is the obligation of researchers wishing to use human tissue that they fully comply with all legislation governing the use of human tissues within their state or territory jurisdiction.

Work using human embryos and gametes (including embryonic stem cells) is governed by the Commonwealth *Research Involving Human Embryos Act 2002* and the NHMRC Ethical Guidelines on the use of Assisted Reproductive Technology in Clinical Practice and Research (National Health and Medical Research Council 2017).

IN SILICO MODELS AND DATA REPOSITORIES

Whilst computer models and chemical databases impose fewer ethical considerations than human tissue banks there are problems in ensuring widespread (and equal) access to computer models, data and databases, standard operating procedures and scientific information services that pertain to the use of non-animal models in science.

The performance of many *in silico* models is highly dependent on the quality and breadth of the training and testing datasets available to the model (Langley et al. 2005). Many private databases held by pharmaceutical companies are currently unavailable to *in silico* models. The access to this wealth of data would greatly increase the capacity of QSAR and CADD models. The use of QSAR-type models for toxicity assessment is expanding, however the addition of data relating to membrane permeability, pharmacokinetics etc. appears to increase predictive ability (Bhatarai et al. 2016). The expansion of databases to include new fields such as these is likely to be necessary for *in silico* models to perform to the required level of confidence for regulatory approval.

4.5 AMBIGUITY IN TERMINOLOGY

There is inconsistency in the accepted definition of terms relating to use or non-use of animals in studies. The potential for confusion between stakeholders may be slowing progress towards non-animal models. Whilst this language challenge is not a focus of this report, awareness of the key terminology confusions may assist future activities through dedicated and agreed definitions and harmonisation of meaning. This language inconsistency has been considered in detail by researchers (Gruber and Hartung 2004) and is summarised below.

- # **Animal-free:** Does this mean no animals are used for the study in question, or does it extend to the non-use of cells or tissues derived from donor animals, or for any precursor studies that were required before the study in question? Does it cover non-animal testing alternatives? This ambiguity means there is no definitive meaning in the statement or claim that a product was tested 'animal-free'.
- # **Alternative test:** An alternative is a method or test that replaces, refines, or reduces an animal experiment. Which *in vivo* experiment has been replaced, reduced or refined should be fully defined for the test to stand as an alternative (some disagree that the word 'alternative' can apply to any test other than one that replaces the use of animals)?
- # **Test:** Tests deliver a defined result; they measure or classify. Tests can be validated, technologies and methods cannot.
- # **Technologies:** Technology refers to a method and approach. Technologies cannot be validated; only specific applications can be validated.

- # **Validated alternative:** This is an alternative test that has been validated under some agreed criteria. Validity has repeatability, reliability, accuracy and an established (strong) relationship with the surrogate variable being assessed by the alternative method (e.g., correlation between the percentage of cell culture killed and the Draize rabbit eye test result).
- # **Approved or accepted alternatives:** A validated alternative is one that has been accepted by authorities as a suitable substitute for an animal test. The regulator can only accept approved alternative tests when assessing applications that use this over traditional (accepted) tests.

This is by no means an exhaustive list of relevant terms, nor would the definitions above be universally agreed. The list is included only to illustrate the problem of ambiguity as identified by one research group (Gruber and Hartung 2004).

The lack of clarity in definitions does not only affect the regulator in making decisions but publications of scientific papers on the replacement of animals in research can also be confused about the meaning of the terms they report. This has potential to slow the advance of science in animal-free research, with flow-on effects to end users of the study papers.

5. REPLACING ANIMAL TESTS

5.1 RISK ASSESSMENT IN CHEMICAL TOXICITY

The risk arising from use of an industrial chemical is not simply a function of the biological toxicity (hazard) of the chemical. The likelihood and extent of exposure are also considerations that determine overall risk. In Australia, risk is assessed by systematically evaluating evidence for potential harm to human health and the environment. This risk is a function of the innate toxicity of the chemical and the likely level of human and environmental exposure arising from its use pattern. This approach considers how the chemical will be used (to determine exposure risk) and this, combined with information on the innate toxicity of the chemical, quantifies the overall risk.

A key starting point is understanding the innate hazards associated with the chemical. This includes the chemical toxicity profile and toxicity dose response. Standardised hazard endpoints are used to assess a chemical's hazard profile; these are presented on the Australian Government Department of Health and Aged Care AICIS web site (Presentation by Endpoints: Section 4 - Health Effects - OECD n.d.). Hazard identification can be complex, often being a combination of information from: previous scientific, regulatory or industry reports; studies ranging from epidemiological surveys to prospective studies including in vivo, in vitro, ex vivo, in chemico and in silico forms (the latter may be conducted at individual or population levels); or read-across extrapolation from assessment of one or more similar chemicals (see below).

Exposure risk can also be viewed in several ways. Exposure risks include occupational and public exposures, intentional (i.e. use) and unintentional exposures and direct versus indirect exposures. Each chemical is examined across its likely exposure pathways by type, intensity, frequency and duration. Often, local exposure risk information must be supplemented by overseas data.

Depending on the chemical hazard and exposure assessment and the availability of data, a qualitative or quantitative hazard risk assessment may be performed. Qualitative assessments mostly are used when there are gaps in data availability; a quantitative risk assessment is preferable in most cases. Given the data challenge, most risk assessments also estimate uncertainty in the findings.

5.2 TOXICITY ENDPOINTS

An important consideration in converting from animal-based to non-animal-based tests for chemical toxicity assessment is identifying suitable (test) endpoints for new approach methods or methodologies⁵ (NAMs). Animal-based tests can (mostly) use the response of the whole organism to determine toxicity, which reduces reliance on understanding the MoA. However, these endpoints are not available for NAMs. The effective use of NAMs requires understanding of the MoA.

Two broad categories of endpoints are used to assess toxicity. The assessment endpoint is concerned with the population or environmental value or parameter that is to be protected. This includes health, behaviour, reproduction, gene stability etc. These are assessed through one or more specific measures of effect (conducted at the individual animal or test level). The measures of effect describe a response of a variable, one that has a recognised relationship to the assessment endpoint (such as impact on survival, growth, reproduction, physiological change, biochemical change, genetic mutation etc.). This is then used as a proxy for the assessment endpoint. The measure of effect may be (for example) a concentration of a chemical or metabolite or cell lethality, and this must be first defined and agreed as suitable for assessing toxicity. Again, the MoA becomes an important prerequisite for NAM selection and interpretation.

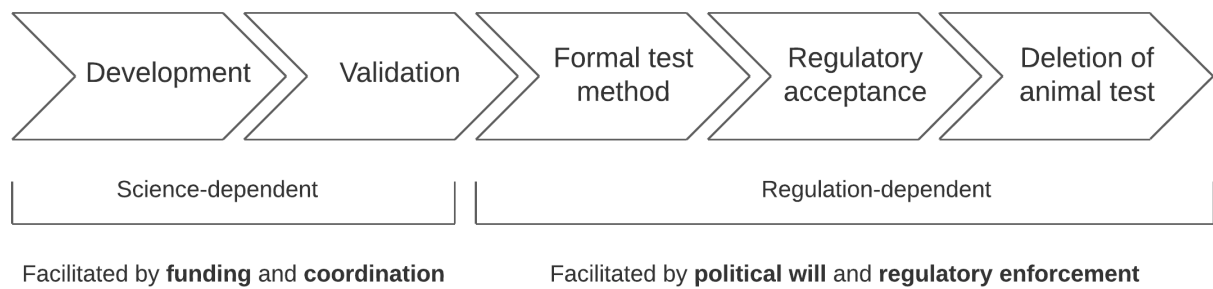
Endpoints are further classified into acute and chronic categories. Acute endpoints typically are evident from a single exposure and accrue within a week of exposure. Chronic endpoints require longer to identify and may take multiple exposures and more time to manifest. The extra time requirement can be a problem for some NAMs, for example where the maintenance of the cellular function (within in vitro NAMs) over time can be challenging. As a result, chronic endpoints are particularly challenging to assess using NAMs. These extend to include mutagenicity, reproductive and developmental toxicity and carcinogenicity. Endpoints used in human toxicology and links to guidelines and recommended approaches are provided by the OECD (Presentation by Endpoints: Section 4 - Health Effects - OECD n.d.). These cover acute toxicity, skin, eye, genotoxicity, carcinogenicity, reproductive toxicity, specific target organ toxicity, repeated exposures, and neurotoxicity. Methods include in vivo and in vitro alternatives, where they exist.

The requirement to define suitable assessment endpoints and the measures of effect from NAMs that relate to the assessment endpoint add a layer of complexity to the replacement of animals in the assessment of chemical toxicity. The acceptability or otherwise of endpoints and their measures by regulators is important; a newly developed NAM needs to be assessed for applicability for use on these endpoints if it is to contribute to toxicological assessments.

5.3 THE PATH TO MARKET FOR NEW APPROACH METHODS

The pathway by which NAMs first come into being, through to their actual usage, is lengthy and bureaucratic. It is well described in a recent book chapter (Taylor 2019), as shown in Figure 1 and summarised below.

⁵ A general and widely-used term to describe any non-animal approach used for chemical hazard and risk assessment



Source: Taylor 2019

Figure 1: Process of acceptance of an alternative test method

The following summarises the process as described by Taylor 2019.

- # **Development:** Where the NAM is ‘created, optimised and partially tested’. This early research takes place in universities, state research institutes and the commercial sector (such as chemical or medical companies), and may be funded by government, private sector interests or charities. Companies may be spun off to further develop and commercialise a new method. The efficiency of this stage can be restricted by academics simply publishing and not further commercialising a new method, and by commercial players keeping new methods in-house, either for competitive advantage or through lack of incentive to seek more widespread use.
- # **Validation:** Where the method is independently assessed for accuracy and reliability. A new method must produce the same results in different laboratories at different times. This is a laborious stage and there may be numerous challenges, for example a finding that the animal test that the NAM is designed to replace was itself never properly validated. Official validation bodies such as the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in the US play an important role in ensuring a rigorous validation process but are not always part of the pathway to market.
- # **Formal test method:** Where the conduct of the method is written up into a highly-specified formal document. This stage can be slow and involve recourse to further validation work. The OECD TG process is one important mechanism for formalisation due to its widespread international recognition but there are others.
- # **Regulatory acceptance:** Where regulatory agencies (usually several in each jurisdiction, sometimes down to sub-national level) decide whether they will accept data generated by the new method. This does not necessarily require formal international recognition for the method but usually does.
- # **Deletion of the animal test:** Where an existing animal-based test is disallowed as an acceptable source of data. Sometimes, a specific intervention such a change of legislation or external campaign is required to prompt this to happen.

These stages can take many years to complete. An example is the replacement of the rabbit skin irritation test. It took seven years for a new, non-animal alternative to move just from formal test method to the point where the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) scheme deleted the *requirement* for the rabbit test and replaced it with the new method. The formal test method for the rabbit test still exists and the rabbit test is still in use in Europe and elsewhere.

5.4 ADAPTATIONS FOR OBTAINING TOXICITY EVIDENCE FROM NAMs

Mostly direct toxicity measurement endpoints cannot be obtained using NAMs. Often there is missing MoA information for individual chemicals. Traditional animal-based tests for toxicity can bypass the need for this information as the animal's response to exposure reflects the desired assessment endpoint, so the MOA need not be known. This is rarely the case for NAMs, which has required the development of new techniques. It is important to note that MoA information mostly underpins use of these adaptations. The most frequently used adaptations are summarised here.

BATTERY OF TESTS

The toxicity profile of a given chemical can be variable. Often AOPs suggest toxicity impacts of a chemical exist across multiple groupings including cell survival, cell function / physiology, development, reproduction, carcinogenesis, mutagenesis, etc. Ideally toxicity risk should be anchored to an AOP; if the AOP can be defined, specific tests (to determine toxicity) can be identified with some confidence. In the absence of a clear (or fully defined) AOP, testing for toxicity across each of the recognised broad groupings is necessary. Animal-based studies explore toxicity across the whole organism and are therefore less reliant on knowing all AOPs for toxicity of a chemical.

NAMs are mostly highly specific. They can assess a single toxic relationship or pathway (such as reactivity with a specific peptide) but are incapable of assessing whole-of-organism toxicity alone. The use of NAMs in the assessment of toxicity risk for chemicals with unknown or incomplete AOPs is therefore problematic. Many tests are required to cover the full gamut of potential adverse responses to exposure. The application of numerous specific tests within a suite of tests that cover the proposed toxicity spectrum is known as a battery of tests. The tests within the battery are interpreted in parallel. One positive test indicates the existence of some chemical toxicity.

The complexity of ensuring completeness within the test battery, and of validation of each test within the battery, is great. Publications warn that this challenge should not be underestimated (Ball et al. 2016). Others warn against the blind application of a battery of tests (Dent et al. 2018). The overarching goal is human and environmental safety and the prescriptive application of (an often insufficient and/or incompletely validated) battery of tests often does not meet this primary objective. Validation of individual tests for determining toxicity is incomplete, and there are gaps in reference datasets (Natsch et al. 2009). This suggests that not all chemicals can be effectively evaluated using a battery-of-test approach. Whilst work is underway on integrating results across the battery of tests (some yielding very high aggregate specificity), existing data holes and individual test gaps reduce the overall sensitivity of the approach, at least for some groups of chemicals. High (aggregate) sensitivity is critical for effective toxicity assessment; herein lies the challenge for replacing animal-based tests with NAMs.

POINT OF DEPARTURE

The point of departure (PoD) is the position on the toxicological dose-response curve that is associated with the onset of toxic effects in the target animal (see Sand et al. 2017 for a more detailed description). The point-of-departure (PoD) concentration is initially established using experimental data. This can subsequently be used within a NAM to set a non-toxic threshold. The dose response of a measured variable to the exposure concentration of a test substance is mapped (using a NAM) and the resultant curve is compared to a similar curve obtained from exposure to a known (toxic) control substance, one that has a pre-identified non-toxic threshold. This comparison then predicts toxicity of the test substance at a given exposure concentration. This is presented graphically in Figure 2.

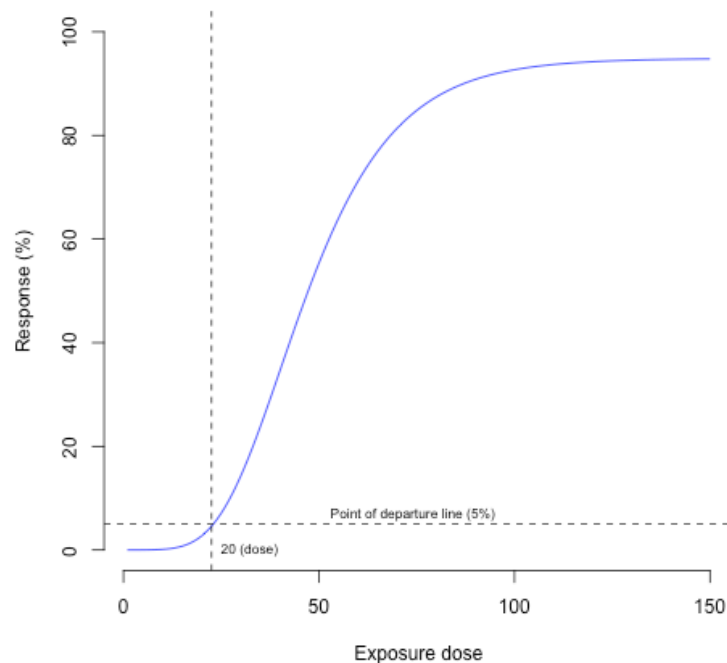


Figure 2: Point of departure – graphical representation

The point of departure (often an inflexion point on the dose-response curve) that identifies the (original) toxicity limit is available for transfer and comparison to test dose-response curves. The reference curve no-observed-adverse-effect level, lowest-observed-adverse-effect level or the statistical benchmark dose point can be used to set the point of departure. A key challenge is to ensure the relationship between the measurement variable and assessment endpoint is recognised and quantified. This is much harder to do using NAM-derived data than for data obtained from animal studies.

The point of inflexion separating the low / no-effect phase from the toxic phase of the curve is typically used to quantify safe exposure levels. Most NAMs cannot map dose-response curves; these data mostly must still come from animal studies.

Models of toxicokinetics describe the absorption, distribution, metabolism and excretion of a chemical and as such are used to determine the internal dose of a chemical arising from an exposure. These can also be used in reverse, to determine the likely exposure dose from internal concentrations. However, applying this one chemical at a time is time consuming, and high-throughput toxicokinetic (HTTK) methods have been developed (Breen et al. 2021). These methods parameterise generic toxicokinetic models to fit many chemicals under evaluation and so speed the analysis. Physiology-based toxicokinetic (PBTk) models simulate chemical concentrations within different tissues of an organism. Specific organism models can then be adapted to other species after adjusting for any species-to-species differences thereby allowing evaluation of chemicals across a wider range of target hosts (Schneckener et al. 2020). These techniques expand the capacity of PoD methods to evaluate potentially toxic chemicals.

READ-ACROSS

Read-across methods are increasingly being adopted to control for specific missing hazard characteristic data of chemicals. Read-across is based upon natural groupings of substances (e.g. those with structural,

mechanistic or biological similarity) and where data exists demonstrating a (measurement) response to a chemical within the group, an identical response is assumed for other chemicals within the group where equivalent data does not exist. Ability to apply read-across can reduce gaps in the knowledge pathway from (NAM)-measured effect to assessment endpoint. The knowledge gaps have placed a high reliance on read-across methods to demonstrate low toxicity of chemicals in registration dossiers. Read-across approaches are often required for repeated-exposure, development and reproductive toxicity assessments (European Chemical Agency 2020).

However, there is often disagreement on the strength, reliability and completeness of read-across-derived associations between the measured effect and the assessment endpoint. Acceptable read-across methods must be underpinned by robust scientific evidence. Common shortfalls in dossiers using read-across include poor documentation of methods; inadequate substance identification; deficiencies in reference studies; inadequate supporting data; insufficient predictive toxicokinetic modelling evidence; and inappropriate or ill-informed toxicological hypotheses. As a result, Good Read-Across Practice (GRAP) guidelines are under development (Ball et al. 2016), as are read-across assessment frameworks (European Chemical Agency 2020).

5.5 WEIGHT OF EVIDENCE

The weight-of-evidence approach uses the totality of scientific evidence to assess if the science supports a particular conclusion (see Committee et al. 2017 for a detailed description). This is more than just a tally of supportive and negative studies, but uses expert scientific judgement to assess, review and integrate all available information to form a meaningful conclusion (on toxicity). This looks at the toxicity hypothesis, toxicity MoA, AOP, study data (including study design and strength of association), and the assessment endpoints (and severity).

The weight-of-evidence approach essentially examines the causal pathway and the evidence supporting or refuting a link between exposure and purported toxicity and the strength of any association. The evidence for causality is extrapolated directly from epidemiology. A gradient of evidence of causality can be determined from the collated evidence from some or all the combination below.

1. **Strength of association:** This is both between exposure and toxicity (forward looking) and toxicity and exposure (backwards looking).
2. **Consistency:** A toxic exposure should result in consistent toxicity response in exposed individuals (or experimental subjects/tests).
3. **Specificity:** The more specific the toxicity response arising from exposure the more likely that exposure produced the observed toxicity.
4. **Temporality:** Exposure must precede the onset of toxicity. This is an essential criterion.
5. **Dose response:** A worsening of toxicity signs as exposure increases (and vice-versa) is strong supportive evidence of a causal link between exposure and toxicity.
6. **Plausibility:** The MoA to produce toxicity signs should be consistent with theory and previous experience.
7. **Coherence:** The cause-effect hypothesis proposed should not contradict established knowledge in the domain.
8. **Experimentally reproducible:** A prospective exposure study should produce predictable toxicity responses in exposed subjects and (predictable) health in unexposed controls.

9. **Analogy:** The toxicity hypothesis should be consistent with toxicities seen from similar chemicals. This is important in toxicology when often multiple chemical exposures may occur concurrently (e.g. from a mix of chemicals). This helps to ensure the correct chemical in the exposure mix is associated with the signs.

There are important considerations in relation to the weight-of-evidence approach for assessing toxicology using NAMs. These are discussed below.

MODE OF ACTION

Any toxicity relationship to chemical exposure must be plausible. Practically, this means at least some information on the MoA and AOPs needs to be available. The MoA also determines the individual NAMs that can be used; they provide an appropriate (and highly specific) set of measurement endpoints that link to the assessment endpoint. Not all MoAs for toxicity events have been elucidated and described, and this impedes selection of appropriate NAMs. Currently, validation of NAMs is limited to those with effective equivalence to (proven) animal tests. Expansion of the suite of useable NAMs will require new (non-animal) based ways to validate them. This is a current area of active research.

Work on *ab initio* chemical assessment is occurring. This approach is underpinned by a hypothesis-driven MoA *in vitro* experiment, the results of which inform *in silico* modelling of exposure risk and outcome. Biokinetic modelling of *in vitro* results can predict points of departure which subsequently help define toxicity risk following exposure. Output includes a threshold of toxicological approach and confidence level in result (collated from underpinning evidence). A recent publication describes the approach, current requirements and workflow in some detail (Berggren et al. 2017).

QUANTITATIVE ASSESSMENT

A dose-response gradient provides very strong evidence of causality, especially if obtained from prospective experimental studies. Dose responses are typically difficult to obtain using NAMs. Most NAM tests provide only evidence supporting (or refuting) toxicity; they cannot quantify the toxicity response. NAM-based tests are therefore of limited value for the setting of safe chemical exposure levels. Consequently, there will be some ongoing reliance on animal testing in toxicity assessment for the foreseeable future. Difficulties in assessing toxicity from use of non-quantitative tests have been discussed (Moné et al. 2020).

ASSESSMENT ENDPOINTS WITH FEW DEDICATED NAMs

Few NAMs exist or are suitable for assessment of repeated dose/exposure toxicity, developmental and reproductive toxicity, endocrine disrupters and carcinogenicity. The EU-ToxRisk project aims to address this shortfall (*EU-ToxRisk - EU-ToxRisk – An Integrated European ‘Flagship’ Programme Driving Mechanism-based Toxicity Testing and Risk Assessment for the 21st century* n.d.). The project applies a collective scientific approach to addressing the paradigm combining advances in *in vitro* and *in silico* toxicology, read-across, battery-of-tests, hazard- and risk-assessment approaches, and AOP to develop effective non-animal model approaches to assessing toxicity in these problematic endpoints (Daneshian et al. 2016). The project uses case studies and focuses heavily on the regulatory pathway.

The objective is to develop an effective mechanism-based integrated approach to assessing toxicity within these difficult endpoints. This is a deliberate move away from ‘black box’ animal-based testing approach that historically predominated. Identifying and mapping the (complete) mechanism of action will likely rely upon analysing data from read-across tests. Any such system will be data heavy and

analytically demanding. The EU-ToxRisk target is to develop methods and approaches for assessing toxicity that are ultimately acceptable to the regulator. Successful read-across systems will likely incorporate information from high-throughput transcriptomics, advanced imaging of cellular pathways (and functioning), *in vitro* systems and advanced mathematical modelling. Development will be dependent upon acceptance (and uptake) of good read-across practice. To date, the EU-ToxRisk pathway has set a target of development of 15 AOPs to allow effective NAM-based assessment. Recent reports suggest up to 20 AOPs may be required to allow effective deployment.

The OECD lists testing and assessment guidance and review documents (*Series on Testing and Assessment / Adopted Guidance and Review Documents - OECD* n.d.). The *OECD Guidelines for the Testing of Chemicals* is available online (*OECD Guidelines for the Testing of Chemicals* n.d.). The European Union has funded EURION (European Cluster to Improve Identification of Endocrine Disruptors) ('Improving Identification of Endocrine Disruptors | EURION' n.d.). The focus is on harvesting synergies between research teams and organisations to develop new testing and screening methods for exploring endocrine-disrupting chemical toxicity. Defining and improving the specificity of tests (through detailed knowledge of the MoA) remains a critical area of focus for the group.

5.6 BARRIERS AND ENABLERS

Several barriers to the implementation of alternatives to animal testing can be discerned. These include (adapted from Taylor 2019 and Balls 1994):

- # **The current scientific paradigm.** To establish the safety or efficacy of a drug or chemical, scientists typically test it against increasingly complex models, from *in vitro* or *in silico* models, to animals, to humans. This is considered critical to ensure all complex, system-level effects are discovered and evaluated. The opposing view to the 'complexity' argument is the question of 'relevance' (whether animal data is relevant to humans), as discussed in section 4.2. A combination of the AOP approach and other methods, and the use of more complex models such as organs-on-chips, can help to address complexity concerns.
- # **Interface with legislation.** Some existing legislation, for example in the classification and labelling of substances, is worded to reflect the data available from animal test results and may not be compatible with the outputs of alternative test methods which may not be directly equivalent. Certain countries have a specific requirement for animal test data for chemical safety assessment, for example (until recently) China (see section 7.3).
- # **Bureaucracy.** There may be an inertia among regulators, who tend to be risk averse and have limited incentives to change from test methods that have established acceptance. As noted in section 5.3, processes of validation and harmonisation are lengthy and provide many opportunities for action to be delayed or deferred (for example, the OECD has an annual cycle for test guideline revision, so missing a deadline for submission means waiting for another year).
- # **Lack of funding.** A Cruelty Free International survey of EU countries showing that direct funding for alternative (3Rs) methods among EU countries was only €18.7 million in 2013, and even in the UK (€11 million) was only 0.04 per cent of the nation's science research and development expenditure (Taylor 2019). Whether this is a reasonable level of expenditure against other priorities is beyond the scope of this report. It should be recognised that a significant proportion of the total investment in this area is likely to be from the commercial sector and harder to quantify. Through Horizon 2020, the eighth of the EU's Frameworks Programmes for Research and Innovation, the EU dedicated €200 million to animal-free toxicology projects in 2020 (*Animals used for scientific purposes - Environment - European Commission* n.d.). This includes public-private partnerships with Cosmetics

Europe and the European Federation of Pharmaceutical Industries and Associations making significant financial contributions to this project.

- # **Entrenchment.** The scientific establishment, including researchers, reviewers of manuscripts and funding application reviewers tends to support existing approaches. Partly, this is a psychological issue (people may feel threatened) and a capacity issue (existing laboratories and facilities may not have the impetus or resources to 're-tool' their environment and scientists to new approaches).
- # **Lack of enforcement.** The EU prohibits the use of animal tests where a recognised alternative is available. In the view of Cruelty Free International and other animal protection organisations, this law is not being enforced in member states of the EU.
- # **Threat of litigation.** Products must be tested to demonstrate safety. Use of new (and less proven or accepted) tests may make organisations susceptible to litigation. This threat may deter uptake.
- # **Unclear process.** Taylor (2019) notes that, even in jurisdictions such as the EU and US, *'Companies with new methods are often unsure about the process, whether they need to submit their method for official validation or directly to the regulatory body, who they should contact, and what information they need to provide'*. This has resulted in a reproducibility crisis, a high failure rate when attempts are made to replicate a published study. This can arise from flaws in the published study design, incomplete description of the underpinning methods, reagent provenance and test settings and components and extending to flaws in statistical analysis. Greater focus on Good Laboratory Practice (GLP) would likely improve reproducibility, greater acceptance of registration dossiers by regulators and reduce unnecessary animal suffering (from ineffective replication of a study). The growth of organisations to champion the 3Rs and to support the validation of test methods (see section 7) has also helped to address this problem.

A recent literature review by NHMRC (National Health and Medical Research Council n.d.) identified 11 'enablers and supports for the uptake and implementation of the 3Rs'. These were:

- # Legislation and regulation;
- # Guidelines and standards;
- # Education and training;
- # Leadership and governance;
- # Improved reporting of experiments in publications;
- # Databases and information sharing;
- # Ethics committee processes;
- # Industry involvement and collaboration;
- # Research support;
- # Policies and frameworks; and
- # Workforce roles.

Whilst this list has been generated through a more research-based, 3Rs 'lens' to that of Taylor (2019) and Balls (1994), it is possible to see how many of these enablers address barriers identified by those authors. For example, all of them can be seen as important to move science beyond its existing paradigm and to challenge entrenched positions.

Finally, the challenges of replacing animal-based testing can be considered from the perspective of purpose (objective of the study) and the approach to study (Gruber and Hartung 2004). This is summarised in Table 5. Studies for regulatory purpose are the most constrained by law. The legal

constraints flow into greater reliance upon standard operating procedures (to ensure the data file meets the obligations for registration). Regulatory processes appear the most inflexible in current form to examining alternative ways to collect data, such as non-animal testing.

Table 5: Differences in objective, structure, design, and method of dissemination of findings between regulatory, medical, and basic research streams

Source: Gruber and Hartung 2004

	REGULATORY TESTING	MEDICAL APPLIED RESEARCH	BASIC RESEARCH
Initiation/reason for science	Legal aspects	Oriented on sickness, effects, and side effects of drugs on humans and animals	Proof of scientific hypothesis
Type of science	Standard operating procedures (SOPs)	Efficiency of e.g., agent discovery	Exploratory
Quality control	Prevalidation / validation (GLP)	Evaluation	Peer-review (journals)
Distribution of results	Report to authorities (regulator)	Limited, since proprietary products ⁶	Publication
Consequences	Permission or non-permission by authorities	Alteration of pharmacopoeias	Acceptance or rejection of study hypothesis

⁶ This is becoming less of a problem as Open Access requirements of some funded research includes distribution of results (and often data) from studies (especially clinical trials)

5.7 TYING IT TOGETHER — THE RISK ASSESSMENT PATHWAY

There are several steps to establishing toxicity arising from the requirements discussed above. The combination of exposure, toxicity MoA, AOP, assessment and measurement endpoint, difficulty in quantifying dose-response, incomplete suite of (specific) NAMs, often missing (analogue) reference data and challenges in development and description of repeatable and reliable methods often result in failure of NAM-reliant dossiers on toxicology to be accepted by regulators.

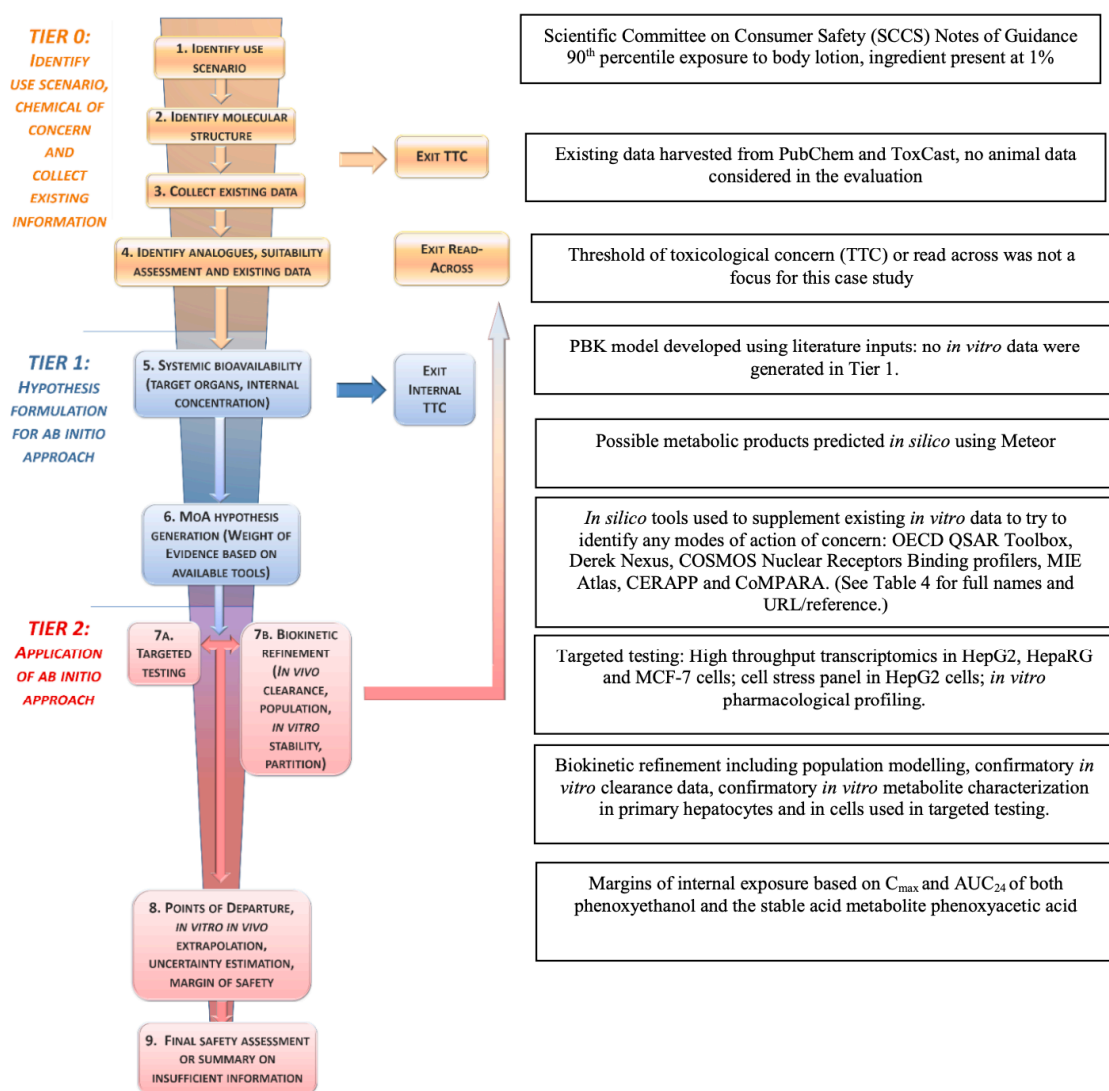
Recently, a working group under the auspices of the International Cooperation on Cosmetics Regulation (ICCR – see section 7.4) developed the Next Generation Risk Assessment (NGRA) approach to incorporating NAMs into an integrated strategy for assessing risk in cosmetic ingredients. NGRA applies an ‘exposure-led and hypothesis-driven’ approach to assessing chemical toxicity. This integrated approach seeks to combine information from all available sources, including *in vitro*, *in silico* and *in chemico*, with the exposure profile and proposed mode of action for toxicity to determine risk. A recent review explores the use of NGRA for cosmetic chemical assessment (Dent et al. 2018).

A case study on phenoxyethanol was submitted for discussion to the OECD Integrated Approach to Testing and Assessment Subgroup meeting. Phenoxyethanol is commonly found in 1 per cent solution in cosmetic body lotions and was tested for systemic toxicity using novel technologies developed as part of the push to replace animals in chemical testing (OECD 2017). The case study used *in silico*, *in chemico*, and *in vitro* data and QSAR modelling and followed the guidelines of the major European Commission research initiative Safety Evaluation Ultimately Replacing Animal Testing (SEURAT-1).

The complex risk assessment cascade from this case study is presented in Figure 3. The complexity represents the challenge of replacing animals in toxicity assessment. Whilst the study confirmed many of the NAMs and techniques developed to replace animals in toxicology studies there remained uncertainties including:

- ⊕ Incomplete understanding of the breadth of biological coverage on the non-animal tools that were used and their assays in evaluating outcomes;
- ⊕ Incomplete *in vitro* studies and missing data sources for the major metabolites (including ‘omics data);
- ⊕ Challenges in interpreting margins of internal exposure (MoIE) from NAM tests; and
- ⊕ Use of *in silico* tools that were not developed specifically for assessing toxicology.

The Australian delegation concluded that the case study was at ‘*preliminary proof of concept state and not currently suitable for our regulatory context*’. Each other commenting nation concluded that the case study data dossier alone would be insufficient for their chemical regulator.



Source: OECD (2021) (OECD 2021)

Figure 3: Example of new risk assessment cascade using an integrated approach to testing and assessment

The OECD identified the following priority areas for development and use of NAMs and the replacement of animal studies in assessing toxicity of chemicals:

1. Development of assessment workflows using non-animal data;
2. Development of *in vitro* and *in silico* information to build physiologically based kinetic (PBK) models;
3. Greater NAM coverage of the biological space;
4. Docking exercises; and
5. More development of MoIEs based on bioactivity data and their interpretation.

The OECD manages the Integrated Approaches to Testing and Assessment (IATA) (*Integrated Approaches to Testing and Assessment (IATA) - OECD n.d.*). The output from IATA is pragmatic, science-based integrated approaches to chemical toxicity hazard identification and characterisation. This

allows participating countries to share and explore novel methodologies for toxicity assessment within a regulatory focus. An iterative approach to advancing answers is applied — new knowledge builds on existing knowledge to improve technique, use or interpretation of results. Combinatorial approaches are supported often combining QSAR, read-across, *in chemico*, *in vitro*, *ex vivo* and ‘omics’ to determine toxicity profiles. The biggest constraint continues to be the lack of mechanistic understanding of induced toxicity; lack of information on MoA and AOP limits assessment of toxicity using NAMs.

The Accelerating the Pace of Chemical Risk Assessment (APCRA) initiative is a cross-country and cross-organisation approach to modernising chemical risk assessment and regulation with a focus on development and application of NAMs (US EPA 2020). A key area is in modernising quantitative risk assessment. The development of NAM-based methods and tools, with special focus on methods applicable to chemicals with limited information (including MoA) is ongoing. A fully operative suite of approaches using NAMs to assess chemical toxicity is not yet available.

Finally, it is important to note the need to engage the regulator in the development of NAMs, especially those proposed for use in registration of chemicals. This is to ensure a NAM has the best chance of being acceptable to the regulator. From the perspective of the regulator, there is a need for:

- # NAMs to be better anchored to human biology (i.e. the measurement end point is linked to the assessment endpoint);
- # Animal-free methods to be better supported by recognised quantitative methods that predict impact of exposure (level) on metabolism and transport in the body, and thereby toxicity risk;
- # Better strategies for defining compounds with low or no toxicity, and specifically to define risk of false negative assessments; and
- # A GLP approach to documentation of NAM development and use and subsequent study conduct and technique to improve reproducibility of studies and provide greater confidence in data generated by studies.

The challenge is often how to best define non-toxic thresholds using NAMs; simply substituting an animal-based test with a NAM may fail when there are complex endpoints and no or incomplete MoA information.

6. ASSESSMENT OF INDUSTRIAL CHEMICALS IN AUSTRALIA

6.1 REGULATION

The oversight of safe and effective chemical use necessitates several functions: policy oversight, risk assessment, risk management and enforcement. These functions are applied across several domains: work health and safety, public health, the environment, product safety, and transport. In Australia, because much of the law in these areas arises from state and territory legislation, governance of chemicals is split between these jurisdictions and the Commonwealth.

The *Industrial Chemicals Act 2019* established AICIS as the body regulating the importation and manufacture (collectively called ‘introductions’) of industrial chemicals to Australia. It replaced the National Industrial Chemicals Notification and Assessment Scheme (NICNAS) on 1 July 2020. AICIS is administered by the Office of Chemical Safety (OCS) within the Department of Health and Aged Care. It is one of four chemical regulators in Australia, alongside the Therapeutic Goods Administration (TGA), Australian Pesticides and Veterinary Medicines Authority (APVMA) and Food Standards Australia and New Zealand (FSANZ).

AICIS maintains the Australian Inventory of Industrial Chemicals. The Inventory is a database of (currently around 40,000) chemicals being manufactured in or imported to Australia. Each entry in the Inventory provides a Chemical Abstracts Service (CAS)⁷ name, CAS number, related names, molecular formula and any regulatory obligations or conditions attached to the chemical.

Any party seeking to introduce an industrial chemical, or product containing an industrial chemical, must be registered with AICIS and must determine the category of AICIS introduction into which the chemical fits. There are five categories:

1. Listed – the chemical is on the Inventory and can be introduced within the terms of the listing.
2. Exempted – for very low-risk introductions, subject only to submission of a once-off declaration after introduction and record-keeping.
3. Reported – for low-risk introductions, subject only to submission of a once-off report before introduction and record-keeping.
4. Assessed – for medium- to high-risk introductions, requiring application for an assessment before introduction, and record-keeping.
5. Commercial Evaluation Authorisation – to allow determination of the chemical's commercial potential, requiring an application before introduction and record-keeping.

Medium- to high-risk introductions are placed in the 'Assessed' category, which requires an application to be made for the chemical or product to be assessed before it is introduced. Very-low- and low-risk introductions fall into other categories that do not require assessment (*Before you start categorising your introduction* n.d.).

There are various types of assessment by AICIS: those with a health focus, environmental focus or both; very-low- to low-risk assessment; and comparable hazard assessment, depending on the indicative risk/s of the chemical or product. Data to support the assessment must be submitted with the application and covers: general information; classification and labelling; manufacture; physical and chemical properties; environmental fate and pathways; ecotoxicological information; toxicological information; residues in food and feeding stuff; guidance on safe use; and the assessment report (*Apply for an assessment certificate* n.d.).

Assessments of industrial chemicals tend to receive less data than those of agricultural and veterinary (agvet) chemicals, therapeutic goods, or foods. Efficacy data is not required for industrial chemicals. On the other hand, the risks posed by industrial chemicals are often more challenging to determine due to the sparsity of underpinning data relative to that available for a therapeutic or agricultural chemical. The challenge is in effectively collating diverse information sources and addressing information gaps. This has required a less prescriptive approach to registration that combines data within a weight-of-evidence approach to assessing toxicity risk, as discussed in section 5.

AICIS specifies acceptable OECD test guidelines and equivalents (for example, EU or US test methods) for the range of human health and environmental hazards to be considered in categorisation (*Appendix - acceptable test guidelines for categorisation* n.d.). *In silico* options are also provided (*Appendix - In silico predictions for categorisation* n.d.). Assessment of environmental data is carried out for AICIS by the

⁷ CAS is a division of the American Chemical Society that maintains a registry of chemical substances. See *Empowering Innovation & Scientific Discoveries* n.d.

Department of Agriculture Water and the Environment (DAWE), while the OCS itself assesses data on risks to human health (public and workers).⁸

There are exemptions to the requirement for registration and categorisation. These include products blended from ingredients sourced only within Australia, naturally-occurring chemicals, chemicals introduced only for personal use, and various other groupings (*Introductions that don't require categorisation and registration* n.d.).

6.2 RESTRICTIONS ON ANIMAL TESTING

Under the *Industrial Chemicals Act 2019*, there are restrictions on the use of test data obtained from animals to support the assessment of certain chemicals by AICIS:

- ⊕ For any new ingredients used exclusively in introduced cosmetics, information from animal testing conducted on or after 1 July 2020 cannot be used to prove safety, except in three exceptional circumstances; and
- ⊕ For any new ingredients with multiple end-uses, including in cosmetics, for which there is no alternative data to animal-test data, approval may be given to use the animal test data on application.

Animal test data means data obtained from a cephalopod or any live vertebrate animal (other than a human being).

There are three exceptions to the restrictions, in cases where new animal test data:

- ⊕ Shows that the chemical has a hazard characteristic and this conflicts with the non-animal test data;
- ⊕ Is the only information that can demonstrate whether a particular environmental hazard exists; or
- ⊕ Is from tests applied to a different (analogue) chemical to that being introduced (*Animal test data - when it can and can't be used and when you need pre-approval* n.d.).

It is notable that the animal testing provisions of the Act can be expected to have the greatest impact on ingredients or products tested overseas, rather than those tested in Australia. The Bills Digest for the Industrial Chemicals Bill 2017 [and associated bills] (Commonwealth Parliament n.d.) notes that very little testing of cosmetics on animals occurs in Australia, largely because of the barriers imposed by the animal research and welfare laws of the states and territories and the NHMRC Code, which require institutions to carefully justify the impact on animal welfare of any such testing. The Digest also notes that, despite the provisions of the new Act, *'it is likely that many companies will continue to undertake animal testing for cosmetic purposes to meet the requirements for sale in other countries'* (this presumably refers to testing undertaken overseas). This is because most assessments meet one of the three exceptions mentioned above.

The passage of the Industrial Chemicals Act was accompanied by several other initiatives to support the ban on animal testing for cosmetics:

- ⊕ A *Voluntary industry code of practice to support the Australian ban on testing cosmetics on animals*, developed by Accord Australasia, with the purpose of informing the cosmetics industry and consumers about the implications of the Act and advertising claims that may be made in relation to animal tests on products ('Voluntary Industry Code on Animal Test Ban' n.d.);

⁸ S. Satya, pers comm

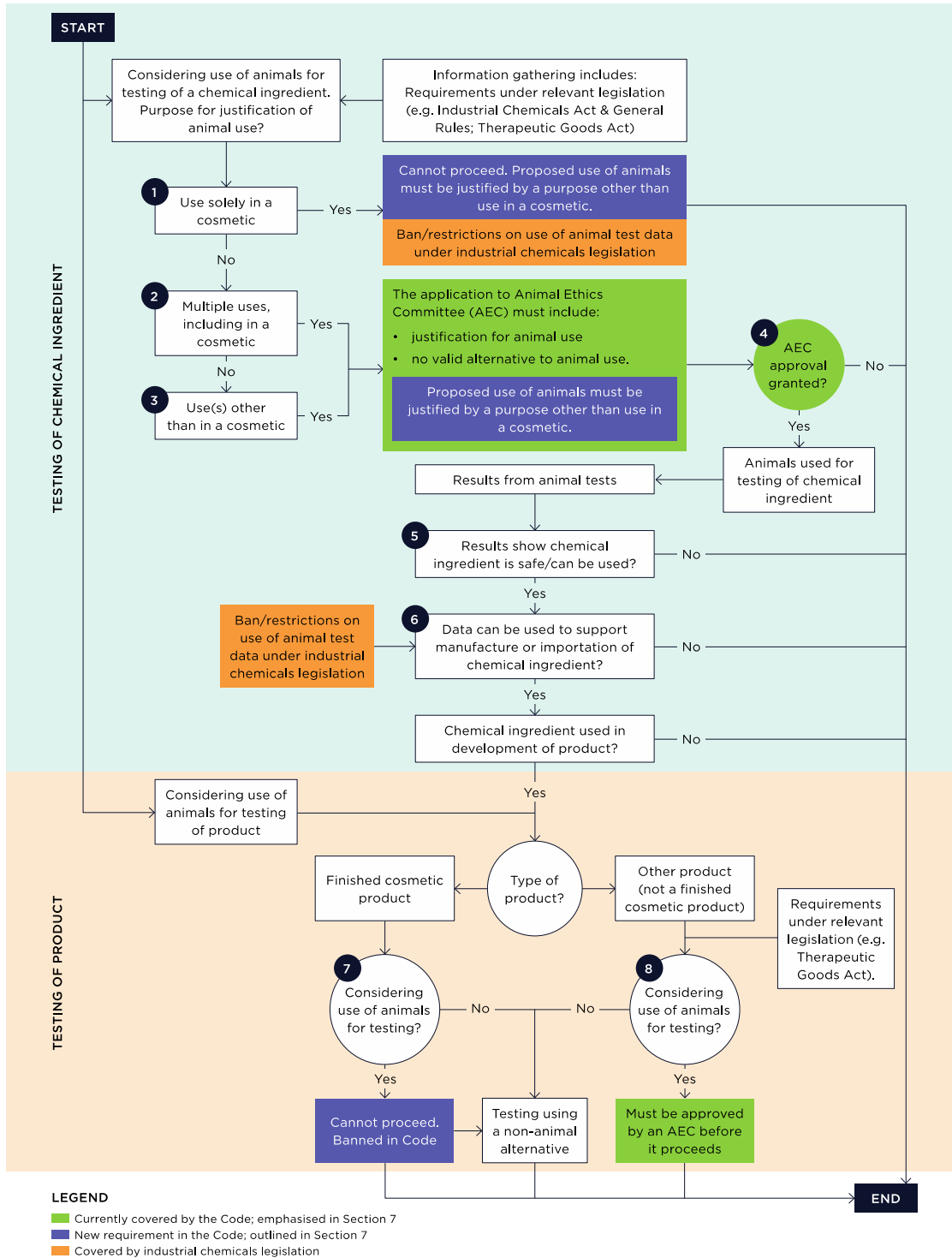
- ⊕ A consumer information package developed by the National Retail Association; and
- ⊕ A revised *Australian code for the care and use of animals for scientific purposes*, led by NHMRC.

It is important to understand that in Australia more general protection mechanisms also exist for the welfare of animals used in science. The first edition of what is now the NHMRC Code was published in 1969 under the title *Code of practice for control of experiments in animals*. The Code was first adopted by legislation under the *NSW Animal Research Act 1985* and has been progressively adopted under the legislation of all other states and territories since that time.

The NHMRC Code *'provides an ethical framework and governing principles to guide the decisions and actions of all those involved in the care and use of animals. It details the responsibilities of investigators, animal carers, institutions, and animal ethics committees, and describes processes for accountability'*. It is endorsed by NHMRC, the Australian Research Council, Commonwealth Scientific Industrial Research Organisation and Universities Australia, and compliance with the Code is a prerequisite for NHMRC funding (National Health and Medical Research Council n.d.).

The latest edition (2021) of the NHMRC Code includes a section on the ban on animal testing for cosmetics. In addition, NHMRC has produced several guidance documents to support the implementation of the ban. This includes a decision tree for the use of animals in testing of chemical ingredients or products (Figure 4). Chemicals that have uses outside of cosmetics must meet animal ethics committee requirements for use of animal studies, including justification that there are no suitable non-animal alternative tests.

Decision tree: Use of animals for testing of chemical ingredient or product



Source: NHMRC (National Health and Medical Research Council n.d.)

Figure 4: NHMRC decision tree on suitability of use of animals for testing of chemicals in Australia

6.3 GENERATION OF DATA FOR ASSESSMENT

The data used to support the introduction of an industrial chemical is generated either in-house by the registrant, or by a contract research organisation (CRO) working on behalf of the registrant. CROs characteristically have expertise in study design and conduct (GLP and Good Clinical Practice accreditation), carry the required laboratory accreditations (e.g., National Association of Testing Authorities), use recognised and approved and validated testing methods, bring expertise in chemical registration, operate at arms-length from the registrant, and are supported by necessary business instruments (such as company registration, insurance etc.).

An analysis of the proceedings from a recent, large international conference on alternatives to animal testing (*WC11 Maastricht | 23 august - 2 september 2021 | Virtual Congress n.d.*), undertaken for this report, showed that many of the large chemical manufacturers were well represented in the authorship of the 1,112 abstracts, suggesting that these companies possess strong in-house capability for testing of their own chemicals.

The test methods to be used in generating a dossier for a given chemical, whether in-house or by a CRO, will be selected to ensure data meets the requirements of the regulator. As noted in section 6.1, AICIS lists acceptable test methods on its website.

A scan of CROs globally was conducted for this report. It suggested that CROs with a NAM focus are mostly concentrated in Europe, but with strong North America and North Asia components. Most companies are small and offer specific solutions and/or tests; few truly global CROs were identified.

The scan, and discussions with various industry participants, also indicated that the CRO sector in Australia is small and offers a limited range of services in respect to chemical registration data. This is not surprising given the relative size of the Australian chemical manufacturing industry and domestic market for cosmetics and other industrial chemicals. The situation stands in contrast to the very active local CRO community for agvet chemical efficacy and animal safety testing to support registration, for example. Likewise, few dedicated NAM-focused regulatory consultants were identified through the preparation of this report. Consultants such as these in the medical and agvet fields facilitate the efficient procurement of testing services on behalf of clients to meet the specific needs of the relevant regulator.

It is pertinent to note that the sole provider of laboratory mice and rats in Australia (Animal Resource Centre) is under threat of imminent closure. This has been a shock to the biomedical research sector (SCIMEX 2021). Whilst one of the drivers may be the imminent reduction in use of animals in research, the lack of preparedness of the Australian research community has been sobering; clearly assuming the status quo will continue lacks necessary insight.

One reason for this difference between the industrial chemicals and agvet/medical industries may be the requirements in the latter case for much more detailed human safety as well as efficacy data relevant to Australia.

7. INTERNATIONAL PERSPECTIVES

The following provides a summary of the major legislative, structural and governance aspects of international efforts to advance the 3Rs, both in individual countries and in international collaboration. It is far from an exhaustive stocktake but, rather, is intended to highlight the main features of this international movement, which may offer pointers for Australia.

7.1 EUROPEAN UNION AND UNITED KINGDOM

Chemicals legislation in the EU is implemented by the European Chemicals Agency (ECHA). ECHA is responsible for:

- # Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) – see below
- # Classification and labelling of chemicals
- # Biocidal products registration
- # Prior informed consent
- # Persistent organic pollutants; and
- # Tasks from laws other than the five main pieces of legislation covering the above, such as worker safety (*Homepage - ECHA n.d.*).

ECHA maintains a database of over 245,000 chemicals. The chemicals database is integrated with the EU Chemicals Legislation Finder (EUCLEF) online service so that users can see how particular chemicals are regulated across the EU (*EU Chemicals Legislation Finder - ECHA n.d.*). Separately, the European Commission maintains a database of information on cosmetic substances and ingredients called 'CosIng' (*CosIng - Cosmetics - GROWTH - European Commission n.d.*).

A ban on testing finished cosmetic products on animals in the EU has been in place since 2004. A testing ban on ingredients or combinations of ingredients of cosmetics was established by the 'cosmetics directive' of 2009, which was replaced by the 'cosmetics regulation' on 11 July 2013. The cosmetics regulation also bans the marketing, in the EU, of finished cosmetic products and ingredients that have been tested on animals. From 2009 to 2013 this ban applied to all tests for human health effects except for repeated-dose toxicity, reproductive toxicity, and toxicokinetics. Since 2013, the ban has covered these aspects as well, regardless of the availability of alternative non-animal tests (*Ban on animal testing n.d.*).

The situation in the EU in relation to animal testing of cosmetics is much more complex than this, however. In 2007, the EU implemented its REACH regulation. REACH applies to all chemical substances. The purpose of REACH is to *'improve the protection of human health and the environment from the risks that can be posed by chemicals, while enhancing the competitiveness of the EU chemicals industry. It also promotes alternative methods for the hazard assessment of substances in order to reduce the number of tests on animals'* (*Understanding REACH - ECHA n.d.*).

REACH requires all chemicals that are manufactured in or imported to the EU, in quantities above one tonne/year, to have registration dossiers submitted to ECHA. Each dossier must contain a *'full (eco)toxicological evaluation, and risk assessment relative to declared use'*. The types of tests required depend in part on the quantity placed into the market. Companies with existing products had until 2018 to meet the new requirements (*REACH 2018 - ECHA n.d.*).

A recent study by the Transatlantic Think Tank for Toxicology (t⁴) has shown that REACH acts in conflict with the cosmetics regulation and its ban on animal testing (Knight et al. 2021). Dossiers on cosmetic ingredients are exempted under REACH from including a full chemical safety assessment for consumer exposure because this is already required under the cosmetics regulation. They are not exempted from a chemical safety assessment for worker exposure during manufacture of the ingredient or the final cosmetic product.

REACH requires that animal testing be used only as a last resort. ECHA notes that it uses a range of mechanisms to minimise animal testing, including the requirement for data sharing between companies,

and requiring alternative methods and approaches as far as possible, including read-across and QSAR. Two of the amendments to the regulation specify the use of *in vitro* test methods for skin / eye irritation and skin sensitisation (*Alternatives to animal testing under REACH - ECHA n.d.*).

Notwithstanding, the introduction of REACH has increased the number of animal tests undertaken. An analysis of the REACH database showed that it had 3,206 chemical dossiers with cosmetics as a reported use, 419 of which reported cosmetics as the only use. Sixty-three of these included the results of *in vivo* tests, to meet REACH requirements for toxicity and worker safety assessments, conducted after the implementation of the cosmetics regulation. In several cases, ECHA rejected data from alternative methods and required the new *in vivo* tests (Knight et al. 2021).

The apparent partial undoing of the good intentions of the cosmetics regulation has been the subject of much criticism. For example, Cruelty Free International estimates that REACH has led to the deaths of over 2 million animals (*Over 2 million animals used in experiments for REACH | Cruelty Free International n.d.*). One cosmetics company appealed an ECHA requirement for new *in vivo* tests in support of two cosmetic ingredients. The ECHA decision was upheld by its court of appeal (Knight et al. 2021).

The EU has also had specific legislation for the protection of animals used for scientific purposes since 1986. The current Directive took effect in 2013 and has been most recently updated by Regulation in 2019, when ambitious goals for reporting and transparency were incorporated. The legalisation is based upon the principle of the 3Rs. The EC website notes that '*The EU Directive is unique in the world because it sets as its ultimate goal the full replacement of use of animals for scientific purposes*' (*Legislation for the protection of animals used for scientific purposes - Environment - European Commission n.d.*; *Animals in scientific research - Environment - European Commission n.d.*).

To support the advancement of the 3Rs, including the development of new test methods, the EU has established the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM). EURL ECVAM conducts research and collaborates in research initiatives, coordinates, and undertakes the validation of alternative methods, promotes knowledge sharing across disciplines and sectors, and promotes the 3Rs internationally (*EU Reference Laboratory for alternatives to animal testing n.d.*). The EU has also funded some large research programs, for example SEURAT-1 (*SEURAT-1 - Towards the Replacement of in vivo Repeated Dose Systemic Toxicity Testing n.d.*) and EU-ToxRisk (*EU-ToxRisk - Case Studies n.d.*).

Several countries in the EU (as well as the UK) also have institutions to support the 3Rs, including Norway, Germany and the Netherlands (*European 3Rs Centres n.d.*). One example is the UK National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs), an autonomous body operating under the Medical Research Council, established in 2004 and dedicated to replacing, refining and reducing the use of animals in research and testing. NC3Rs receives core funding from the UK's Medical Research Council and Biotechnology and Biological Sciences Research Council, and funding for specific programs from several charitable and commercial organisations. Its annual budget is around £10 million.

NC3Rs is unique because it not only supports research and skills development but also funds and works with academic institutions and industry to connect people in different spheres. An example is the CRACK IT program, which sets 3Rs 'challenges' to be solved by collaborations between sponsors, innovators and funders (*CRACK IT Challenges | Innovation Platform n.d.*).

NC3Rs works across 5 'Ps':

- ⊕ Practice in the biosciences, including funding in 3Rs research and supporting its commercialisation
- ⊕ Procedures on animals, focused on refining animal research and ensuring welfare is a priority

- # People in the biosciences, supporting early career researchers and improving opportunities for training in the 3Rs
- # Places conducting animal research, supporting institutions to actively promote the 3Rs; and
- # Policy related to animal research, with an emphasis on global harmonisation in regulatory testing.

In the Netherlands, the National Committee for the protection of animals used for scientific purposes (NCad) delivered a report in 2016 recommending opportunities to transition to non-animal research (Ministerie van Landbouw 2016). The report argues that the use of laboratory animals in regulatory safety testing can be phased out without compromising safety levels. It outlines six elements of a transition strategy that include working at the international level to review the regulatory risk assessment process, encouraging multidisciplinary collaboration on innovation in the 3Rs and prioritising the monitoring, evaluation and reporting of progress towards the phasing out of animal procedures.

7.2 UNITED STATES

The United States Federal Food, Drug and Cosmetic Act does not require animal testing to prove that cosmetics are safe, but neither does it prohibit the use of animals for cosmetics testing (Food and Drug Administration 2022a). However, a number of states, including California, Hawaii, Illinois, Maine, Maryland, Nevada and Virginia, have passed such legislation (*Cosmetics testing FAQ | The Humane Society of the United States* n.d.). This created difficulties for companies wanting to market products both into China, which required animal test data to establish safety (China has since amended its laws – see below), and into jurisdictions that ban the sale of cosmetics that have been tested on animals. For this reason, laws in California, Nevada and Illinois provide exemptions for cosmetics that were tested on animals to comply with regulations of a foreign government (*US states join global push to ban animal-tested cosmetics* 2021).

On the testing of industrial chemicals more broadly, the US Congress in 2016 passed the Frank R. Lautenberg Chemical Safety for the 21st Century Act, which amends the longstanding Toxic Substances Control Act (TSCA). Among many other provisions, the amended TSCA directs the US EPA to:

- # Reduce and replace, to the extent practicable and scientifically justified, the use of vertebrate animals in the testing of chemical substances or mixtures; and
- # Promote the development and timely incorporation of alternative test methods or strategies that do not require new vertebrate animal testing (US EPA 2017).

Consistent with the TSCA, in 2018, the US EPA published its 'Strategic Plan to Promote the Development and Implementation of Alternative Test Methods Within the TSCA Program'. The US EPA also publishes a 'List of Alternative Test Methods and Strategies (or New Approach Methodologies [NAMs])' that '*do not require new vertebrate animal testing and are scientifically reliable, relevant, and capable of providing information of equivalent or better scientific reliability and quality to that which would be obtained from vertebrate animal testing*' (US EPA 2019).

The US FDA also has a program of work to advance alternative methods (Food and Drug Administration 2022b).

The US has a governance structure to advance the development and acceptance of alternatives to animal testing, which includes:

- # The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), which is 'focused on the development and evaluation of

alternatives to animal use for chemical safety testing'. The NTP is part of the Department of Health and Human Services (*NICEATM: Alternative Methods* n.d.).

- # The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), a permanent committee of the National Institute of Environmental Health Sciences (NIEHS) under NICEATM. The ICCVAM comprises representatives from 17 US federal regulatory and research agencies that require, use, generate, or disseminate toxicological and safety testing information. It issues recommendations for replacing and reducing animal use and compiles a list of methods for chemical safety testing that are accepted by US and international regulatory authorities as replacement, reduction, or refinement alternatives to required animal tests (*About ICCVAM* n.d.).
- # The Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), which advises NICEATM and ICCVAM on their activities (*Advisory Board & Committees* n.d.).

Activities in this area are guided by 'A Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States', published by ICCVAM in 2018. The Roadmap has three strategic goals:

1. **Connect end users with the developers of NAMs.** This strategy includes activities to identify anticipated testing requirements, encourage the establishment of grant review criteria tailored to the development of alternative methods and develop mechanisms to improve communication between end users and researchers.
2. **Foster the use of efficient, flexible, and robust practices to establish confidence in new methods.** This strategy includes activities to clearly delineate testing requirements and context of use, promote the use of new approaches for establishing confidence and utilise public-private partnerships to promote cross-sector communication and cooperation.
3. **Encourage the adoption and use of new methods and approaches by federal agencies and regulated industries.** This strategy includes activities to provide clear language regarding the acceptance of NAMs, collaborate with international partners to facilitate global harmonisation and regulatory acceptance, explore processes to incentivise and promote the use of NAMs and identify appropriate metrics for prioritising activities, monitoring progress, and measuring success (*A Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States* n.d.).

Among other things, NICEATM and ICCVAM provide several resources for test method developers, including a data sharing facility and the Bibliography on Alternatives to the Use of Live Vertebrates in Biomedical Research and Testing (ALTBIB), providing access to PubMed citations on animal testing alternatives.

Another organisation of note in the US is the Johns Hopkins Bloomberg School of Public Health Center for Alternatives to Animal Testing (CAAT) in Maryland. CAAT plays a similar role to the UK's NC3Rs but is funded through philanthropic and commercial sponsorship. CAAT supports research (externally and internally), provides training, and develops resources to promote humane research. It produces the journal ALTEX – Alternatives to Animal Experimentation. CAAT also has a European branch (CAAT-Europe), based at the University of Konstanz in Germany.

7.3 OTHER COUNTRIES

In 2021, **Mexico** became the first North American country and the 41st country worldwide to ban the use of animal testing for cosmetics (*In major win for animals, Mexico bans animal testing for cosmetics* 2021).

There is currently no national legislative mandate to phase out animal testing in **Canada**. There are, however, coordinated efforts to advance the 3Rs. The Canadian Centre for Alternatives to Animal Methods (CCAAM) and the Canadian Centre for the Validation of Alternative Methods (CaCVAM) are based at the University of Windsor. CCAAM supports research, training and education, and regulatory aspects of NAMs. CaCVAM is similar in scope and purpose to ECVAM in the EU, ICCVAM in the US and similar organisations in other countries (*Canadian Centre for Alternatives to Animal Methods* n.d.).

In 2015, **New Zealand** introduced an Animal Welfare Amendment Bill to ban animal testing of cosmetics, or ingredients for exclusive use in a cosmetic. The ban was largely symbolic, as there had never been any animal testing of cosmetics in NZ. The ban does not apply to imports (Commonwealth Parliament n.d.).

The **Japanese** Center for the Validation of Alternative Methods (JaCVAM) is part of the country's National Institute of Health Science. JaCVAM was established to promote the 3Rs through the validation and endorsement of NAMs (*JaCVAM* n.d.). The **Korean** Center for the Validation of Alternative Test Methods (KoCVAM) has a similar remit (*KoCVAM* n.d.).

Until recently, **China** has specifically required that cosmetics sold into its large and lucrative market must be tested on animals. As of May 2021, however, China has changed its laws to allow companies to apply for an exemption to the animal testing requirements, where they wish to market 'general cosmetics' including shampoo, bodywash, makeup and lipstick. 'Special cosmetics' (some of which would be classified as therapeutic goods in Australia, such as sunscreens or anti-hair loss products, but also hair dyes and perming products) still require animal testing ('China Proposes End to Tests on Animals for Many Imported Cosmetics - News' 2021).

The changes to Chinese regulations make little difference to Australia, which restricts the use of animal test data rather than products that have been tested on animals. They may however encourage chemical and ingredient and cosmetic manufacturers to move more quickly away from animal testing.

India, Israel, Norway, Iceland and Switzerland have passed laws on animal testing of cosmetics similar to those of the EU (*Cosmetics testing FAQ | The Humane Society of the United States* n.d.).

7.4 INTERNATIONAL COOPERATION AND HARMONISATION

There are several bodies facilitating international cooperation on efforts to advance the 3Rs, including the harmonisation of NAMs.

The International Cooperation on Alternative Test Methods (ICATM) was established in 2009 to facilitate '*international cooperation in the critical areas of validation studies, independent peer review, and development of harmonized recommendations*'. The Memorandum of Cooperation underpinning ICATM includes EURL ECVAM (EU), ICCVAM (US), Health Canada, JaCVAM (Japan) and KoCVAM (Korea). China and Brazil have participated in ICATM since 2015 as observers and Singapore and Taiwan have also participated in ICATM events (*International Cooperation on Alternative Test Methods* n.d.).

Another organisation with a pivotal role in the harmonisation of test methods is the OECD. The OECD's Environment Directorate oversees a range of areas of work, including the development and publication of Test Guidelines (TG), the Mutual Acceptance of Data (MAD) system and hazard assessment.

The OECD's TG program is overseen by the Working Group of National Co-ordinators of the TGs programme (WNT). The WNT comprises representatives of regulatory authorities in OECD member countries (including Australia) and countries adhering to MAD. The WNT is in turn overseen by the OECD Chemicals and Biotechnology Committee (since 2020), which endorses new test guidelines for approval

by the OECD Council (Environment Directorate 2009). Several other OECD Working Groups (for example on Biocides) also have input.

The process of developing a new TG involves input from each of the represented countries as well as consultation with a range of experts and interested parties. Since 2002, animal protection has a formal voice in this process through the International Council on Animal Protection in OECD Programmes (ICAPO), comprising representatives from ten NGOs advocating for more humane research, including Cruelty Free International, European Coalition to End Animal Experiments, Humane Society of the United States, PETA International Science Consortium and Physicians Committee for Responsible Medicine (International Council on Animal Protection).

The MAD system requires that participating countries accept data from tests that are carried out by other participant countries if they are performed in accordance with OECD TGs and the OECD Principles of Good Laboratory Practice (GLP). Whilst its purpose is primarily to increase efficiency, the MAD system has a 3Rs purpose, in that it aims to reduce the duplication of testing and therefore use of animals where animal tests are applied (*Mutual Acceptance of Data (MAD) - OECD n.d.*).

The OECD's work is highly influential in shaping the way that national and multinational regulators such as the EU approach their testing regimes. This includes Australia. Indeed, key personnel from Australia are very active participants in OECD discussions.

In respect to cosmetics, the International Cooperation on Cosmetics Regulation (ICCR) is a forum of cosmetics regulatory authorities from Brazil, Canada, Chinese Taipei, the EU, Japan, the Republic of Korea, and the US. According to its terms of reference, '*ICCR provides a multilateral framework to maintain and enable the highest level of global consumer protection by working towards and promoting regulatory convergence, while minimizing barriers to international trade*'. ICCR discussions also include industry peak bodies of the respective member countries (*About Us n.d.*). ICCR produces white papers, general principles, and recommendations on various regulatory issues. This includes an 'Inventory of validated alternatives to animal testing applicable for cosmetic products and their ingredients in all ICCR regions' (*Topics & Documents n.d.*).

8. OPPORTUNITIES AND CHALLENGES FOR AUSTRALIA

It appears that most data dossiers in support of industrial chemical introductions are generated overseas by overseas-based chemical companies or CROs. This is not surprising given the smaller size of the Australian chemical manufacturing industry and domestic market for cosmetics and other industrial chemicals. Likewise, it is not surprising that there appear to be relatively few CROs serving the non-therapeutic chemicals industry, notably the NAM-focused cosmetics sector.

Whilst the relative underdevelopment of the CRO and consultancy sector might be inevitable in the circumstances, it makes it difficult for an Australian chemical manufacturer or importer to obtain local data from NAMs. This means that not all toxicology studies can be conducted at arms-length, nor does a generic toxicological test necessarily provide the data uniquely required by Australia. For example, Australia may require toxicological data relevant to ecosystems found only in Australia.

This is further compounded by lack of harmonisation between importing countries of the requirements for industrial chemical registration. Countries may differ in their categorisation of chemical products or components, requiring different data on toxicity. For example, in the case of cosmetics, more extensive data on 'functional or special cosmetics' (such as hair repair products) is required than for general cosmetics. Repeated-dose toxicity data requirements can differ between countries. Australia often requires this information for new ingredients, but this is unavailable from NAM tests. In the situation where analogue data is unavailable, registration cannot proceed. This is often the case for UVCB chemicals. The differences between jurisdictions for toxicity data requirements, the ability to use animal-based study data and the size of the market opportunity all determine the appetite of chemical manufacturers and importers to seek access to a market with a new chemical. Greater harmonisation of requirements and acceptability of tests is a logical next step to streamline and improve the performance of the chemical registration process.

The lack of a commercial testing sector also has implications for the translation of Australian research outcomes. Australia has a strong basic science sector and there appear to be numerous, world-class research groups in Australia developing alternative test methodologies, some of which have the potential to replace existing animal models for industrial chemical testing. Some such researchers were identified during the preparation of this report.⁹ However, the path from research to commercialisation is far less clear in Australia. As noted in section 5, this path is long and laborious, even in those jurisdictions such as the EU which appear the most progressive in moving away from animal testing and which have the largest domestic markets. Any provider seeking to deliver a new test method to the market faces substantial costs in acquiring the instruments, reagents, skills, and necessary accreditations to deliver the method.

Universities and other research institutions may not have strong interest in taking on such challenges where the demand is limited. This may be changing with a greater focus on industry engagement, but the fact remains that such organisations often lack the commercial nous, resources, and other enablers (such as registrations and insurances) to offer chemical testing as a for-profit service. Alternatively, a research institution may opt to license a third party to commercialise a test it develops, but this path too assumes

⁹ These interviewees have not been listed, as a systematic cross-sectional consultation with stakeholders outside the SRG was not part of the report methodology

there is an active commercial sector willing to take on the licence. It also requires a degree of commercial insight and proactivity on the part of the research leader and the commercial arm of the institute.

In short, there needs to be sufficient commercial return for providers to take on NAMs and offer them in Australia. It is not at all clear that this exists in Australia for the testing of chemicals. This may be due to a combination of effects including there being a patchwork of NAMs, often developed by numerous organisations, but with an insufficient suite residing within any single organisation to support commercialisation (especially for CROs); the small market size; the lack of understanding among participants of others in the sector; and perhaps other factors (such as differing skill sets required to develop and deploy).

Another observation from interviews for this report is that Australian researchers are unlikely to identify themselves *primarily* as being part of a scientific community addressing the replacement of animals in research. Instead, they may identify themselves as researchers in a particular disease field (e.g. pulmonary) who happen to develop a non-animal model that facilitates their studies. This is undoubtedly not a universal rule. The University of New South Wales has recently implemented a 3Rs grant scheme (*UNSW 3Rs Grant Scheme* | *UNSW Research* n.d.). NHMRC and ANZCCART also promote and provide valuable supporting resources on 3Rs approaches, but their role is not to facilitate the development or commercialisation of NAMs.

More generally, the interviews for this report revealed a poor overall appreciation of the entire 3Rs / NAM domain in respect to industrial chemicals in Australia and its global context. There appear to be few who understand the Australian pipeline for NAM development (and indeed it is not clearly defined) and fewer still who understand how to commercialise a NAM.

It seems clear that further intervention is needed to galvanise progress towards reduced reliance on animal test data. Indeed, this appears to be true across all biomedical research. Australia lacks several coordinating, cross-disciplinary mechanisms that exist in other countries to promote the adoption of the 3Rs and to increase the options available under a 3Rs approach, especially the development and validation of NAMs. There are numerous groups in Australia working in this domain. Paradoxically, while Australian individuals and organisations are active at the global level in delivering world-class research, or in OECD working groups, there is little coordination of effort within Australia.

There are opportunities for Australia to take advantage of its relationships with international jurisdictions that have larger industrial chemical sectors and more advanced institutional responses to the challenge of moving away from animal testing. These are:

- ⊕ Promoting funding streams to support research in the 3Rs and specifically NAM development and commercialisation. These would help ensure any unique Australian needs for NAMs are effectively and efficiently addressed now and into the future.
- ⊕ Promoting other activities to advance the 3Rs in Australia, including in all biomedical and other fields that use animals. These activities might include:
 - ◆ Coordinating the exchange of information between stakeholders such as policy-makers, regulators and the scientific community
 - ◆ Providing support for researchers, including signposting or administering grant schemes and postgraduate programs
 - ◆ Facilitating central access to resources such as databases (of technical reports, original research data, gene expression profiles and so on), and
 - ◆ Coordinating or delivering training for researchers, regulators and others.

The effectiveness and sustainability of such an initiative are likely to be maximised by involving both public and private stakeholders (such as industry), given the apparent market failure in the commercialisation of NAMs in Australia.

- # Promoting a system to support the adoption of internationally-validated NAMs and support validation of alternative test methods with a specific focus on unique Australian needs and research outcomes. This would also support Australian chemical companies that seek to export and require access to internationally-accepted NAMs to support overseas registration. It would be beneficial for Australia to participate in ICATM, either as an active member or seek opportunities as observer (in the same way that China and Brazil participate) for increasing Australia's influence in global decision-making in respect to NAM development.

To determine and identify the means to achieve these opportunities, there is a need to develop a strategy or 'roadmap' that articulates Australia's needs and ambitions in relation to the 3Rs. The strategy would also consider what data is available or required to set performance targets and monitor progress. The US roadmap provides one possible model in this respect.

There are potential benefits for Australia from advancement in alternatives to animal testing. Greater coordination of activities within Australia may help to overcome market failure and stimulate both research in NAM development and commercialisation of NAMs and service delivery. There may also be economic benefits for Australian chemical companies who are interested in approaching global markets. A coordination strategy would help Australia to be better placed to address its unique needs in relation to chemical assessment and regulation, which is particularly important for Australia as a 'data taker'. Last, but not least, animal welfare outcomes are likely to be improved more rapidly as the 3Rs gain a greater focus.

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APPENDIX 1: MEMBERSHIP OF THE PROJECT STAKEHOLDER REFERENCE GROUP

The project stakeholder reference group comprised participants from the following organisations:

REGULATORS AND COMMONWEALTH AGENCIES

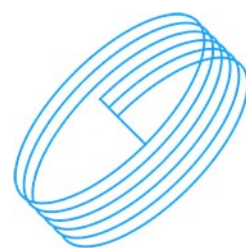
- ✦ Australian Pesticide and Veterinary Medicines Authority (APVMA)
- ✦ Australian Industrial Chemicals Introduction Scheme (AICIS)
- ✦ Food Standards Australia New Zealand (FSANZ)
- ✦ National Health and Medical Research Council (NHMRC)
- ✦ Department of Agriculture, Water and Environment (DAWE)

PEAK BODIES

- ✦ Australian & New Zealand Council for the Care of Animals in Research and Teaching (ANZCCART)
- ✦ Australian Society of Cosmetic Chemists (ASCC)
- ✦ Accord Australasia
- ✦ Australian Veterinary Association (AVA)
- ✦ Universities Australia
- ✦ Society of Environmental Toxicology and Chemistry Australasia (SETAC)
- ✦ Australasian College of Toxicology and Risk Assessment (ACTRA)

NON-GOVERNMENTAL ORGANISATIONS

- ✦ RSPCA Australia
- ✦ Humane Research Australia (HRA)
- ✦ Humane Society International (HSI)
- ✦ Medical Advances Without Animals (MAWA)



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