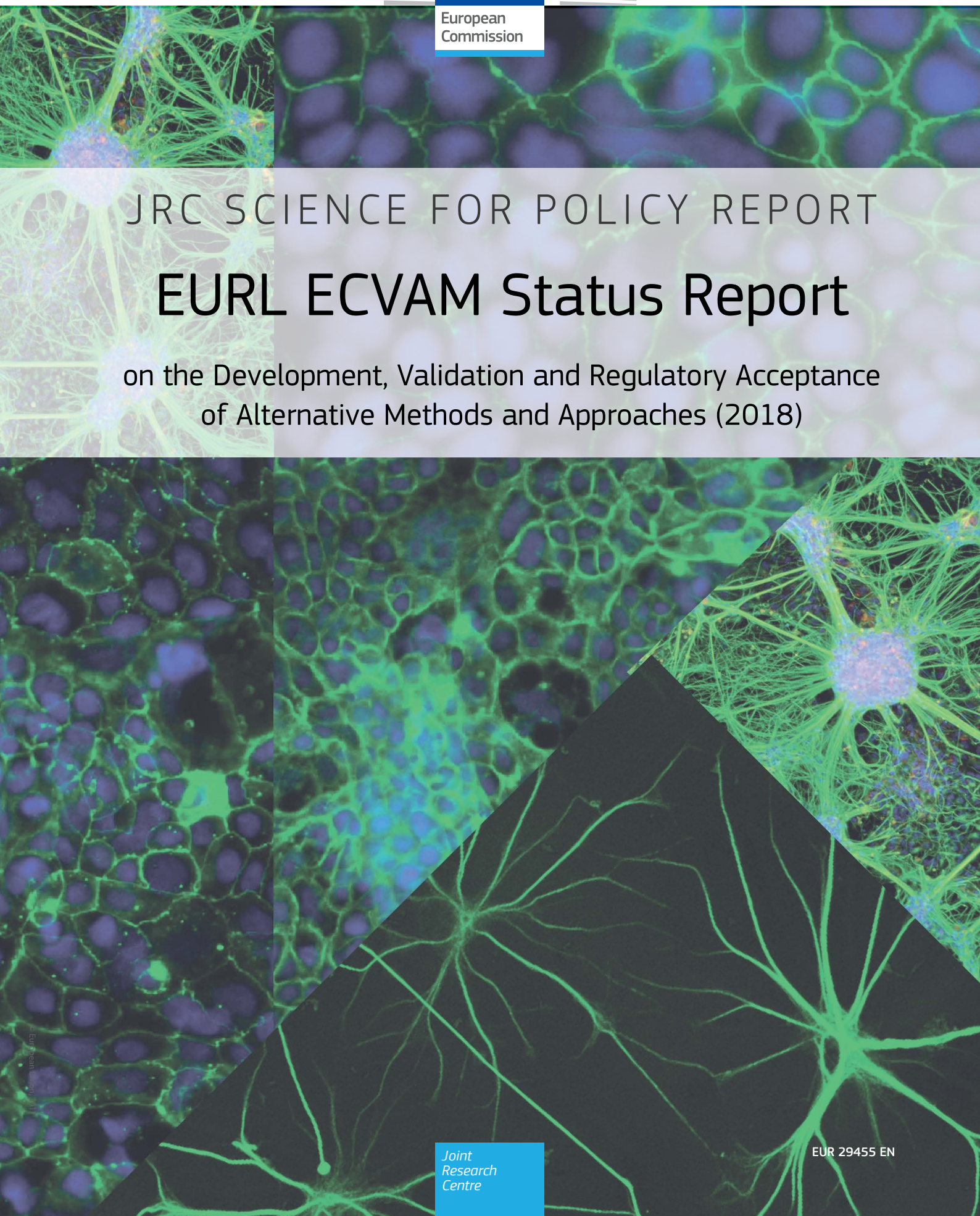

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JRC SCIENCE FOR POLICY REPORT

EURL ECVAM Status Report

on the Development, Validation and Regulatory Acceptance of Alternative Methods and Approaches (2018)

Joint Research Centre

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Editors: Zuang V. & Dura A.

Status of Alternative Approaches to Animal Testing

Abstract
Replacement, Reduction and Refinement (Three Rs) of the use of animals for scientific purposes is required by EU legislation. Integrated Approaches to Testing and Assessment that incorporate multiple data streams derived from new sources such as *in vitro* methods and computational (*'in silico'*) modelling are advancing to translate mechanistic understanding of toxicity into safety testing strategies avoiding traditional animal testing for chemicals safety testing. In addition, effective knowledge sharing activities on alternatives in the areas of basic and applied research in life sciences as well as education and training are growing in scope, oureach and impact.

Printed in Italy

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Executive Summary

The European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) is an integral part of the European Commission's Joint Research Centre (JRC). This annual EURL ECVAM status report provides updates on the progress being made in the development, validation and regulatory acceptance of alternative-to-animal methods and approaches and their dissemination.

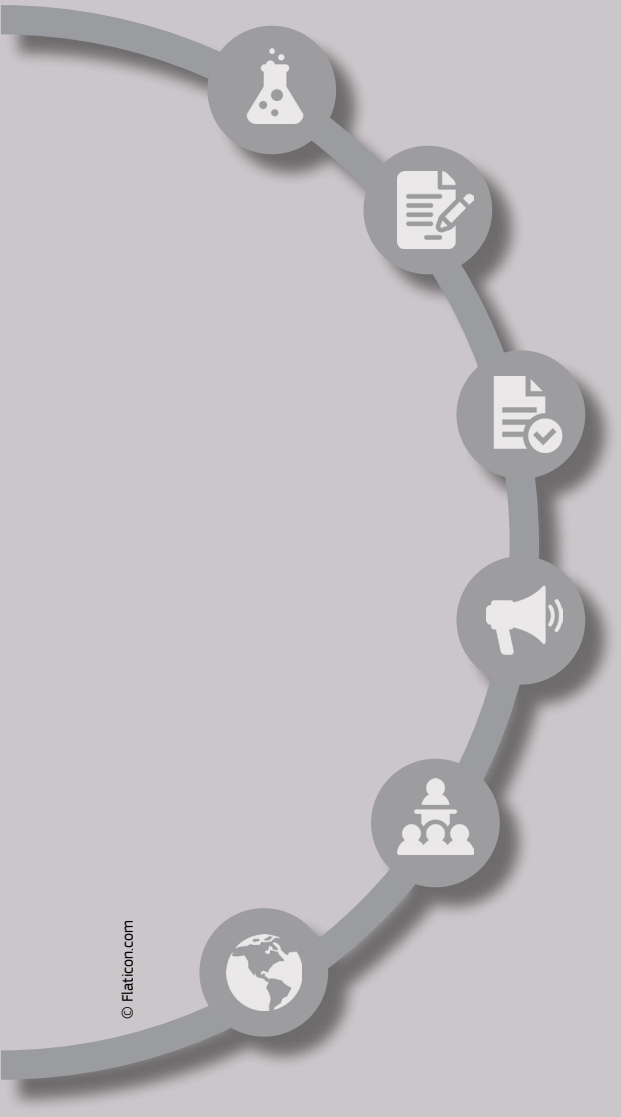
In the areas of toxicology and safety assessment, significant progress has been made in demonstrating how *in vitro* and computational methods can be combined to 'read-across' toxicological properties between similar chemicals to avoid unnecessary animal testing. Alternative methods are also being increasingly employed for the identification of endocrine disruptors and the assessment of chemical mixtures. However the use of alternative methods alone to fully characterise complex toxicological properties of chemicals, such as chronic systemic health effects, remains a formidable challenge. Recommendations have been published on how to improve multi-centre validation studies of alternative methods for the quality control of vaccines and how the data generated can be used for product specific validation by vaccine manufacturers. Recent advances in the ecotoxicology field include the development of a Threshold of Toxicological Concern approach (eco-TTC) for environmental safety assessment and the use of integrated non-animal methods to predict the bioaccumulation potential of chemicals in fish. Addressing the biomedical research domain, EURL ECVAM launched a major study to review the use of alternative methods and models for understanding biology and disease and for developing novel approaches to diagnosis and therapy.

The validation of alternative methods intended for regulatory use has progressed on a number of fronts including novel methods for predicting the skin sensitisation potential of chemicals, assessing the leaching (bioelution) of chemicals from metal alloys, and for determining acute toxicity in fish. Two EURL ECVAM validation studies deal with methods for the identification of endocrine disruptors and involve the EU Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL). Several multi-stakeholder discussions on validation took place in various fora which indicate that although well-established validation principles are still relevant today, the process of validation needs to be continually adapted to keep pace with scientific and technological progress. Reflecting the potential application and impact of emerging organ-on-chip devices, EURL ECVAM conducted an international survey of developers and stakeholders to identify the issues determining end-user confidence and to solicit opinions on how best to approach validation to facilitate uptake and acceptance. The EURL ECVAM Scientific Advisory Committee (ESAC) was renewed during 2018 and one of its first mandated tasks is to deliver an opinion on the scientific validity of antibodies and non-antibody affinity reagents generated using animal-free technologies used in research and diagnostics.

In the context of international regulatory acceptance and promotion of alternative methods for safety assessment, several initiatives at the Organisation of Economic Cooperation and Development (OECD) are breaking new ground and delivering impact. In addition to new test guidelines and guidance documents to support non-animal approaches to assess chemical toxicity in fish, the OECD published guidance on Good *In Vitro* Method Practices (GIVIMP) to ensure the reliability and integrity of *in vitro* data intended for regulatory use. The OECD project to develop a Guideline for 'Defined Approaches' for skin sensitisation assessment which combine both *in vitro* and computational methods has made steady progress and a draft has recently undergone commenting by OECD member country experts. The Adverse Outcome Pathway (AOP) programme is growing and 2018 saw the publication of a second set of AOPs for complex endpoints that were endorsed by OECD expert groups. Further initiatives on Integrated Approaches to Testing and Assessment (IATA) included a new cycle of IATA case studies and the launch of a project to map relevant guidance documents. Incorporation of alternative approaches into regulatory frameworks has also been addressed by other international bodies such as the International Cooperation on Cosmetics Regulation (ICCR) and the United Nations sub-committee on the Globally Harmonised System (GHS) of classification and labelling of chemicals.

Finally, sharing of expert knowledge remains a central pursuit to advance alternatives and a variety of collaborative initiatives are ongoing within the European Partnership for Alternative Approaches to Animal Testing (EPAA) and the International Cooperation on Alternative Tests Methods (ICATM). In addition, EURL ECVAM has been working with its network of EU and Member State regulatory bodies (PARERE) and its stakeholder forum (ESTAF) to identify opportunities for enhancing knowledge sharing in the fields of regulatory toxicology, biomedical research and education and training.

1 Introduction



1. Introduction

The European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) is an integral part of the European Commission's Joint Research Centre (JRC). The EURL ECVAM status report provides updates on the progress made in the development, validation and regulatory acceptance and use of alternative methods and approaches and their dissemination. The status report describes research, development and validation activities, as well as initiatives that promote the regulatory and international adoption and use of alternative approaches and their dissemination.

The mandate of EURL ECVAM is described in Directive 2010/63/EU (EU, 2010) on the protection of animals used for scientific purposes and includes a number of duties (Article 48 and Annex VII) to advance the Replacement, Reduction and Refinement (the Three Rs) of animal testing. The current activities of EURL ECVAM build on over 25 years of JRC support to the Three Rs (EC, 1991) and include participation in research projects; coordination and undertaking of validation studies; dissemination and sharing of information on the Three Rs; and the promotion of dialogue towards the international acceptance and uptake of alternative methods and approaches. Besides Directive 2010/63/EU, EU chemicals legislation such as REACH (EC, 2008) and the Cosmetics Regulation (EC, 2009) have had an important impact on the Three Rs by increasing the pace and the number of methods being developed, validated and proposed for international adoption.

Other pieces of EU chemicals and products legislation refer to alternative approaches and allow them to be used in hazard and risk assessment, including: Regulation (EC) No 1272/2008 on Classification, Labelling and Packaging of substances and mixtures (CLP); Regulation (EC) No 1107/2009 concerning the placing of plant protection products on the market; Regulation 283/2013 on data requirements for active substances; Regulation 284/2013 on data requirements for plant protection products; Regulation 528/2012 concerning the making available on the market and use of biocidal products; Directive 2001/83/EC (and its amendments) on the Community code relating to medicinal products for human use; and Directive 2001/82/EC (and its amendments) on the Community code relating to medicinal products for veterinary use. In addition, the Commission Communication on the combined effects of chemicals - chemical mixtures (EC, 2012) and the EU strategy on Endocrine Disruptors (EC, 2018) are also important drivers of EURL ECVAM's work since they present significant opportunities for the use of non-animal methods to address safety assessment challenges.

The EURL ECVAM status report describes primarily, but not exclusively, all the activities it has undertaken or has been involved in since the publication of the last report in November 2017. It is intended to inform EURL ECVAM stakeholders and any interested parties on on-going activities in the field of alternative approaches and serves multiple purposes. This includes providing input to the annual Commission report on the progress made in the development, validation and regulatory acceptance of alternative methods and approaches that is prepared in the framework of Regulation 1223/2009 on cosmetic products. It also supports the monitoring and implementation of provisions on alternatives included in Directive 2010/63/EU on the protection of animals used for scientific purposes and in other EU legislation related to chemicals and products.

2 Research and Development Activities on Alternative Methods and Approaches

2. Research and Development Activities on Alternative Methods and Approaches

2.1 EU-ToxRisk

EU-ToxRisk is a European collaborative project funded by the EU Framework Programme for Research and Innovation, Horizon 2020, to advance mechanism-based toxicity testing and risk assessment. The JRC has a formal collaboration agreement in place with the consortium to support its science programme and to aid in the eventual translation of research results into application. For more information on the project, a detailed description has been provided in the EURL ECVAM status report 2017 (see section 2.2 in Zuang *et al.*, 2017).

With the aim of encouraging early engagement of the regulatory hazard and safety assessment community, EURL ECVAM launched a consultation within the Preliminary Assessment of Regulatory Relevance (PARERE) network to get their opinion on the regulatory relevance of safety assessment case studies being developed within the consortium. The EU-ToxRisk case study strategy and an overview of four representative case studies were shared with the PARERE members, including:
1) Prediction of microvesicular liver steatosis - a read-across case study with (un-)branched carboxylic acids,
2) Valproic acid analogues and developmental and reproductive toxicity (DART) liabilities,
3) Prediction of Parkinsonian-like liabilities based on AOP aligned testing linked to mitochondrial toxicity and
4) Repeated-dose toxicity: Popcorn Lung – read-across on diketones.

Overall, the PARERE opinion was that such engagement was very beneficial for all parties and that New Approach Methodologies (NAMs; ECHA, 2016) have indeed a lot of potential for use in regulatory toxicology, although to what extent this would be possible in the short-term was unclear (see also section 4).

EURL ECVAM is actively supporting the consortium in a number of areas including: providing training and advice on the development of Adverse Outcome Pathways (AOPs); the centralised provision of reference chemicals for cross-laboratory generation of key datasets; the use of modelling for *in vitro* to *in vivo* extrapolation; and the definition and design of new case studies.

Collaboration also covers the inclusion of selected protocols developed within the EU-ToxRisk consortium into the EURL ECVAM Database on alternative methods (DB-ALM). Currently, the following protocols have been revised and published, with more protocols in the pipeline:

- cMINC assay to test migration inhibition compounds on human iPSC-derived neural crest cells (NCCs).
- UKN3b assay to test compound-derived neurite integrity impairment in human mature dopaminergic neurons.
- Automated CALUX reporter gene assay procedure.

READ MORE

- EU-ToxRisk: www.eu-toxrisk.eu
- Horizon 2020: europa.eu/!Fm38rr

2.2 European Research Projects on Chemical Mixtures

Several EU funded Horizon2020 and FP7 research projects that are relevant in the context of chemical mixtures are currently ongoing.

In 2017, the projects EDC-MixRisk, EuroMix, EU-ToxRisk, HBM4EU, and SOLUTIONS joined forces together with EURL ECVAM, other Commission Services (DG Research and Innovation, DG Environment) and European agencies (EFSA, ECHA, EEA) to organise a joint workshop on "Advancing the Assessment of Chemical Mixtures and their Risks for Human Health and the Environment". The workshop was prepared in collaboration and finally held 29 to 30 May 2018 at JRC Ispra, bringing together 60 experts in the field of chemical mixtures (see box 2.1 and fig. 2.1). The aim of the workshop was to discuss among researches and regulators mixture risk assessment from the different perspectives, to evaluate the progress achieved and identify further gaps to be addressed.

The workshop provided a forum to discuss the latest advancements in science, as well as research and policy needs, in order to make progress in mixture risk assessment and management. The topics of the workshop included hazard and exposure assessment, data and tools, and risk analysis and governance. To consider also the wider international perspective, experts from Japan, United States (US) and the Organisation for Economic Cooperation and Development (OECD) joined to present the way they are tackling mixtures. Although much progress has been achieved over the recent years, the participants stated that it is evident that more needs to be done to better address the combined exposure of multiple chemicals, both in terms of intentional mixtures (manufactured products such as pesticide formulations and cosmetic products) and unintentional mixtures (*e.g.*, surface water pollutants). One of the major gaps continues to be the lack and availability of data. The Information Platform for Chemical Monitoring, IPCHeM, is addressing the gap for chemical monitoring data. However, another big challenge remains in the accessibility and quality of data on (eco) toxicological properties and on the types of use of chemicals. In the group and plenary discussions, ideas were brought up on improving governance aspects to better protect public health and environment from hazardous chemical mixtures. The workshop outcome and future research needs will be summarised and published later in 2019. An overview of the current EU projects on mixtures and mapping of their activities to the different aspects of mixture risk assessment can be found in Bopp *et al.*, 2018a.

> ***The aim of the workshop was to discuss mixture risk assessment from the different perspectives, to evaluate the progress achieved and identify further gaps to be addressed***

Another aspect of this collaboration was to discuss the use of the European Commission's Information Platform for Chemical Monitoring (IPCHeM) as a tool to provide occurrence data and to facilitate the exposure assessment for multiple chemicals.

A dedicated workshop with the above-mentioned project partners, held at JRC Ispra in December 2017, gave the opportunity to present and discuss the most recent enhancements and tools of IPCHeM. It was useful to gather requirements for revising and extending IPCHeM's functionalities and tools to best meet the needs of users

interested/working on risk assessment of combination effects of chemicals (Dalla Costa *et al.*, 2018).

READ MORE

- EDC-MixRisk: edcmixrisk.ki.se
- EuroMix: www.euromixproject.eu
- EU-ToxRisk: www.eu-toxrisk.eu
- HBM4EU: www.hbm4eu.eu
- SOLUTIONS: www.solutions-project.eu
- IPCHeM: ipchem.jrc.ec.europa.eu
- Horizon 2020: europa.eu/!pc39Fj
- 7th Framework Programme: europa.eu/!QH99KW
- Towards improved safety assessment of combined exposure to chemicals - News item: europa.eu/!xv48tN

2.3 European Research Projects on Endocrine Disruptors

A call[1] was recently launched under the EU Horizon 2020 framework programme for research and innovation, for new screening and testing methods to identify endocrine disrupting substances. According to the call text, the proposals should focus on methods where regulatory needs are greatest, addressing targets such as the thyroid axis, developmental neurotoxicity, metabolic disorders and non-genotoxic carcinogenicity. The outcome of the projects should contribute to international activities on Endocrine Disruptors (ED) at OECD level (see sections 5.1, 5.3.13 and 5.3.14) and, in order to facilitate regulatory uptake, the inclusion of a validation step in the proposals was considered essential. It is envisaged that JRC/EURL ECVAM will provide support to the selected projects on the translation of research results into regulatory application.

READ MORE

- New testing and screening methods to identify endocrine disrupting chemicals (call SC1-BHC-27-2018): europa.eu/!rj96gx

1 Call number: SC1-BHC-27-2018

2.4 Acute Systemic Toxicity

The need for mechanistic based approaches and methodologies that could potentially replace animal use for acute systemic regulatory-required testing is well recognised (Prieto *et al.*, 2014; Hamm *et al.*, 2017; ICCVAM, 2018; Clippinger *et al.*, 2018a). With the ultimate aim of supporting the development of Adverse Outcome

Figure 2.1 Workshop on "Advancing the Assessment of Chemical Mixtures and their Risks for Human Health and the Environment" held in May 2018 at JRC Ispra.

Box 2.1

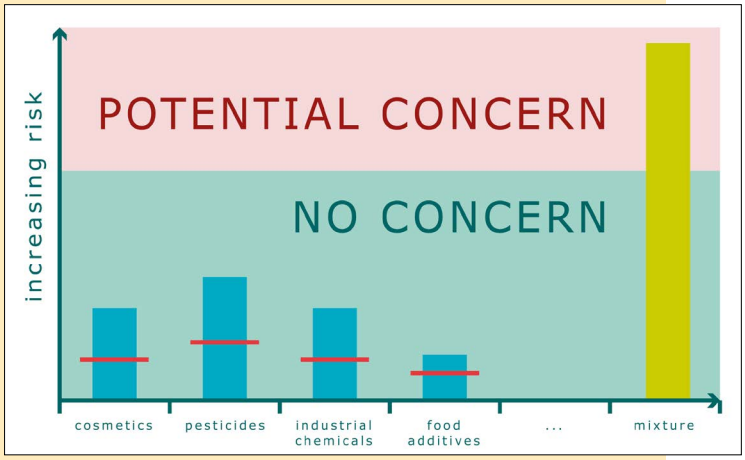
Chemical mixtures – EU research consortia take stock of the science-policy landscape

The JRC has worked with five EU funded research consortia, EC department for Research and Innovation, the European Environment Agency and the European Food Safety Authority to produce a paper (Bopp *et al.*, 2018a) which identifies research and policy needs to deal with the assessment and management of potential risks posed by chemical mixtures to human health and the environment.

There are several major research projects currently ongoing at European level that aim to improve the assessment and management of combined exposures to multiple chemicals, including the European FP7 and Horizon 2020 research consortia: SOLUTIONS, EuroMix, HBM4EU, EDC-MixRisk and EU-ToxRisk. The paper was prepared in the context of the Joint Horizon 2020 workshop, "Advancing the Assessment of Chemical Mixtures and their Risks for Human Health and the Environment" which the JRC co-organised and hosted in May 2018 (see fig. 2.1). The workshop brought together the research community with Commission services and EU Agencies to assess the state of the science, to map the research strategies of EU consortia against policy needs, and to prioritise areas for collaboration at the science-policy interface.

Every day we are exposed to low levels of hundreds of different man-made chemicals present, for example, in our food, consumer products, and the air we breathe. Our environment too is exposed to a high number of chemical mixtures, originating from numerous sources (Carvalho *et al.*, 2014). This combined exposure to multiple chemicals can lead to health and environmental effects, which might be overlooked in regulatory assessments. This is because current safety assessment practice is primarily based on understanding the potential risk posed by single substances, rather than their "real life" combinations.

To better protect human health and the environment through the assessment of chemical mixtures, a number of scientific and policy challenges need to be addressed. These have been described in a series of publications, including a JRC policy brief (Bopp *et al.*, 2018b), JRC technical report (Kienzler *et al.*, 2014), and scientific paper (Kortenkamp & Faust, 2018). The European Commission is investing significantly in scientific and technical developments, primarily through its Framework Programme for Research and Innovation.



Pathways (AOPs) and IATA in the area of acute systemic toxicity, EURL ECVAM analysed mechanistic information collected on eight organs (nervous system, cardiovascular system, liver, kidney, lung, blood, gastrointestinal system and immune system) identified as relevant for acute oral toxicity and using a set of chemicals inducing acute toxicity after oral exposure (see box 2.2).

As anticipated in the EURL ECVAM status report 2017 (see section 2.9 in Zuang *et al.*, 2017), from the analysis performed, it can be concluded that cytotoxicity is an important determinant of acute systemic toxicity. Furthermore, the nervous and the cardiovascular systems are the most frequent targets for chemicals inducing acute oral toxicity. Changes in neurotransmission and altered ion flow appeared as important mechanisms often associated with acute neurotoxicity and cardiotoxicity, respectively. However, no clear relationship emerged between specific mechanisms of target organ toxicity and the level (category) of toxicity. Mechanistic information and kinetic considerations are clearly useful to explain *in vitro* misclassifications obtained with the cytotoxicity assay. Nevertheless, the number of acute oral toxicity categories under the EU Classification, Labelling and Packaging (CLP) Regulation (EC, 2008) and the associated LD_{50} ranges, which are not based

As anticipated in the EURL ECVAM status report 2017, from the analysis performed, it can be concluded that cytotoxicity is an important determinant of acute systemic toxicity

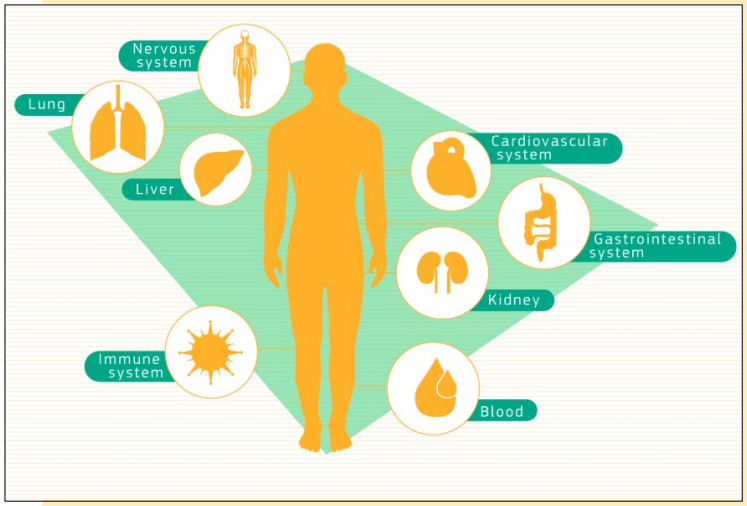
Figure 2.2 Workshop on current practices for acute inhalation toxicity testing co-hosted by PETA International Science Consortium (PISC) and the US NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) in September 2016.

Box 2.2

How do chemicals cause acute oral toxicity via organ-specific mechanisms?

EURL ECVAM scientists collected and analysed mechanistic information on the effects of chemicals on eight organs identified as relevant for acute systemic toxicity in humans. The ultimate aim is the replacement of the use of animals in the regulatory assessment of acute oral toxicity. This knowledge is expected to support the development and application of adverse outcome pathways (AOPs) and mechanistically relevant new approach assessment methodologies.

The replacement of animals in acute systemic toxicity testing, which is a core component of the safety assessment of substances, remains a considerable challenge. This is partially due to the incomplete mechanistic understanding of the key acute toxicity pathways in humans, some of which are specific for different cell types (*e.g.,* neuronal, cardiac, liver or kidney), while others are broadly applicable (*e.g.*, general cytotoxicity). Therefore, improving the knowledge of the numerous mechanisms involved would be useful to developers of test methods and other predictive tools as well as to validation and regulatory bodies.



EURL ECVAM analysed the relevant literature and confirmed that general cytotoxicity is an important determinant of acute systemic toxicity. While the nervous and the cardiovascular systems are the most frequent targets, a clear pattern was not found with regard to which specific mechanisms of target organ toxicity are representative of compounds in the different potency classes. For this analysis, the potency classes were based on the EU regulatory system for classifying chemicals for acute oral toxicity, namely the Classification, Labelling and Packaging (CLP) categories.

At present, the regulatory classification is based on animal (mainly rodent) studies. Building on all the collected information, it is worth trying to develop an alternative way of classifying chemicals for acute oral toxicity based mainly on cytotoxicity and kinetic information, and complemented, if needed, with relevant organ-specific mechanisms of toxicity (Prieto *et al.*, 2018).

Box 2.3

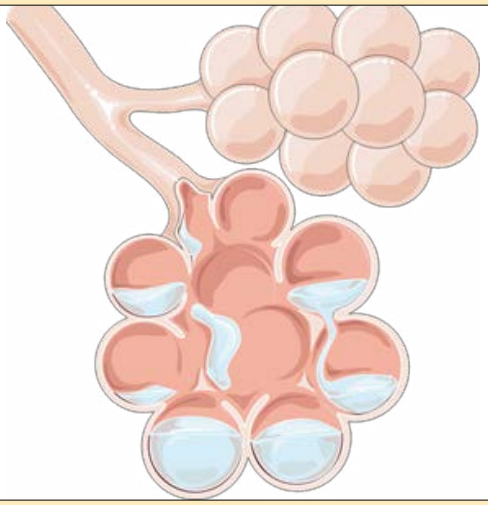
New approaches needed to assess the effects of inhaled substances on human health

In 2016 EURL ECVAM joined a working group, together with other international experts, to assess acute inhalation toxicity (see fig. 2.2). They discussed how existing knowledge can be used to design effective non-animal testing approaches (Clippinger *et al.*, 2018b). Experts described Adverse Outcome Pathways (AOPs) and the toolbox of non-animal approaches that could be used to investigate relevant mechanisms leading to acute inhalation toxicity. The review proposes a decision tree to help guide consideration of exposure parameters and the design of an integrated strategy for inhalation testing.

Some important challenges need to be addressed on the way towards meaningful progress in implementing non-animal approaches:

- Curating data in user-friendly databases
- Evaluating of existing QSAR models and developing new ones
- Advancing mechanistic dosimetry models for *in vitro* to *in vivo* extrapolation
- Developing and sharing AOPs
- Optimising *in vitro* systems
- Designing and testing integrated approaches

These mechanistic based non-animal approaches will provide a predictive tool and likely more information to risk assessors than a median lethal concentration (LC_{50}) or other *in vivo* observations.



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on a particular mechanistic rationale, cannot be ignored when evaluating alternative approaches for the purposes of regulatory classifications. Work is continuing to explore alternative, mechanistic-based criteria for classifying chemicals for acute oral toxicity based mainly on cytotoxicity and kinetic information and, if needed, complemented with relevant organ specific mechanisms of toxicity (Prieto *et al.*, 2018).

Current testing requirements and major needs in relation to alternative approaches for acute inhalation toxicity testing were discussed during an international workshop in 2016 (Clippinger *et al.*, 2018a) attended by EURL ECVAM (see fig. 2.2). In response to one of the workshop's recommendations, an expert working group reviewed the state-of-the-science concerning mechanistic information or relevant AOPs and assays available and emerging to assess acute inhalation toxicity and how the existing knowledge could be used to design effective non-animal testing approaches (Clippinger *et al.*, 2018b; see box 2.3).

2.5 The VAC2VAC Project

The VAC2VAC project - "Vaccine batch to vaccine batch comparison by consistency testing" brings together 21 public and private partners including the JRC represented by EURL ECVAM. This five years project (2016 – 2021) is funded under the Innovative Medicines Initiative 2 (IMI 2), a joint undertaking of the EU Horizon 2020 Research and Innovation Programme and the European Federation of Pharmaceutical Industries and Associations (EFPIA).

VAC2VAC

The focus is on the use of the consistency approach for quality control of established vaccines for human and veterinary use. For this purpose, VAC2VAC partners are developing, optimising and evaluating non-animal methods for routine batch quality, safety and efficacy testing of vaccines, in collaboration and consultation with regulatory agencies. EURL ECVAM is participating in the project as leader of the work package related to validation, and supports project activities related to international dissemination, harmonisation and regulatory acceptance of consistency approaches.

In order to discuss ways of improving multi-centre validation studies and making use of the data generated for product-specific validation purposes, EURL ECVAM organised with VAC2VAC partners a workshop in 2017

at the JRC, Ispra, Italy (see fig. 2.3). The summary of the discussions and recommendations agreed upon by 30 experts from veterinary and human vaccine manufacturers, official medicines control laboratories, academia, translational research organisations, and vaccinology alliances have been published in 2018 (Halder *et al.*, 2018a; see box 2.4).

Recommendations encourage manufacturers to play a more active role by identifying suitable non-animal methods, providing relevant samples, or by sponsoring of studies. Both multi-centre validation studies and product-specific validation are technically demanding and time and resource intensive, availability of sufficient resources for validation studies and implementation of new methods is crucial. Moreover, the availability of critical reagents and reference preparations should be secured.

There are several new documents available supporting the substitution of animal tests for the quality control of vaccines and the use of data generated in multi-centre validation studies for product-specific validation

There are several new documents available supporting the substitution of animal tests for the quality control of vaccines (Council of Europe, 2018) and the use of data generated in multi-centre validation studies for product-specific validation (EMA, 2017).

READ MORE

- VAC2VAC: www.vac2vac.eu
- Innovative Medicine Initiative (IMI): www.imi.europa.eu
- European Federation of Pharmaceutical Industries and Associations (EFPIA): www.efpia.eu
- Horizon 2020: europa.eu/!pc39Fj
- JRC hosts the VAC2VAC workshop on validation of non-animal methods for quality control of vaccines - News item: europa.eu/!nc73BM

2.6 EURL ECVAM Laboratory Studies

EURL ECVAM hosts a specialised laboratory for *in vitro* toxicity testing. A detailed description of the laboratory capabilities is provided in the EURL ECVAM status report 2017 (see section 2.5 in Zuang *et al.*, 2017). The laboratory includes a High Throughput Screening facility that covers all general activities related to non-routine or non-standardised processes and procedures, linked mainly to method optimisation and high throughput and high content screening. The laboratory hosts also a Good Laboratory Practice (GLP) facility that covers primarily

Figure 2.3 VAC2VAC workshop on validation of non-animal methods for quality control of vaccines held at the JRC Ispra in February 2017.

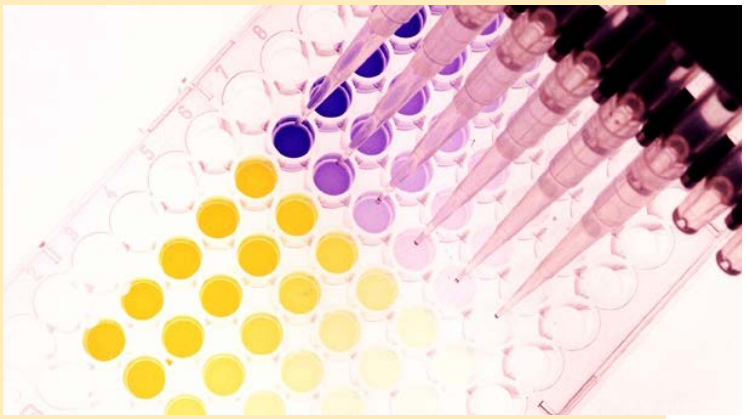
Box 2.4

Optimising validation studies of non-animal methods for vaccine testing

A new report (Halder *et al.*, 2018a) summarises the outcome of a workshop organised by EURL ECVAM and VAC2VAC partners on the design of multi-centre validation studies.

The VAC2VAC project aims to develop and validate non-animal based methods for the quality control of vaccines. In order to incorporate a new method into a monograph or a guideline used for regulatory purposes, the method has to be shown to be reproducible and to have the ability to detect vaccine batches of insufficient quality. Moreover, any new method introduced for the quality control of a vaccine needs to undergo product-specific validation. In the light of this, workshop participants coming together in early 2017 discussed ways of optimising the design of validation studies and making use of the data generated for product-specific validation purposes (see section 2.4 in Zuang *et al.*, 2017 and see also fig. 2.3).

Recommendations encourage manufacturers to play a more active role by identifying suitable non-animal methods, providing relevant samples, or by sponsoring of studies. Since multi-centre validation studies and product-specific validation are technically demanding and time and resource intensive, availability of sufficient resources for validation studies and implementation of new methods is crucial. Moreover, the availability of critical reagents and reference preparations should be secured. There are several new documents available or under development which support the substitution of animal tests for the quality control of vaccines and the use of data generated in multi-centre validation studies for product-specific validation.



formal validation studies to assess the performance of standardised methods. The laboratory directly supports the EURL ECVAM mandate as stipulated in Directive 2010/63/EU and also serves collaboration and internal projects. Examples of experimental studies, implemented in-house, are given below.

The AR-CALUX *in vitro* method, developed for the detection of compounds with androgenic and anti-androgenic potential (see section 4.1), has been implemented in EURL ECVAM's High Throughput Screening facility. The method's protocol developed for manual implementation has been adapted for automated operation in quantitative High-Throughput Screening (qHTS) format. Besides the higher throughput achieved, and owing to a higher range of concentrations tested, this implementation allows to combine both the pre-screen and the comprehensive testing, eliminating the requirement of a preliminary range finding experiment to determine the proper concentration range. To this end, modifications of the manual protocol were made to allow for an optimal use of the automated equipment and these included a plate layout allowing the testing of about 40 chemicals per run. The acceptance criteria were also revised accordingly. About 60 chemicals were tested in agonistic and antagonistic mode.

The EURL ECVAM Laboratory is also engaged in progressing research and development of new alternative methods to animal testing. Nowadays, automated screening technologies and informatics are available to screen high numbers of chemicals to identify important biological processes that may be disturbed or disrupted. At EURL ECVAM, a pilot study for the generation and use of High-Throughput Transcriptomics data using Targeted RNA-Sequencing has been conducted, with the aim of supporting chemical grouping and read-across. This pilot study aimed at producing transcriptomics (RNA sequencing) data from hepatic (HepaRG) cells treated with 20 selected chemicals at seven concentrations. The data generated (approx. 20 million data points) was complemented with High Content Imaging data using automated fluorescence

The AR-CALUX in vitro method, developed for the detection of compounds with androgenic and anti-androgenic potential, has been implemented in EURL ECVAM's High Throughput Screening facility

microscopy. The study has been executed in a qHTS format for an initial assessment of the performance of the targeted RNA-sequencing technology and the feasibility of its application in *in vitro* toxicity testing. A follow-up experiment is planned to continue to build a deeper understanding in the application of RNA sequencing with a special focus on time course data.

READ MORE

- EURL ECVAM Laboratory: europa.eu/!PQ33Rv

2.7 Fish Toxicity and Bioaccumulation Research and Development Projects

Several R&D projects related to fish toxicity and bioaccumulation, which are of specific interest to EURL ECVAM, are described below.

2.7.1 Use of a Fish Cell Line-Based Cytotoxicity Assay for Acute Fish Toxicity Testing

An ISO guideline (ISO/DIS 21115) using the RTgill-W1 cells for water quality testing was adopted in 2018 (ISO, 2018). The RTgill-W1 cell line assay is also under assessment by EURL ECVAM (see section 3.6).

READ MORE

- ECO8.3-NC3Rs-EAWAG: Round-robin test of the RTgill-W1 cell line assay to study its robustness in establishment and inter-laboratory comparability: cefic-lri.org/projects/eco8-3-nc3rs-eawag-round-robin-test-of-the-rtgill-w1-cell-line-assay-to-study-its-robustness-in-establishment-and-inter-laboratory-comparability

2.7.2 Development of Adverse Outcome Pathways for Chronic Fish Toxicity Testing

Several research groups are working on the identification and description of potential AOPs relevant to chronic fish toxicity, which is currently assessed with a fish early life-stage (FELS) test (OECD, 2013a). Summaries of the outcome of two Cefic Long-range Research Initiative (LRI)-funded project (LRI-ECO20-UA; LRI-ECO20.2) are available on the Cefic LRI website.

Two FELS relevant AOPs (AOP on AhR activation and AOP on thyroperoxidase and/or deiodinase inhibition leading to impaired swim bladder inflation in fish during early life stages) have been included into the OECD AOP work plan.

READ MORE

- Cefic's Long-range Research Initiative (LRI): cefic-lri.org
- ECO20-UA: Development of an alternative testing strategy for the fish early life-stage test for predicting chronic toxicity: cefic-lri.org/projects/lri-eco20-ua-development-of-an-alternative-testing-strategy-for-the-fish-early-life-stage-test-for-predicting-chronic-toxicity
- ECO20.2: Development of an alternative testing strategy for the fish early life-stage test for predicting chronic toxicity - assay validation: cefic-lri.org/projects/eco20-2-development-of-an-alternative-testing-strategy-for-the-fish-early-life-stage-test-for-predicting-chronic-toxicity-assay-validation

2.7.3 Threshold of Toxicological Concern in Aquatic Toxicity Assessment

The ecological Threshold of Toxicological Concern (eco-TTC) has been proposed for environmental risk assessment as extension to the well-established human safety TTC concept (Belanger *et al.*, 2015).

An international collaboration/working group under the Health and Environmental Sciences Institute (HESI) has been established who developed the EnviroTox, a database of approximately 91,000 unique ecotoxicological records, 4,000 chemicals and 1,500 species from three trophic levels (fish, invertebrates, algae/plants). An on-line analytical tool has been developed, which aims to calculate threshold values based on statistical distributions of two types, Predicted No Effect Concentration (PNEC) distribution or ecotoxicological data distribution, according to particular research criteria. The EnviroTox database and the analytical tool are now finalised and have been made publicly available in November 2018.

An international collaboration/working group under the Health and Environmental Sciences Institute (HESI) has been established who developed the EnviroTox

EURL ECVAM is contributing to this initiative, and worked in particular on the evaluation of the existing aquatic mode of action classification frameworks, their overlap and possibilities to move forward (Kienzler *et al.*, 2017).

READ MORE

- Health and Environmental Sciences Institute (HESI): hesiglobal.org
- EnviroTox Database & Tools: envirotoxdatabase.org

2.7.4 Aquatic and Terrestrial Bioaccumulation

2.7.4.1 Development of a Biotransformation Database

As announced in section 2.6.6 in Zuang *et al.* (2017), EURL ECVAM launched in 2016 the development of a biotransformation database involving an external contractor. The project was finalised in 2018.

The database (organised in four Excel sheets) contains *in vivo* and *in vitro* biotransformation data from various species (mouse, rat, fish) and provides a valuable data source for model developers (*e.g.*, for *in vitro* to *in vivo* extrapolation models, kinetic models, models to predict exposure and internal concentration in an organism) as well as for chemical assessors. The database can be downloaded from the JRC Data Catalogue (Halder *et al.*, 2018b and see also section 6.1.4).

READ MORE

- EURL ECVAM Fish *In Vitro* Intrinsic Clearance Database: data.jrc.ec.europa.eu/dataset/jrc-eurl-ecvam-fish-in-vitro-intr-clear-db

2.7.4.2 Development of a Tiered Testing Strategy for Fish Bioaccumulation Testing Based on in vitro Approaches

This Cefic LRI-funded project (LRI-ECO34) started in 2016 and combines various *in vitro* approaches using fish cell lines to estimate chemical uptake and biotransformation with toxicokinetic and quantitative structure activity relationship models. The aim is to develop a tiered approach for the assessment of the bioaccumulation potential of chemicals. The principle of this work is further described in Stadnicka-Michalak *et al.*, (2018).

READ MORE

- LRI ECO34: A tiered testing strategy for rapid estimation of bioaccumulation by a combined modelling – *in vitro* testing approach: cefic-lri.org/projects/eco34-a-tiered-testing-strategy-for-rapid-estimation-of-bioaccumulation-by-a-combined-modelling-in-vitro-testing-approach

2.7.4.3 Integrating Bioaccumulation Assessment Tools for Mammals

This Cefic LRI-funded project (LRI-ECO44) started in 2018 and aims at developing a toxicokinetic modelling framework for bioaccumulation assessment in mammals, combining *in vitro* and *in vivo* physiologically-based toxicokinetic (PBTK) data, field collected bioaccumulation data, and quantitative structure activity relationship models. Within the context of this project, toxicokinetic models for mammals will also be developed and the state of the science and available data streams will be synthesised in order to develop an integrated testing strategy for priority chemicals.

READ MORE

- LRI ECO44: A toxicokinetic mammalian modelling framework for bioaccumulation assessment: cefic-lri.org/request-for-proposals/lri-eco44-a-toxicokinetic-mammalian-modelling-framework-for-b-assessment

2.8 Review of Non-animal Methods Used for Biomedical Research

According to the last report on animal use in EU Member States (EC, 2013), more than 60% of animals were used for research and development in the fields of human and veterinary medicine, and in biological studies of fundamental nature. However, the significant failure rate in translating results of preclinical animal studies into human disease treatments remains very concerning (Langley, 2014; Holmes *et al.*, 2011).

To address these concerns, EURL ECVAM has launched a study to review non-animal models and methods already in use for basic and applied research, starting with respiratory tract diseases and neurodegenerative disorders (see section 2.7 in Zuang *et al.*, 2017). A second call for tender was published on 1 October 2018, in five additional research areas, namely, cardiovascular diseases, breast cancer, immunogenicity testing for advanced therapy medicinal products, autoimmune diseases and immune oncology models (see box 2.5).

EURL ECVAM has launched a study to review non-animal models and methods already in use for basic and applied research, starting with respiratory tract diseases and neurodegenerative disorders

The intention is to understand when research approaches involving alternatives have been successful and how it is possible to benefit from the significant advances made in recent years in novel non-animal procedures and techniques (*e.g.*, *in vitro*, *in silico*) development, providing mechanistic information and modelling human-specific processes and conditions. These new methodologies represent a huge resource for enhancing the comprehension

of human-specific biological processes and pathologies. The outcome of the study will be important to create a basis to advance uptake, implementation and promotion of non-animal methodologies in biomedical sciences by performing systematic reviews on selected human disease areas and thereby contributing to the reduction of the reliance on animal use and the betterment of science.

The first review is expected to be completed during 2019 and the information gathered will be made publicly available. It will cover existing and in-development non-animal approaches including, but not confined to, *in vitro, in silico* and *ex vivo* approaches.

READ MORE

- Reviewing the use of alternative methods in biomedical research - News item: europa.eu/!Fw33hq

2.9 Chemistry Framework

The Chemistry Framework is being developed to improve the way in which sets of reference chemicals, *i.e.*, the chemicals that are used to validate the reliability (reproducibility) and relevance of an alternative method, are assessed. The approach is to explore how individual chemicals can be considered as representing regions in a 'similar chemical space', and thus represent more than a single chemical in a validation study.

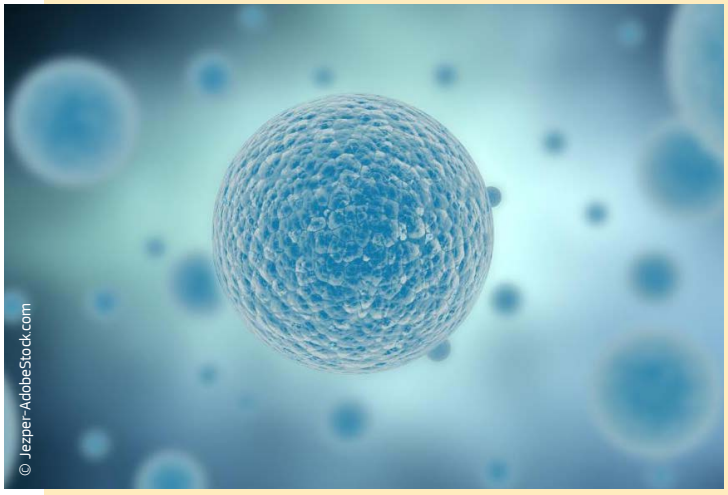
The framework focuses on the biological and physicochemical properties of chemicals in addition to their structure. For instance, in the context of skin sensitisation (Asturiol *et al.*, 2016), a given chemical might represent generically a chemical that is soluble in water and has the properties to react with proteins. If this chemical is predicted correctly (*e.g.*, as sensitiser) by a method being validated, then the method can be regarded as applicable to chemicals with similar properties, and therefore there would be no need to test other chemicals that have similar properties but a different structure, in that same method. Thus the Chemistry Framework is essentially applying the principles of grouping and read-across, not to predict the toxicological properties of a chemical, but instead to define the (chemical) applicability of a method.

The chemistry framework will facilitate the comparison of two methods that have been validated with different validation sets of chemicals

Box 2.5

Alternative methods in biomedical research: second call for tender reviewing methods in 5 new areas

EURL ECVAM has launched a second call for tender to review alternative methods and models being used for research in the areas of cardiovascular diseases, breast cancer, immunogenicity testing for advanced therapy medicinal products, autoimmune diseases and immune oncology models. This new set of studies is aimed to continue the collection of successful non-animal procedures and techniques (*e.g.*, *in vitro*, *in silico*) in five new areas of biomedical research, to broaden the knowledge on the possible benefits from the significant technological advances made in recent years in novel non-animal methods development, providing mechanistic information and modelling human-specific processes or tissues.



The call will cover comprehensive information on the status and applications of innovative and alternative methods in biomedical research, on the basis of extensive reviews of scientific literature and related information sources.

The outcome of the study, together with the data obtained from the previous review (see section 2.7 in Zuang *et al.*, 2017), will be valuable to advance uptake, implementation and promotion of non-animal methodologies in biomedical sciences. This second review is expected to be completed during 2020 and the information gathered will be made publicly available.

It is intended that the framework facilitates, for example, the comparison of two methods that have been validated with different validation sets of chemicals. It should also enable the definition of generic validation sets, consisting of a list of properties and effects that a method must be capable of identifying to be considered scientifically valid. A manuscript describing the basis of the chemistry framework is in preparation.

2.10 Integration of Data across Toxicity Endpoints to Explore New Ways for Carcinogenicity Testing

Knowledge about underlying and recently identified mechanisms involved in the development and promotion of cancer, as well as tools to analyse them (mechanism-based tests, epigenetics, -omics) have considerable potential to advance safety assessment.

For this reason, a methodology to guide the evaluation of carcinogenic hazard of chemicals is being developed in order to facilitate the uptake and the integration of new mechanistic data by combining information across different toxicity endpoints rather than considering them individually. Such an approach is also expected to help with the identification of potential information gaps and, at the same time, avoid redundant animal testing. A poster describing ongoing work at EURL ECVAM was presented at the European Association on Cancer Research Conference EACR25 in July 2018 (Madia *et al.*, 2018).

A paper on the rationale behind this integrated approach and its potential application to other systemic toxicity endpoints is under finalisation.

READ MORE

- 25th Biennial Congress of the European Association for Cancer Research (EACR25): www.eacr25.org

2.11 Preclinical Assessment for Advanced Therapy Medicinal Products

New scientific progress in cellular and molecular biotechnology has led to the development of Advanced Therapies Medicinal Products (ATMPs), such as gene therapy, somatic cell therapy, and tissue engineering. They offer the promise of providing innovative treatments for patients, particularly in disease areas where conventional approaches are inadequate (Milone & Bhoji, 2018). As a consequence, the field of new drug development is moving its focus from well-defined small-molecule compounds towards ATMPs. However, the risk associated with their administration without an appropriate safety and efficacy assessment and the subsequent detrimental consequences for patients have been reported (Cuende *et al.*, 2014; Zheng *et al.*, 2018).

Because of the novelty, complexity and technical specificity of ATMPs, specially tailored and harmonised guidance is needed to evaluate their safety and efficacy, which addresses both commercial product manufacturers and hospitals. Thus there is a growing need to understand what the challenges associated with the progress of ATMPs through the medicines life-cycle are. Based on the internal scientific expertise, as well as on the collaboration with the wider scientific and regulatory communities, EURL ECVAM is exploring opportunities to contribute to the harmonisation of requirements for the safety (and efficacy) assessment of ATMPs. Through surveying various stakeholders, gaps and opportunities have been identified that will be the subject of further investigation.

Advanced therapies medicinal products (ATMPs) offer the promise of providing innovative treatments for patients, particularly in disease areas where conventional approaches are inadequate

2.12 Relationship between Children's Health and Exposure to Environmental Chemicals

EURL ECVAM recently met with the Norway Institute for Public Health (NIPH) to discuss the topic of *Children's health and exposure to environmental chemicals* (see fig. 2.4) where consideration was given to integration of multiple sources of information including mechanistic knowledge, biomonitoring data, epidemiological studies, *in silico* as well as *in vitro* data to support decision making. As a result of this collaboration, a review on early life exposure to air pollution particulate matter as a risk factor for attention deficit and hyperactivity disorder (ADHD) has been published (Myhre *et al.*, 2018). Chemicals that are known to trigger specific developmental neurotoxic (DNT) effects belong to a variety of chemical classes including industrial chemicals, persistent organic pollutants (POPs), metals and pesticides. Effects resulting from the combined

NIPH and EURL ECVAM have initiated studies where mixtures of DNT chemicals are being evaluated using in vitro assays anchored to common key events identified in AOPs

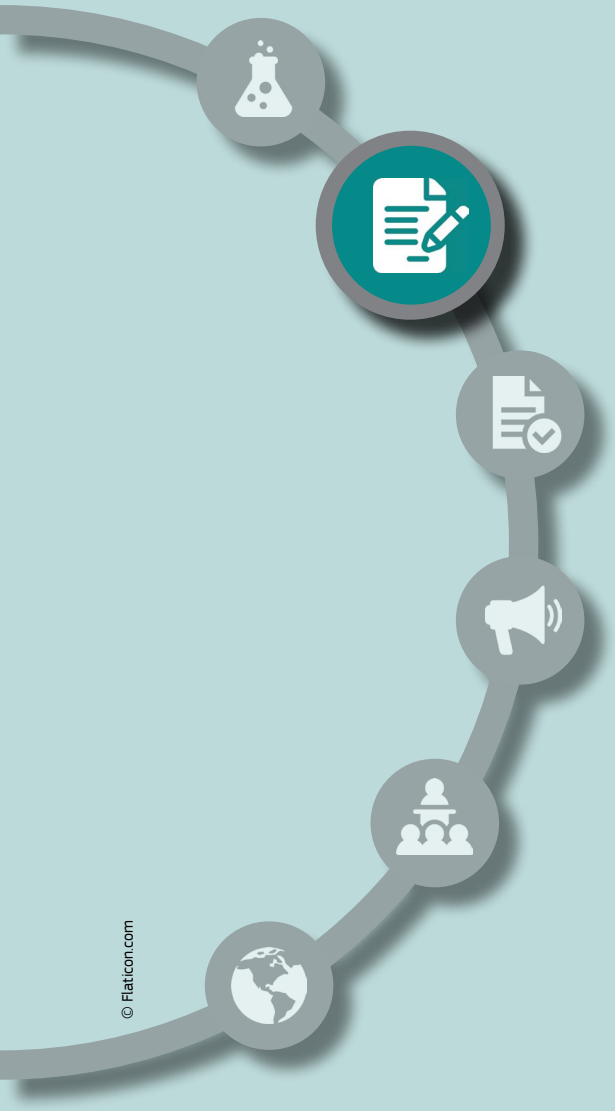
Figure 2.4 Workshop on Children's health and exposure to environmental chemicals organised between EURL ECVAM and the Norway Institute for Public Health (NIPH) at the JRC Ispra in June 2018.

exposure to these chemicals across different regulatory domains are not currently considered. NIPH and EURL ECVAM have initiated studies where mixtures of DNT chemicals which were found in human samples (*e.g.*, breast milk or children's blood) and belong to heterogeneous classes of chemicals are being evaluated using *in vitro* assays anchored to common key events identified in AOPs where impairment of learning and memory in children was defined as the adverse outcome.

READ MORE

- Norwegian Institute of Public Health (NIPH): www.fhi.no/en

3 Test Method Submissions



3. Test Method Submissions

A new pre-submission (TR MARCoNI) and three new full submissions (GARD, Bioelution and RTgill-W1) were received by EURL ECVAM since the last EURL ECVAM status report published in November 2017 (see section 3 in Zuang *et al.*, 2017). The assessments of the new pre-submission and of the full submission of the GARD were concluded this year, while the assessment of the two other new full submissions will progress into 2019. The assessment of the γH2AX/pH3 pre-submission (see section 3.1) and of the SENS-IS full submission (see section 3.3) were also concluded.

The assessment of the γH2AX/pH3 pre-submission was completed in February 2018 and the test submitter was informed that EURL ECVAM does not intend to progress the submission further at the present time (see section 3.1). The assessment of the TR MARCoNI pre-submission was completed in March 2018 and the test submitter was invited to provide a full submission (see section 3.2).

The assessment of the full submission of the SENS-IS assay was finalised with some shortcomings being identified. The test submitter was invited to address those and submit a revised full submission (see section 3.3). The assessment of the GARD full submission was put on hold pending outcomes of discussions that are on-going at the OECD (ESAC, see section 3.4).

Full submissions of the Bioelution (see section 3.5) and RTgill-W1 cell line (see section 3.6) assays were received in 2018 and their assessments by EURL ECVAM will progress into 2019.

Following a preliminary EURL ECVAM assessment and PARERE consultation on the pre-submissions, the test submitters of the Toxtracker® and of the EDITOX assays were invited in 2017 to provide a full submission (see sections 3.2 and 3.4 in Zuang *et al.*, 2017) but no full submissions were received for these two assays in 2018.

Finally, EURL ECVAM updated its Test Pre-submission Form and its Test Submission Template in 2018 with the intention of making them more straightforward to complete and to request additional information on the use of animal derived serum and animal derived antibodies, including their replacement by suitable non-animal alternatives.

READ MORE

- TSAR: Tracking System for Alternative methods towards Regulatory acceptance: tsar.jrc.ec.europa.eu
- EURL ECVAM test method submission: europa.eu/!ww88Mh

3.1 γH2AX/pH3 Assay

In August 2017, EURL ECVAM received a pre-submission for the assessment of a method based on γH2AX/pH3 biomarkers for the prediction of genotoxic potential of a range of substances. The combination of two particular biomarkers is proposed to detect early events in the DNA damage response and to discriminate structural and numerical chromosome damage. Increased levels of γH2AX phosphorylation at serine 139 measure the recruitment of DNA repair machinery to DNA double strand breaks. The induction of Histone 3 (pH3) is proposed by the test submitter as an indicator of mitotic index and cell proliferation status. Specifically, aneugenic chemicals are considered to increase the mitotic index, thus pH3 tends to be reduced by cytotoxic conditions, including cytotoxicity associated with many DNA-reactive chemicals.

The submitter claimed that the integration of information derived from the detection of the two biomarkers was more predictive of genotoxicity than the common Micronucleus *in vitro* and Ames tests. For this reason, the test method was proposed to be used as screening and early selection test of candidate molecules before entry into drug development, or as a follow-up of positive results within the current available regulatory battery of *in vitro* genotoxicity assays, to provide insight into the mechanism of action and help read across approaches.

The combination of two particular biomarkers is proposed to detect early events in the DNA damage response and to discriminate structural and numerical chromosome damage

EURL ECVAM acknowledged that the types of assays/biomarkers described within the pre-submission have the potential to address the MoA of genotoxicity and may be relevant in a regulatory context. However, based on the information provided, the test method's definition, as well as its protocol and prediction model characterisation did not meet EURL ECVAM's criteria to allow a proper assessment of the test method. The test submitter was informed in February 2018 that EURL ECVAM does not intend to progress the submission further.

READ MORE

- γH2AX/pH3 Assay: tsar.jrc.ec.europa.eu/test-method/tm2017-01

3.2 TR MARCoNI

In October 2017, EURL ECVAM received a pre-submission of an *in vitro* Thyroid receptor microarray assay for real-time Nuclear Receptor-coregulator interaction. This test method quantitatively measures the interaction profile of a compound-activated thyroid receptor with a range of nuclear receptor co-regulators. Nuclear receptor co-regulator recruitment is a key biological step between receptor binding and transactivation, and currently there are no available tests covering this specific interaction. The information provided in the test pre-submission was considered sufficient to understand the principle of the test method, and its biological and general mechanistic relevance. The TR MARCoNI method could be integrated in a testing strategy and combined with additional methods suitable to capture different modes of action of thyroid hormone disruptors.

The TR MARCoNI method could be integrated in a testing strategy and combined with additional methods suitable to capture different modes of action of thyroid hormone disruptors

In March 2018, EURL ECVAM completed the assessment of the TR MARCoNI pre-submission, and invited the test submitter to progress the test method to a full submission, taking into account some issues raised by EURL ECVAM. In particular, it will be relevant to know the following: whether the TR MARCoNI method can detect thyroid disruptors that are not detected by other methods (*e.g.*, receptor binding, transactivation or proliferation); the reproducibility of the method; (potential) issues with Intellectual Property Rights; and additional information on compounds that have tested positive in this specific method.

READ MORE

- TR MARCoNI: tsar.jrc.ec.europa.eu/test-method/tm2017-02

3.3 SENS-IS

EURL ECVAM has finalised the evaluation of all the information received in the SENS-IS test method submission (toxicogenomic analysis on 3D reconstituted epidermis for measuring skin sensitisation potency; see section 3.3 in Zuang *et al.*, 2017). The evaluation highlighted some shortcomings that impeded EURL ECVAM from reaching a conclusion on the validity of the test method. The test submitter was invited to address all the identified issues, eventually with a revised

submission, so as to allow EURL ECVAM to complete its assessment and to consider if the test method can be progressed to peer-review by the ECVAM Scientific Advisory Committee (ESAC).

An additional consideration is the fact that SENS-IS is protected by a patent with a very wide coverage, which is likely to make it very difficult for third parties to develop and validate similar methods on the basis of performance standards. However, this is currently a requirement at OECD level for the adoption of methods based on Intellectual Property Rights (IPR) as Test Guidelines in order to avoid commercial monopolies (OECD, 2005). Although it is recognised that IPR are necessary to stimulate and protect innovation, there is also the need for the OECD Test Guidelines Programme to define principles and acceptable best practice for uptake of methods protected by patent and subject to licensing for commercial application. The JRC is currently involved in an expert group set up by the OECD to discuss this matter, but the deliberations are still on-going (see section 5.3.16).

EURL ECVAM has finalised the evaluation of all the information received in the SENS-IS test method submission

READ MORE

- SENS-IS: tsar.jrc.ec.europa.eu/test-method/tm2011-11

3.4 GARD

The SenzaGen-led validation of the Genomic Allergen Rapid Detection (GARD) skin test for sensitisation hazard assessment was finalised (see section 4.5 in Zuang *et al.*, 2017) and the information package was submitted to EURL ECVAM in January 2018. Knowing that additional work was conducted to generate information on the GARD potency, EURL ECVAM communicated to SenzaGen AB that the evaluation of the GARD could only be initiated once all the information was made available. EURL ECVAM received the submission on the validation of the GARD potency in July 2018.

There are a number of challenging scientific, technical and legal aspects that require particular attention before proper evaluation of GARD can commence. These are as follows: a suitable agreement needs to be put in place and appropriate arrangements made to provide EURL ECVAM access to the web-based GARD Data Analysis Application (GDAA) software hosting the data analysis and the Support Vector Machine-based prediction model, and its source codes; it is unclear if a web-based prediction software (to process a user's experimental data) that is under the control of the method provider would be acceptable to regulatory authorities and implementable under Good Laboratory Practice (GLP); and finally, similar to SENS-IS, elements of the GARD method are protected by patent which may impede the development and validation of similar methods.

Most of the issues described above require resolution at the level of the OECD and the JRC (EURL ECVAM) is actively involved in the relevant discussions and expert groups. In addition, good progress is being made within the OECD project to develop a set of three Defined Approaches for skin sensitisation into a new Test Guideline, two of which address potency-based classification (see section 5.3.8). EURL ECVAM will wait for the eventual outcome of the discussions at the OECD before taking a decision on whether to commence evaluation of the GARD.

Good progress is being made within the OECD project to develop a set of three Defined Approaches for skin sensitisation into a new Guideline, two of which address potency-based classification

READ MORE

- GARD: tsar.jrc.ec.europa.eu/test-method/tm2011-09

3.5 Bioelution Assay

As reported in the EURL ECVAM status report 2017 (see section 3.1 in Zuang *et al.*, 2017) the bioelution test method is an *in vitro* extraction method that provides the fraction of a substance that dissolves under surrogate physiological conditions (*i.e.,* simulated gastric fluid) and is potentially available for absorption into systemic circulation (bioaccessible concentration). The test method was proposed by the European non-ferrous metal association (Eurometaux) to support read across and grouping under REACH for systemic endpoints and to assign CLP classifications. In the context of hazard classification, the test method has been proposed as a refinement to the current approach to alloy classification.

The pre-submission underwent PARERE consultation and, in general, a positive feedback was received from the experts with regard to the potential use of the test method in a regulatory context. The regulatory

application of the bioelution test method has also been discussed in other fora (*i.e.,* ECHA, CARACAL) as reported by Zuang *et al.* (2017). Based on all the information retrieved, the pre-submission was positively evaluated in 2017, and the test submitter was invited to proceed with a full submission. As a follow-up, a full test submission was received in February 2018 together with clarifications on the questions that were raised in the pre-submission assessment report. During the reviewing process of the full submission, several issues were identified and the test submitter was invited to address them. The evaluation process will be finalised once the additional information will be provided.

In the context of hazard classification, the bioelution assay method has been proposed as a refinement to the current approach to alloy classification

READ MORE

- Bioelution Assay: tsar.jrc.ec.europa.eu/test-method/tm2016-02

3.6 RTgill-W1 Cell Line Assay

The RTgill-W1 (rainbow trout gill cell line) cytotoxicity assay was initially submitted to EURL ECVAM in early 2014 (see section 3.1 in Zuang *et al.*, 2015). The assay has been developed within the CEllSens project (Tanneberger *et al.*, 2013) and a ring trial evaluating the transferability and within-laboratory reproducibility of the test method has been organised by the Swiss Federal Institute of Aquatic Science and Technology (EAWAG) within the Cefic LRI project. Recently, Natsch *et al.*, (2018) further demonstrated the suitability of the RTgill-W1 cell line for fish acute toxicity predictions of fragrance chemicals.

Recently, Natsch et al., (2018) further demonstrated the suitability of the RTgill-W1 cell line for fish acute toxicity predictions of fragrance chemicals

EURL ECVAM received a full submission of this assay in September 2018 and is currently evaluating it.

READ MORE

- RTgill-W1: tsar.jrc.ec.europa.eu/test-method/tm2014-01
- ECO8.3-NC3Rs-EAWAG: Round-robin test of the RTgill-W1 cell line assay to study its robustness in establishment and inter-laboratory comparability: cefic-lri.org/projects/eco8-3-nc3rs-eawag-round-robin-test-of-the-rtgill-w1-cell-line-assay-to-study-its-robustness-in-establishment-and-inter-laboratory-comparability

4 Validation of Alternative Methods and Approaches

4. Validation of Alternative Methods and Approaches

4.1 Endocrine Disruption - AR-CALUX Test Method

The AR-CALUX method is a reporter-based assay where an increase or decrease in expression of luminescence is measured in osteosarcoma cells, stably transfected with a human androgen receptor, when presented with chemicals that have (anti)androgenic potential.

The method was submitted by the Dutch company Bio-Detection Systems (BDS) to EURL ECVAM for a validation study. This study is carried out with three participating laboratories of EURL ECVAM's network of specialised laboratories, the EU Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL, see section 4.8). EURL ECVAM has obtained complete data sets for assessing transferability, reproducibility (within-laboratory and between-laboratory) and predictive capacity. The validation report and its review by ESAC is foreseen for 2019, to be followed by the submission of a draft test guideline to OECD.

EURL ECVAM has the lead in developing an OECD Performance Based Test Guideline (PBTG) for Androgen Receptor Transactivation Assays (ARTAs) and related Performance Standards, if applicable. This TG will be based on ARTAs either already validated (the AR STTA using the AR-EcoScreen cell line, led by Japan and issued as TG 458, see annex 2) or in the process of a validation (the ARTA using the 22Rv1/MMTVGR- cell line, led by Korea, see annex 2) and the EURL ECVAM ongoing validation study of the *in vitro* method AR-CALUX.

4.2 Thyroid Hormone Disruption

EURL ECVAM is coordinating a validation study of a set of mechanistically informative alternative methods related to the detection of thyroid hormone disruption, in collaboration with EU-NETVAL and the developers of such methods.

In 2017, EURL ECVAM launched a call to EU-NETVAL members for participation in the validation study. A total of 17 alternative methods have been identified by EURL ECVAM as candidates taking primarily into account the information reported in an OECD scoping document on *in vitro* and *ex vivo* methods for the identification of modulators of thyroid hormone signalling (OECD, 2014a), but also an OECD Detailed Review Paper (OECD, 2006), and feedback received at various meetings [(*e.g.*, the EU-NETVAL meeting of 2016, the meeting of the OECD Validation Management Group-Non-Animal of 2016, and the DG ENV/ANSES Thyroid Disruptor workshop (DG ENV, 2017)]. The methods cover the main possibilities of interaction with the thyroid signalling pathway (see also annex 2), *i.e.*, (1) Central regulation

[synthesis/release of hypothalamic thyroid releasing hormone (TRH), its delivery and action on pituitary thyrotrophs, and the synthesis/release of thyroid stimulating hormone (TSH)], (2) Thyroid Hormone (TH) synthesis [thyroperoxidase (TPO) activity, activation of the sodium iodide symporter (NIS)], (3) Secretion and transport in serum of THs [Transthyretin (TTR) and Thyroxine binding globulin (TBG)] (4) Metabolism and excretion of THs (deiodinase-1 activity, glucuronidation, sulphation), (5) Local cellular concentrations [TH membrane (MCT8) transporters], and (6) Cellular responses [activation of the TH nuclear receptors (TRalpha and beta transactivation)]. Furthermore, the validation study will also consider (7) Relevant short term *in vitro* methods integrating multiple Modes of Action (MoA, through the measurement of the intrafollicular T4-content in zebrafish embryos) and (8) Integrative cellular *in vitro* methods (cellular proliferation and differentiation regulated by the activation of the TH nuclear receptors; see annex 2).

This validation study consists of two parts: part one defines the methods and assesses their transferability and reliability, and part two is focused on the overall relevance of the combination of methods. After the launch of the call, fourteen EU-NETVAL facilities have been assigned to work on the 17 methods described in the call. The work done in the context of this validation study will comply with the recently published OECD Guidance on Good *In Vitro* Method Practices, GIVMP (OECD, 2018a; see section 5.3.7 and box 5.3). For methods based on the use of cell lines, EURL ECVAM is currently assessing both the authentication and the absence of contamination of methods, which is a fundamental prerequisite before transfer of materials to the EU-NETVAL partners and the beginning of the validation activities.

Those methods which perform best will be selected for further assessment in support of EU and OECD strategies to address the potential risks to human health and the environment posed by thyroid disruptors

For each of the methods, outline protocols are being drafted including definition of the test systems used and also the control and reference items. EURL ECVAM is also putting in place the necessary legal instruments, such as *e.g.* collaboration agreements and material transfer agreements, so that any existing intellectual property rights are properly managed in the validation study. Those methods which perform best will be selected for further assessment in support of EU and OECD strategies to address the potential risks to human health and the environment posed by thyroid disruptors.

READ MORE

- EURL ECVAM call to EU-NETVAL members for participation in the validation study for the detection of thyroid disruptors - News item: europa.eu/!Nv43dW
- Meeting of the EU Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) - News item: europa.eu/!Tm94Qu

4.3 Vaccine Quality Control – EDQM Biological Standardisation Programme

Most of the validation studies on alternative methods for vaccine quality control are carried out within the framework of the Biological Standardisation Programme (BSP) of the European Directorate for the Quality of Medicines & HealthCare (EDQM; Council of Europe) and are co-sponsored by the European Commission.

Several validation studies are currently ongoing which assess alternative methods for the safety and potency testing of human and veterinary vaccines, *e.g.,* a serological assay for the potency testing of whole-cell pertussis vaccines; the BINACLE assay for *in vitro* detection of toxicity in tetanus vaccines, and, in collaboration with the European Partnership on Alternative Approaches to Animal Testing (EPAA), BSP130 Phase III Validation of *in vitro* methods for the testing of *Clostridium septicum* vaccine (see section 5.7.4) and BSP148 Validation of a rabies *in vitro* potency assay (see section 5.7.5). Lang *et al.*, (2018) summarises the work carried out by the European Pharmacopoeia and progress achieved with regard to the implementation of the 3Rs in European Pharmacopoeia monographs.

READ MORE

- EDQM Biological Standardisation Programme (BSP): www.edqm.eu/en/work-programme-bsp

4.4 Genotoxicity Testing - Micronucleus Test and Comet Assay in Reconstructed Skin Models

During the 2017 International Workshop on Genotoxicity Testing (IWGT), one workgroup conducted a status review of available genotoxicity data generated in liver, lung and skin 3D tissue models. These models offer a more '*in vivo*-like' behaviour for key parameters like cell viability,

proliferation, differentiation, morphology, gene and protein expression, and function. Therefore, they can provide a valuable complement to the classical '2D' cell culture based assays. These novel 3D tissue-based genotoxicity assays, can be used as second tier assays to follow up on positive results from standard *in vitro* assays. However, it is important that for each tissue model, the full range of genotoxic damage (leading to mutagenicity, clastogenicity, and aneugenicity) can be detected.

The workgroup found that many more data were available for the 3D skin model assay (which has been in use for over ten years) than for the 3D liver and lung models. Review of the 3D liver spheroids focused on micronucleus (MN) data, and it was suggested that an assay covering DNA damage leading to gene mutations (such as the comet assay) should be developed for the liver spheroids. After review of data from the 3D lung tissue (all from the comet assay), it was acknowledged that the 3D lung comet assay would detect chemicals that induce DNA damage leading to gene mutation and chromosomal damage, and the development of 3D lung assays that can detect aneugenicity is strongly encouraged. The limited proliferation of the lung cells makes the micronucleus (MN) assay currently problematic and needs therefore more investigation. Further development of 3D liver and lung-based genotoxicity assays was encouraged since they could be a complement to the 3D skin based assays, each allowing exposure-specific assessment.

3D tissue models can provide a valuable complement to the classical '2D' cell culture based assays

The validation work on the 3D skin comet and Reconstructed Skin Micronucleus (RSMN) assays, coordinated by Cosmetics Europe, has been finalised and the relative manuscripts are in preparation. The combination of the two methods (using 56 chemicals) was shown to be highly predictive. The IWGT workgroup believes that the 3D skin comet and RSMN assays are now sufficiently validated to move towards the development of individual OECD Test Guidelines. Further discussion on the way forward to accelerate the implementation of these assays in regulatory decision making will take place in a workshop that will be organised by Cosmetics Europe in November 2018.

READ MORE

- International Workshop on Genotoxicity Testing (IWGT): www.iwgt2017.org

4.5 Genotoxicity Testing - Hen's Egg Test for Micronucleus Induction (HET-MN)

The hen's egg test for micronucleus induction (HET-MN) may represent a good supplement to the genotoxicity assays in 3D skin models because it uses the complex model of the incubated hen's egg, which mimics systemic exposure. It has been proposed to be used as a follow-up to positive results in the two-test *in vitro* genotoxicity battery, as proposed by the Scientific Committee on Consumer Safety (SCCS) for the testing of cosmetic ingredients (SCCS, 2018).

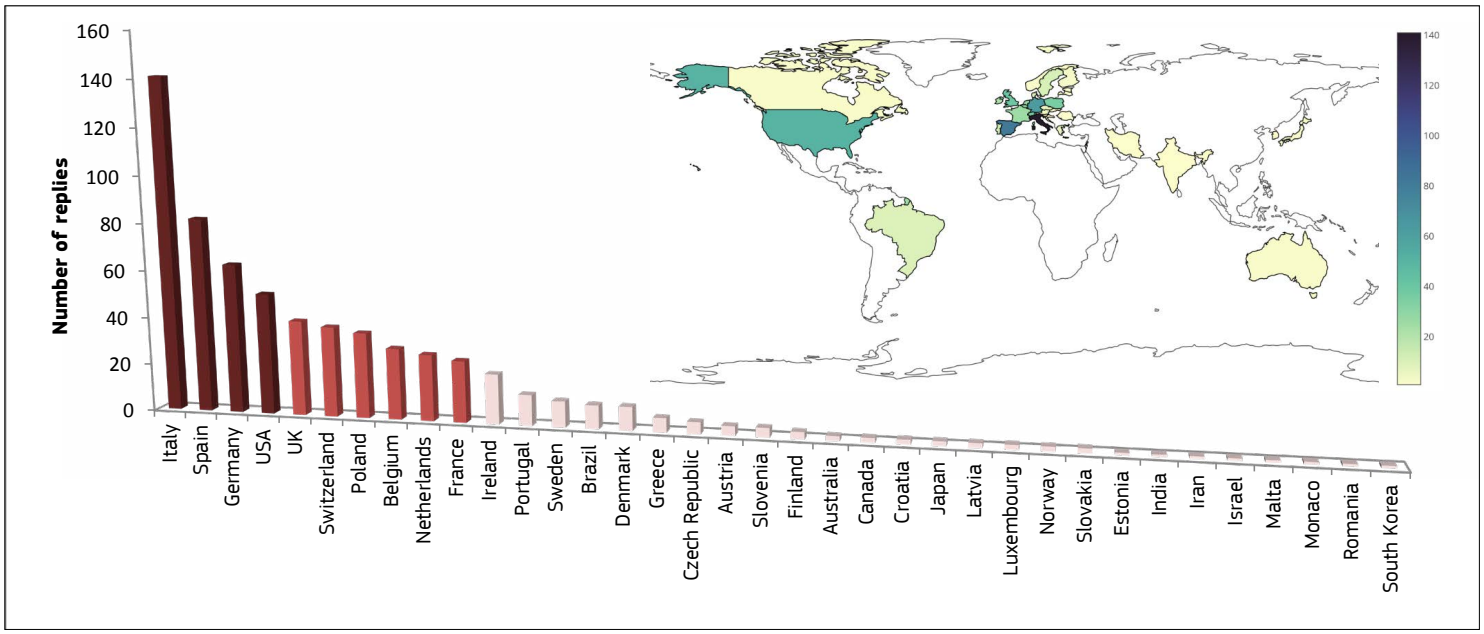


Figure 4.1 Number of survey respondents per country.

A validation study is currently under finalisation by a German consortium (Greywe *et al.*, 2012). The experimental work was carried out in three laboratories with testing of more than 30 chemicals (Riesinger *et al.,* in preparation; Maul *et al.*, in preparation).

4.6 EURL ECVAM Survey on Issues Influencing End-user Confidence in Complex *in vitro* Models

During May 2018, EURL ECVAM conducted a survey to collect scientific opinions on how to assess the relevance and fitness-for-purpose of complex *in vitro* models such as organ-on-chip devices. This is to understand how to best facilitate their uptake and application in a variety of areas, such as for risk assessment and research. As this new generation of models and methods matures, it is important to facilitate their translation from research and development into useful application by considering the scientific and technical issues that influence end-user confidence and uptake.

The purpose of this survey was to get a better understanding of the current level of uptake and satisfaction regarding complex *in vitro* models, and the current thinking around how best to establish their validity for use in research and testing. The survey sought the opinion of all those interested in complex *in vitro* models and their potential applications irrespective of how familiar one may be with the subject area. For a better interpretation of the data, the questions took into account the different levels of expertise and interests of the responders to assess their views on the credibility and relevance of these models. Responses from 646 people worldwide (see fig. 4.1) from academia (54%), industry (23%), public service (17%) and others (6%) were received.

The first results of the survey were presented at the 20th International Congress on In Vitro Toxicology (ESTIV 2018) which took place in Berlin in October 2018

The first results of the survey were presented at the 20th International Congress on *In Vitro* Toxicology (ESTIV 2018; see box 4.1) which took place in Berlin in October 2018. The whole set of findings will be made publicly available once the responses have been fully analysed. The intention is to combine the results with information gathered from other sources to eventually inform validation strategies and technology-transfer initiatives for expediting the uptake and use of complex *in vitro* models

Box 4.1

EURL ECVAM survey on issues influencing end-user confidence in complex *in vitro* models

Complex *in vitro* models such as 3D cell cultures, bioprinted tissues, bioreactor cultures or microphysiological systems (*e.g.*, organ-on-chip devices) aim to represent higher-level anatomical and physiological aspects of human biology in practical experimental setups. Methods based on complex *in vitro* models have the potential to not only provide an attractive alternative to animal models, but also offer the promise of advancing scientific methodologies underpinning research, development and testing in a variety of sectors.

At the 20th International Congress on *In Vitro* Toxicology (ESTIV 2018), held from 15 to 18 October 2018 in Berlin, EURL ECVAM released the first results of a major internal survey launched in May 2018 on complex *in vitro* models, which include 3D cell cultures, bioprinted tissues, and microphysiological (organ-on-chip) systems. The survey provided valuable insight into what developers and end-users are thinking regarding the validation of such models to ensure their successful translation into useful application. A total of 646 responses were received from 36 countries, 76% of those surveyed believe that it is possible to establish the validity of complex *in vitro* models without knowing their specific application. Among those, 60% of respondents believe that validation is useful to increase acceptance and uptake.

and methods in a variety of fields such as chemical risk assessment and biomedical research.

READ MORE

- Survey on issues influencing end-user confidence in complex *in vitro* models for use in research and testing: europa.eu/!Nm89my
- 20th International Congress on *In Vitro* Toxicology (ESTIV2018): www.estiv2018.com

4.7 EURL ECVAM Scientific Advisory Committee Peer Reviews

4.7.1 Renewal of ESAC

EURL ECVAM renewed its Scientific Advisory Committee (ESAC) in 2018 (see fig. 4.2). Renewed every three years, ESAC is composed of external scientists who are appointed on the basis of their scientific expertise and who act independently in the public interest. ESAC advises EURL ECVAM on scientific and technical issues related to the protection of animals used for scientific purposes. Nine experts were formally appointed on 16 April 2018 as the new core members of the ESAC. More details on the new ESAC including its membership, Terms of Reference and Rules of Procedure can be found on the Register of Commission Expert groups. The new ESAC will meet in plenary at the JRC in Ispra, Italy, on 4 to 5 December 2018 and on 3 to 5 June 2019.

ESAC is composed of external scientists who are appointed on the basis of their scientific expertise and act independently

READ MORE

- EURL ECVAM Scientific Advisory Committee (ESAC): europa.eu/!gp34jf
- ESAC in the Register of Commission Expert groups: europa.eu/!qm67uC

4.7.2 Upcoming ESAC Reviews

In 2018, the ESAC has been mandated to review the scientific validity of antibodies and non-antibody affinity reagents produced using animal-free technologies (see section 4.7.2.1). ESAC is expected to discuss and endorse its Opinion on this topic during the plenary meeting scheduled for 4 to 5 December 2018.

Preparations are currently underway to mandate ESAC to review and deliver an opinion on the scientific validity of the AR-CALUX test method for detection of androgenic activity of chemicals in 2019 (see section 4.1). The Opinion on the AR-CALUX is expected to be discussed

Figure 4.2 Core members of the new ESAC, European Commission staff and members of the International Cooperation on Alternative Test Methods (ICATM) from Brazil and Korea.

and endorsed by the ESAC during its plenary meeting scheduled for 3 to 5 June 2019.

Conditional on a positive evaluation by EURL ECVAM of the current full test method submissions on the bioelution assay for testing of metals, inorganic metal compounds and complex metal-containing materials in synthetic gastric fluid (see section 3.5) and on the RTgill-W1 cell line assay to predict acute fish toxicity of chemicals (see section 3.6), these methods may also be considered for ESAC peer review in 2019.

4.7.2.1 Scientific Validity of Antibodies and Non-antibody Affinity Reagents Produced Using Animal-free Technologies

Affinity reagents are binding molecules that have a high specificity for their unique target (antigen). They are crucial for research, diagnostics, therapeutic and regulatory applications. Based on their recognition properties and binding specificity, protein-based antibodies are currently still the most important tools for the specific detection of proteins or other molecules. However, with developments in protein and genetic engineering, new alternative binders are being introduced, such as aptamers, affimers, DARPINs, etc. These alternative binders can be based on peptides, proteins, ribonucleic acids, or single-stranded DNA. Although these new molecules are already being applied in diagnostics, antibodies are still the molecules of choice for many applications.

In many cases, antibody production is still relying on animal-based methods. More recently developed animal-free technologies involve the use of large collections of recombinant forms of miniaturised antibodies such as single-chain variable fragment (scFv) and fragment antigen binding (Fab), or antibody analogues. These approaches require the ability to present large binder libraries on the surface of various available display systems (*e.g.*, phage display), which permit the selection of peptides or proteins with high affinity and specificity for virtually any target. In line with the legal requirements of EU Directive on the protection of animals used for scientific purposes (2010/63/EU), animals should not be used in procedures, where a non-animal alternative exists, which provide the same or higher level of information as obtained from animal procedures.

> ***In line with EU Directive 2010/63 animals should not be used in procedures, where a non-animal alternative exists, which provide the same or higher level of information as obtained from animal procedures***

In April 2018, EURL ECVAM mandated ESAC to review the available evidence and deliver an opinion on the scientific validity of antibodies and non-antibody affinity reagents produced using animal-free technologies for use in research, regulatory testing and diagnostics.

Figure 4.3 The ESAC Working Group on Affinity Reagents at the JRC Ispra.

Taking into consideration the available evidence, the ESAC will provide an opinion on the suitability of existing animal-free technologies to produce affinity reagents with equal or better quality (purity, activity, specificity, affinity, stability) than that offered by antibodies produced using the conventional animal-based methods. In addition, ESAC will explore the scientific benefits of using animal-free affinity reagents and assess whether there are any production and/or application scenarios for which these are not fit-for-purpose and animal-derived antibodies are still indispensable. In case exceptions are identified, a strategy to tackle them should be proposed.

An ESAC Working Group was created to conduct the detailed review. Two core ESAC members agreed to participate in the Working Group and six new ad-hoc ESAC members were selected from the DG RTD Experts Management Internal (EMI) database on the basis of their expertise on the subject matter, and were formally appointed on 19 October 2018 to participate in the Working Group. More details on the current ESAC membership can be found on the Register of Commission Expert groups.

The ESAC Working Group met at the JRC in Ispra, Italy, on 8 to 9 November 2018 (see fig. 4.3) to advance the detailed review and draft a Working Group Report and an ESAC Opinion for discussion at the ESAC plenary meeting scheduled for 4 to 5 December 2018.

READ MORE

- ESAC in the Register of Commission Expert groups: europa.eu/!qm67uC

4.8 Update on EU-NETVAL

The first EU-NETVAL validation project involves three test facilities from Sweden, UK and France. Using the *in vitro* AR-CALUX method, experimental data is being generated to support the development of an OECD Test Guideline and associated performance standards (if applicable) for Androgen Receptor Transactivation Assays (ARTA) for the detection of compounds with (anti)androgenic potential (see section 4.1).

During this validation, procedures were developed to ensure efficient communication and transfer of the method to the selected EU-NETVAL facilities and to perform the *in vitro* method under GLP conditions, since this is a requirement for TGs to satisfy the terms for Mutual Acceptance of Data by Member Countries.

In June 2017, EURL ECVAM launched a validation study to assess 17 *in vitro* methods for the detection of thyroid disruptors (see section 4.2 and annex 2). EU-NETVAL test facilities will interact with the *in vitro* method developers during the initial phase of the validation study for defining and establishing the methods in a way that they can be used in a GLP environment. This will be followed by an assessment of their reliability and the generation of reference data that can be used

Figure 4.4 Meeting of the EU Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) held in October 2017 at the JRC Ispra.

to support an analysis of their optimal combination in an eventual test battery.

The 17 methods were selected based on an OECD review paper (OECD, 2014a), and with input from previous meetings and workshops (see section 5.1). The methods which perform well may be selected for overall relevance assessment (when used in combination), with a set of selected reference chemicals, in view of their eventual use in a regulatory context. *In vitro* method developers and experts who have shown interest, or have in-depth knowledge on *in vitro* method best scientific and quality practices, are involved. The validation study will comply with the recently published OECD Guidance on Good *In Vitro* Method Practices (GIVIMP) that was led by EURL ECVAM (see section 5.3.7 and box 5.3).

Invited experts and in vitro method developers gathered with EU-NETVAL members to share experiences and knowledge on specific in vitro methods for the detection of thyroid hormone disrupting chemicals

An EU-NETVAL meeting took place on 10 to 11 October 2017 at the JRC, Ispra (see fig. 4.4) to facilitate the sharing of expertise and practical knowledge for the advancement of *in vitro* methods in animal-free safety assessments. Invited experts and *in vitro* method developers gathered with EU-NETVAL members to share experiences and knowledge on specific *in vitro* methods for the detection of thyroid hormone disrupting chemicals. This will support the newly launched validation study on *in vitro* methods for detecting thyroid disruptors described above (see section 4.2). Experts were invited to host sessions addressing the practical management of test items and test systems, as well as exchanging information on the process of in-house validation (GIVIMP; OECD, 2018a) and on the use of non-TG methods such as the miniaturised Ames assay. These face-to-face knowledge sharing opportunities are essential for promoting the use of *in vitro* methods.

Box 4.2

European Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL)

The European Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) was set up by EURL ECVAM in response to the provision of Directive 2010/63/EU on the protection of animals used for scientific purposes, which requests that EU Member States assist the European Commission in the validation of alternative methods. EU-NETVAL is a large network of highly qualified test facilities across Europe, coordinated by EURL ECVAM to support the *in vitro* method validation process.

EU-NETVAL covers a wide range of expertise and competences and includes laboratories experienced in advanced *in vitro* procedures, biological test systems and measurement techniques which are considered important to address specific aims and objectives identified in EURL ECVAM's strategies to achieve impact in the 3Rs for regulatory safety assessment. Currently, there are 37 members (including the European Commission's own *in vitro* GLP test facility) of EU-NETVAL representing fifteen countries in the network. Facilities are selected, using pre-defined eligibility criteria, to carry out tasks detailed in the terms of reference, endorsed by the National Contact Points of Directive 2010/63/EU.

With the EU-NETVAL activities and applying the modular approach to validation (Hartung *et al.*, 2004), EURL ECVAM demonstrates that validation is a flexible scientific process aiming to establish confidence that the methods are fit for a particular purpose.

Figure 4.5 Participants in the joint meeting of the Preliminary Assessment of Regulatory Relevance (PARERE) network and ECVAM Stakeholder Forum (ESTAF) at the JRC Ispra in November 2017.

As a follow-up to the practical training sessions at the 2016 EU-NETVAL meeting, which included a session on *in vitro* methods for assessing the skin sensitisation potential of chemicals [DPRA (OECD, 2015), KeratinoSens™/LuSens (OECD, 2018b) and h-CLAT (OECD, 2018c) OECD TG methods)], EU-NETVAL members reported on their own experiences of using these methods. Feedback from the users of these methods is important for assessing the uptake of alternatives and for addressing potential issues for future users.

READ MORE

- EU Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL): europa.eu/!jD93bV
- Meeting of the EU Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) – News item: europa.eu/!Qn48GP

4.9 Meeting of the Preliminary Assessment of Regulatory Relevance (PARERE) Network and the ECVAM Stakeholder Forum (ESTAF)

EURL ECVAM hosted the annual meeting of the Preliminary Assessment of Regulatory Relevance (PARERE) network followed by a joint meeting of PARERE and the ECVAM Stakeholder Forum (ESTAF) on 27 to 29 November 2017 (see fig. 4.5).

The trans-sectorial PARERE network provides advice to EURL ECVAM on the regulatory relevance and suitability of alternative approaches proposed for validation, whilst both PARERE and ESTAF networks contribute to EURL ECVAM strategies and recommendations as part of the validation process.

The first part of the PARERE meeting provided updates on test submissions related to the Bioelution (see section 3.5), the ToxTracker and the EDITOX methods (see section 3) on which PARERE had been consulted. It also included a session on toxicokinetic data in regulatory frameworks where the EU regulatory agencies EFSA, EMA and ECHA provided their perspectives on the use and integration of toxicokinetic data in regulatory safety decisions. EURL ECVAM presented its activities in the toxicokinetics area which was followed by a Q&A session on Absorption, Distribution, Metabolism and Excretion (ADME) and later, by a written consultation of PARERE on these projects. The regulatory value of all of these projects was acknowledged, as were the commonalities in their applications (*e.g.*, providing ways of performing interspecies extrapolation and assessing human relevance of animal data).

In the morning of 28 November, the more advanced case studies of the EU-ToxRisk project (see section 2.1) were presented, as well as the outcome of the PARERE

consultation on the preliminary regulatory relevance of these case studies. The PARERE members appreciated that EU-ToxRisk had taken up the comments from the first meeting in 2016 and that the case study descriptions improved significantly since then. Furthermore, it was recognised that EU-ToxRisk had made huge progress over the last year. More information on the outcome of the PARERE meeting of 2017 can be found at: https://europa.eu/!mk78yT.

In addition to providing updates and highlights from EURL ECVAM, the joint meeting with both PARERE and ESTAF representatives focused on exploring the status of Three Rs relevant knowledge sources and sharing practices. Building on the outcomes of the study on the Three Rs knowledge sharing (Holley *et al.*, 2016), the participants in the joint meeting took part in a workshop to identify good practices and explore opportunities in three specific areas: Research; Education and training; and Regulatory Testing.

Motivations and goals of basic researchers in pursuing the Three Rs may differ significantly from other areas. However, this may represent an opportunity to stimulate an open and frank discussion between experts in *in vivo* and alternative methods. The need for filling existing gaps and engaging academic researchers are clearly beneficial and would provide more opportunities for knowledge exchanges. EURL ECVAM has already followed up on this by organising a workshop with different actors in the field of biomedical sciences (see section 6.2.1).

There are many opportunities to improve the delivery and access to education and training that are relevant to the Three Rs

It is evident that education and training are fundamental to driving progress in the development and uptake of the Three Rs. However, the current provision of resources in the Member States is not well defined and shows apparent differences in the level of coverage and content. Nevertheless, there are many opportunities to improve the delivery and access to education and training that are relevant to the Three Rs. As a starting point, a review of education and training resources has

Box 4.3

Preliminary Assessment of Regulatory Relevance (PARERE) network and ECVAM Stakeholder Forum (ESTAF)

The Preliminary Assessment of Regulatory Relevance (PARERE) network was established by EURL ECVAM further to a provision of Directive 2010/63/EU that requires that Member States nominate a single point of contact to provide advice on the regulatory relevance and suitability of alternative approaches proposed for validation. This trans-sectorial network is composed of regulators nominated by the EU Member States, representatives from EU regulatory agencies such as the European Medicines Agency (EMA), the European Chemicals Agency (ECHA) and the European Food Safety Authority (EFSA), and relevant Commission services. In order to expedite the process of regulatory acceptance of alternative methods, it was considered that regulators operating within all sectors of relevance to alternative methods should be involved as early as possible in providing a preliminary view on the potential regulatory relevance of methods and approaches submitted to EURL ECVAM for validation or peer review or evaluation. PARERE has some additional tasks which are described on the EURL ECVAM website: europa.eu/!gF94hp**.** PARERE members are consulted on several occasions over the year, either on the regulatory relevance of individual methods or approaches that are submitted to EURL ECVAM or on other topics such as *e.g.* EURL ECVAM strategy documents in different areas, EURL ECVAM Recommendations, EURL ECVAM's projects at the OECD and case studies being developed within research projects funded by the EU Framework Programme for Research and Innovation, Horizon 2020.

The ECVAM Stakeholder Forum (ESTAF) comprises stakeholder organisations from academia, industry and civil society (e.g., animal welfare groups), and the OECD. It was established to strengthen participatory approaches and to support communication and cooperation with the stakeholder community. The forum provides input in a number of areas of EURL ECVAM's work including strategies to implement the Three Rs and EURL ECVAM Recommendations that are typically issued on conclusion of a validation study. Due to the valuable scientific, technical and societal expertise of its various stakeholders, the ESTAF represents a precious network for sharing Three Rs knowledge, encouraging dialogue and exchanging information on topics related to alternative methods and approaches.

An open invitation for stakeholder organisations with an interest in joining the forum is available on the EURL ECVAM website: europa.eu/!JG48wW

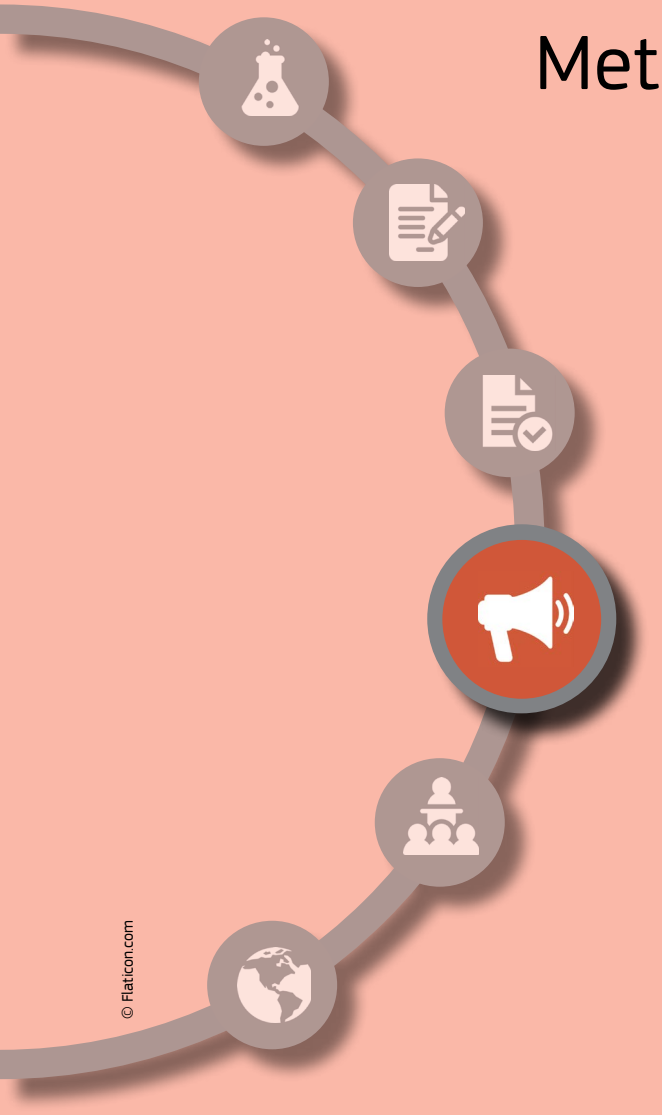
been undertaken by EURL ECVAM in order to map the available provision of resources with Three Rs relevance (see section 6.3.1). Knowledge sharing in the area of regulatory testing already takes place, but there needs to be a better coordination and communication. For example, new developments in technology and scientific knowledge gaps represent a significant barrier in the use and uptake of the Three Rs. As a result there is insufficient application or even reflection on the appropriate use of alternative approaches within the regulatory context of diverse sectors. More details of the joint meeting of PARERE and ESTAF are available at https://europa.eu/!wr94Rm..

The next PARERE meeting will take place on 27 to 28 November 2018 and will include updates from the PARERE network and a discussion on critical elements within test submissions as well as relevant work within some of the OECD working groups. On the second day, a workshop on the Adverse Outcome Pathways (AOP) framework will introduce the scope and the content of the OECD AOP framework and will discuss how this framework could be moved forward with the PARERE network. The next joint meeting of PARERE and ESTAF will take place on 28 to 29 November 2018. It will focus on a workshop to discuss the validation of alternative methods, this time from a regulatory and industry perspective, and to gather opinions on key questions and ways forward. Similar questions had been discussed during the workshop of the International Cooperation on Alternative Test Methods (ICATM) involving regulators from the different countries of the ICATM partners (see section 7.1).

READ MORE

- Preliminary Assessment of Regulatory Relevance (PARERE): europa.eu/!gF94hp
- ECVAM Stakeholder Forum (ESTAF): europa.eu/!wU38uj
- EURL ECVAM's stakeholder forum and regulatory advisors share opinions on better knowledge sharing - News item: europa.eu/!dN98Pw

5 Promoting the Regulatory Acceptance and International Adoption of Alternative Methods and Approaches



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5. Promoting the Regulatory Acceptance and International Adoption of Alternative Methods and Approaches

5.1 Activities Related to the Identification of Endocrine Disruptors

In support of the EU Community Strategy for Endocrine Disruptors and the OECD programme on testing and assessment of endocrine disruptors, the JRC through EURL ECVAM has been supporting the European Commission's Directorate Generals for Environment, and Health and Food Safety on several activities aimed at improving ED detection (see also box 5.1).

READ MORE

- DG SANTE - Endocrine Disruptors: europa.eu/!bV43dk

5.1.1 Revision of the Community Strategy

In 1999, the European Commission published a Community Strategy for Endocrine Disruptors (COM(1999) 706) (EC, 1999) with the objectives of identifying the problem of endocrine disruption, its causes and consequences, and to identify appropriate policy action on the basis of the precautionary principle, in order to respond quickly and effectively to the problem, thereby alleviating public concern. A new Communication 'Towards a more comprehensive European Union framework on endocrine disruptors' has recently been published (EC, 2018), which takes stock of the actions undertaken in the EU so far and outlines areas where further action is needed with a view to minimising exposure to endocrine disruptors and promoting the substitution of endocrine disrupting chemicals with less harmful chemicals. The proposals of the Commission, following up on the key strands of the 1999 Strategy, revolve around three principles: 1) addressing the gaps in knowledge by taking forward the science; 2) linking science and regulation: ensuring an effective EU legal framework; 3) deepening international cooperation.

READ MORE

- Endocrine disruptors: A strategy for the future that protects EU citizens and the environment – Press release: europa.eu/!mR37Ug

5.1.2 Guidance Document on the Identification of Endocrine Disruptors in the Context of the EU Regulations on Biocides and Plant Protection Products

Scientific criteria for the determination of endocrine disrupting properties for the Biocides [(EU) 2017/2100] and Pesticides [(EU) 2018/605] Regulations, were recently

adopted which will be applicable from June 2018 and November 2018, respectively. EURL ECVAM has been supporting the Commission on scientific aspects of the process for criteria development, including the drafting of a Guidance Document (GD) accompanying these criteria, in support of the relevant agencies, EFSA and ECHA.

This GD assists applicants and assessors of competent regulatory authorities on how to apply these criteria (ECHA and EFSA, 2018). The GD describes how to gather, evaluate and consider all relevant information for the assessment, conduct a mode of action analysis, and apply a weight of evidence approach, in order to establish whether the ED criteria are fulfilled. The GD has been based upon the suite of *in vitro* and *in vivo* test guidelines currently available, including *in silico* tools, as described by the recently updated OECD's Conceptual Framework for the Testing and Assessment of Endocrine Disruptors and accompanying guidance (OECD, 2018d; see section 5.3.13). The assessment strategy pursued in the guidance requires the generation of supplementary data only when necessary to avoid further animal testing.

The GD describes how to gather, evaluate and consider all relevant information for the assessment, conduct a mode of action analysis, and apply a weight of evidence approach, in order to establish whether the ED criteria are fulfilled

READ MORE

- ECHA - Guidance on identifying endocrine disruptors published: echa.europa.eu/it/-/guidance-on-identifying-endocrine-disruptors-published
- EFSA - Guidance on identifying endocrine disruptors published: www.efsa.europa.eu/en/press/news/180607

5.2 Activities in the OECD Working Party on Hazard Assessment

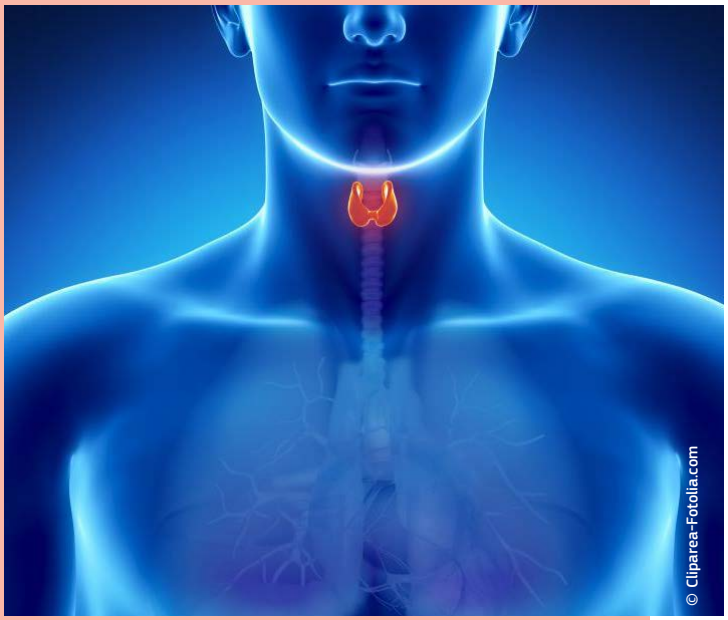
5.2.1 OECD Integrated Approaches to Testing and Assessment Case Studies Project

The IATA Case Studies Project under the OECD Working Party on Hazard Assessment (WPHA) aims to support efforts of the OECD Member Countries to increase the use of alternative methods within IATA. The project is investigating the practical applicability of IATA by discussing case studies, based on a draft template (OECD, 2014b,c), to create a common understanding of using the approaches. Findings and conclusions are summarised in considerations documents, which highlight the major issues discussed and lessons learned from the IATA approaches of the case studies, to contribute to the development of further guidance and respective tools.

Box 5.1

Activities on the development and validation of assays for the identification of endocrine disruptors

The adoption of the ECHA-EFSA GD will allow the implementation of scientific criteria for the determination of endocrine disrupting properties for the Biocides [(EU) 2017/2100] and Pesticides [(EU) 2018/605] Regulations (see section 5.1.2). However, there is still much work to be done in the development and validation of assays for the detection of endocrine disruptors. In particular, EURL ECVAM has embarked upon a project with its network of validation laboratories (EU-NETVAL) to characterise the performance of 17 alternative methods that investigate interference with different aspects of thyroid physiology (either by interference with synthesis, action or clearance; see section 4.2) and is offering support to a number of H2020 funded projects focussed on development and validation of novel methods for screening and testing for EDs which are about to start (see section 2.3).



The first and second cycle comprised case studies focused on grouping and read-across for different hazard endpoints (OECD, 2016a-d; OECD, 2017a,b), one specifically supported by toxicogenomics data (OECD, 2017c), a pesticide cumulative risk assessment and assessment of lifestage susceptibility (OECD, 2017d), as well as the JRC/BIAC chemical safety assessment workflow based on exposure considerations and focusing on non-animal methods, integrating multiple data streams for safety assessment decisions (OECD, 2017e) and based on the SEURAT-1 cross-cluster *ab initio* case study (Berggren *et al.*, 2017).

The third cycle started in 2017 and reviewed an IATA workflow using interchemical and intrachemical comparison of data from traditional and alternative approaches (OECD, 2018e), the prioritisation of chemicals using an IATA-based Ecological Risk Classification (OECD, 2018f), a read-across case study with metabolism playing a key role in toxicity (OECD, 2018g) and a JRC case study on grouping and read-across for nanomaterials - genotoxicity of nano-TiO_2 (OECD, 2018h), based on the Nanocomput project (Worth *et al.*, 2017a). The aim of this case study was to determine the genotoxic hazard potential of two nano-TiO_2 target substances by reading across *in vitro* comet assay results. It is illustrating the applicability of the workflow for grouping and read-across proposed in the REACH guidance update for nanomaterials (ECHA, 2017a) and identifying sources of uncertainty, exploring the extent to which ECHA's Read-Across Assessment Framework (RAAF) (ECHA, 2017b) captures the different sources of uncertainty for nanoforms, pointing out nanospecificities to be considered. Furthermore, the relevance of cheminformatics methods in grouping of nanoforms was explored. The four case studies of the 2017 cycle mentioned above and its considerations document (OECD, 2018i), updating the learnings from the previous cycles (OECD, 2016e; OECD, 2017f) with the new experience and issues identified for further discussion, were endorsed at the WPHA meeting in June 2018 and have been published on the OECD website. Uncertainty assessment was one focus of the discussions.

The IATA Case Studies Project under the OECD Working Party on Hazard Assessment (WPHA) aims to support efforts of the OECD Member Countries to increase the use of alternative methods within IATA

The fourth cycle is reviewing a case study on read-across for testicular toxicity of ethylene glycol methyl ether (EGME)-related chemicals, including considerations of metabolism and AOP information, as well as a Defined Approach for identifying estrogen receptor (ER) active chemicals, consisting of an integrated battery of *in vitro* high throughput screening (HTS) assays and a computational model of ER pathway activity.

READ MORE

- Integrated Approaches to Testing and Assessment (IATA) - Case studies project: www.oecd.org/chemicalsafety/risk-assessment/iata-integrated-approaches-to-testing-and-assessment.htm

5.2.2 Information Exchange Seminar for IATA-related Projects

A half-day "Information Exchange Seminar for IATA Related Projects" was held on 14 November 2017 as a webconference. Members from past or ongoing OECD IATA-related projects were invited to participate to exchange information and experience in IATA related activities and identify possible synergies.

An overview of OECD IATA related definitions and of 11 OECD IATA-related projects were presented. EURL ECVAM contributed with presentations on the guidance documents on Defined Approaches (OECD, 2016f,g) and on the project to establish a guideline for defined approaches relating to skin sensitisation. Furthermore, EURL ECVAM gave an overview of currently available guidance documents and templates related to IATA and IATA components in view of noting gaps or possible overlaps, with specific notice of the consideration of assessment and reporting of uncertainties in the guidance documents.

READ MORE

- Webinar: Information exchange seminar for IATA related projects – November 2017: youtu.be/PXZHJzDGljo

5.2.3 Mapping of Guidance Documents Related to IATA and IATA Components

The scoping exercise of mapping IATA-related guidance carried out by EURL ECVAM showed that the number of documents from different sources, directly or indirectly related to guidance on IATA, is proliferating but fragmented and sometimes duplicated across countries, sectors and topic areas. These results and previous discussions in the IATA Case Studies Project Group gave rise to a project under the WPHA, led by EC (through JRC-EURL ECVAM) in a project team with Canada, Germany

and the Netherlands. The aim of the project is to compile a comprehensive overview document on the concepts and available guidance for IATA and their components as well as for connected cross-cutting topics such as data quality and uncertainties.

5.2.4 Chemical Mixtures and Combined Exposure

Every day we are exposed to low levels of hundreds of different manmade chemicals present for example in our food, consumer products and the air we breathe. Our environment is also exposed to a near-infinite number of chemical mixtures derived from numerous sources. However, current safety assessment practice is primarily based on understanding the potential risk posed by single substances rather than their "real life" combinations, thus potential combination effects might be overlooked.

Combined exposure to multiple chemicals can lead to adverse effects on human health or the ecosystem, even if single substances in the mixtures are below their individual safety thresholds. While manufactured products such as pesticide formulations or cosmetic products are covered in current chemical legislation, unintentional mixtures which are coincidentally formed such as mixtures of contaminants *e.g.*, in indoor air, are not consistently addressed. Their composition is often unknown and changes over time, making them difficult to regulate. The assessment of unintentional mixtures is therefore usually limited to specific legislative sectors only, such as pesticide residues in food. EURL ECVAM is investigating recent progress in considering combined exposures to multiple chemicals to help translate best science into best assessment practice. The latest policy brief (Bopp *et al.*, 2018b), puts together issues around the topic, including the specific challenges that will further inform discussions of the working group of Commission services and EU agencies on the combination effects of chemicals.

EURL ECVAM is investigating recent progress in considering combined exposures to multiple chemicals to help translate best science into best assessment practice

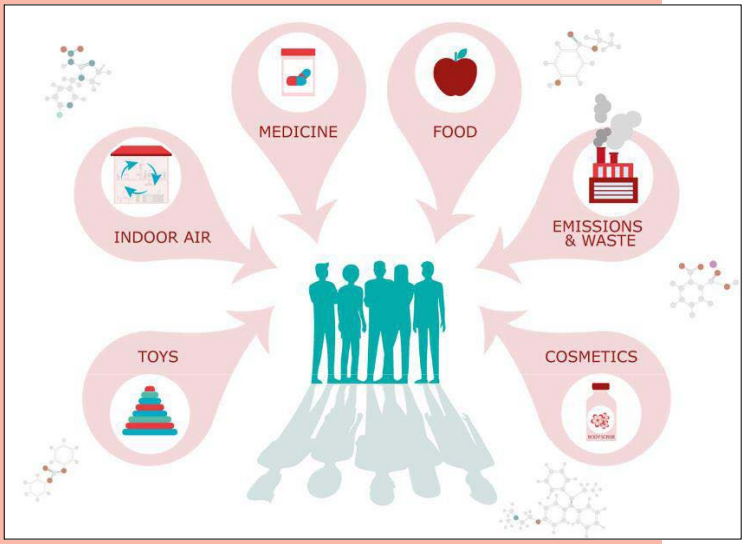
Box 5.2

Chemical mixtures: how to address the safety of combined exposures to multiple chemicals for people and the environment

Different methods are already being used to predict or assess mixture toxicity. Chemical mixtures are assessed either by testing the whole mixture (*e.g.,* in effect-based monitoring of surface water) or by predicting the combined risk based on concentration and effect information of the individual components in the mixture (*e.g.,* in the assessment of dioxins and dioxin-like compounds in food and feed).

With a view to harmonising assessment methodology, international bodies, such as the World Health Organisation (WHO) and the Organisation for Economic Cooperation and Development (OECD) have developed guidance on the assessment of risks from combined exposure to multiple chemicals. However, there are still many gaps concerning availability and interpretation of data, and there is no harmonised assessment approach across different sectors of legislation.

EURL ECVAM has been analysing the available methodologies for chemicals testing. Due to the large number of chemical combinations, it is practically unfeasible to test all possible mixtures experimentally, in particular in animal studies for ethical and practical reasons. Therefore smart strategies are needed to address the data gaps and to assess the potential hazards using new tools that rely less on *in vivo* testing and incorporate instead alternative experimental and computational tools. Their main strengths lie in their integrated use in smart combinations, allowing a better, mechanistically based prediction of mixture effects.



EURL ECVAM is performing research on the use of alternative (non-animal) methods and new strategies to assess the combination effects of chemicals. Having reviewed current EU regulatory requirements and mixture assessment practices (Bopp *et al.*, 2015, 2016; Kienzler *et al.*, 2014, 2016), it is currently exploring links between mixtures and possible subsequent diseases, interaction effects of chemicals, as well as the use of biomonitoring data in exposure assessment. EURL ECVAM also recently reviewed the role and availability of Physiologically Based Kinetic (PBK) models in addressing chemical mixtures (Desalegn *et al.*, 2018).

EURL ECVAM collaborates with five European research consortia focusing on chemical mixture assessment for the environment (SOLUTIONS), human health (EuroMix, HBM4EU), endocrine disruption (EDC-MixRisk) and alternatives to animal testing (EU-ToxRisk) (Bopp *et al.*, 2018a; see section 2.2). As international harmonisation is essential in this context, EURL ECVAM actively supports the OECD project on combined exposure (led by the OECD Working Party on Hazard Assessment in collaboration with the Working Party on Exposure Assessment) and supports the development of consistent assessment approaches for combined exposure to chemical mixtures at international level. The draft guidance on "Considerations for Assessing the Risks of Combined Exposures to Multiple Chemicals" was endorsed in October 2018.

READ MORE

- SOLUTIONS: www.solutions-project.eu
- EuroMix: www.euromixproject.eu
- HBM4EU: www.hbm4eu.eu
- EDC-MixRisk: edcmixrisk.ki.se
- EU-ToxRisk: www.eu-toxrisk.eu

5.2.5 Guidance Document on Physiologically Based Mathematical Models

New approach methodologies (NAM) combining *in vitro* data, *in silico* data and physiologically based kinetic (PBK) modelling have the potential to play a significant role in reducing animal testing. It is necessary to investigate the strengths, uncertainties, and limitations of PBK models developed using NAM data, for establishing a higher degree of confidence in the regulatory application of such models (Paini *et al.*, in press). With this in mind, the EC (through EURL ECVAM) and the US Environmental Protection Agency (EPA) are co-leading an expert working group at the OECD to develop a guidance document on the characterisation, validation and reporting of physiologically based models that are based on data derived from non-animal methods to facilitate regulatory applications. A reporting template was developed to capture and highlight the uncertainties in the several steps of development and application of PBK models. In addition, following this template, a few well-documented case studies were discussed at a face-to-face meeting of the working group in Paris in September 2018. It is expected that these case studies will inform and illustrate the guidance which should be finalised in 2019.

New approach methodologies (NAM) combining in vitro data, in silico data and physiologically based kinetic (PBK) modelling have the potential to play a significant role in reducing animal testing

5.3 Activities in the OECD Test Guidelines Programme

5.3.1 Summary of the Outcome of the 30th Meeting of the Working Group of National Coordinators of the OECD Test Guidelines Programme

At the 30th meeting of the Working Group of National Coordinators of the OECD Test Guidelines Programme (WNT) held at the OECD headquarters in Boulogne, Paris on 24 to 27 April 2018 (see fig. 5.1), eight Test Guidelines were approved of which two were new TGs and six were updated TGs.

The new and updated TGs included:

- New Test Guideline 319A on the "Determination of *in vitro* intrinsic clearance using cryopreserved rainbow trout hepatocytes" (co-led by the EC through JRC-EURL ECVAM, and the US; see section 5.3.3 and annexes 1 and 2).
- New Test Guideline 319B on the "Determination of *in vitro* intrinsic clearance using rainbow trout liver S9 sub-cellular fraction" (co-led by the EC through JRC-EURL ECVAM, and the US; see section 5.3.3 and annexes 1 and 2).
- Updated Test Guideline 438 (and updated Guidance Document 160, see below) on the Isolated Chicken Eye Test Method for identifying i) chemicals inducing serious eye damage and ii) chemicals not requiring classification for eye irritation or serious eye damage, including histopathological examination as additional endpoint to identify UN GHS cat.1 non-extreme pH (2<pH<11.5) detergents and surfactants and modified decision criteria for chemicals requiring classification for eye hazard (taking into consideration

the variability of the Draize eye test; see annexes 1 and 2).
- Updated Test Guideline 492 on Reconstructed human Cornea-like Epithelium (RhCE) test methods for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage with the inclusion of the Labcyte CORNEA MODEL 24 EIT test method (see annexes 1 and 2).
- Updated Test Guideline 442D on *in vitro* skin sensitisation addressing key event 2 on keratinocyte activation with the inclusion of the LuSens test method, and the Keratinosens test method using animal-free serum (see annexes 1 and 2).
- Updated test Guideline 442B on the Local Lymph Node Assay (LLNA) including the BrdU FCM method (see annex 2).
- Updated Test Guideline 408 on 90 day Oral Repeated Dose Toxicity Study including endocrine-related endpoints (see section 5.3.15).
- Updated Test Guideline 414 on Prenatal Development Toxicity Study including endocrine-related endpoints (see section 5.3.15).

Three new Guidance Documents and four updated ones were approved as well and included:
- Updated Guidance Document 23 on Aqueous-phase Aquatic Toxicity Testing of Difficult Test Chemicals [US, EC (through EURL ECVAM) and ICAPO-led].
- New Guidance Document 280 on Determination of *in vitro* intrinsic clearance using cryopreserved hepatocytes (RT-HEP) or liver S9 sub-cellular fractions (RT-S9) from rainbow trout and extrapolation to *in vivo* intrinsic clearance [EC (through EURL ECVAM) and US-led].
- Updated Guidance Document 160 on the collection of eye tissues for histological evaluation and collection of data (see annex 2).
- Updated Guidance Document 39 on inhalation toxicity studies.
- Updated Guidance Document 150 on Evaluation of Chemicals for Endocrine Disruption using Standardised Guidelines (EC through EURL ECVAM was member of the Steering Group, see section 5.3.13).
- New Guidance Document 286 on Good *In Vitro* Method Practice (JRC through EURL ECVAM-led, see section 5.3.7).
- New Guidance Document on Efficacy of Biocide Treated Articles.

In addition, modifications were also made to some existing Test Guidelines on chronic toxicities such as TGs 443, 451, 452, 453 and GD 116 to include a sentence on adequacy of dose/concentration selection to fulfil regulatory needs. Fourteen new projects were adopted on the OECD Workplan. More information can be found on the OECD website of the TGP.

The following chapters mainly focus on TGs for which the EC (through JRC-EURL ECVAM) has the lead or co-lead. However, some projects with relevance to the alternative field led by other Member Countries are also briefly described. Beside those, EURL ECVAM participated in

Figure 5.1 30th meeting of the Working Group of National Coordinators of the OECD Test Guidelines Programme (WNT) held at the OECD headquarters in Boulogne, Paris in April 2018.

numerous OECD expert groups and validation management groups and commented on several other draft TGs and GDs led by other OECD Member Countries.

READ MORE

- OECD Guidelines for the testing of chemicals and related documents: www.oecd.org/chemicalsafety/testing/oecd-guidelines-testing-chemicals-related-documents.htm

5.3.2 OECD Test Guidelines on Determination of *in vitro* Fish Intrinsic Hepatic Clearance

The OECD project on the development of new OECD TGs on the determination of *in vitro* Fish Intrinsic Hepatic Clearance (under the lead of USA and the EC represented by JRC EURL ECVAM) aimed at standardising two *in vitro* methods using either rainbow trout S9 fraction (Johanning *et al.*, 2012) or cryopreserved rainbow trout hepatocytes (Fay *et al.*, 2015) to determine *in vitro* fish intrinsic hepatic clearance.

The project built on work carried out within the framework of the HESI project "Bioaccumulation". HESI coordinated a multi-laboratory ring trial (2014-2016) to assess the reliability, transferability, and predictive value of the two *in vitro* methods (Nichols *et al.*, 2018). The two OECD TGs, the associated guidance document and the ring trial report underwent OECD public consultation in 2017. OECD WNT approved the documents at its 30th meeting in April 2018, and the documents are now available on the OECD website (OECD, 2018j-m). Moreover, EURL ECVAM supported this project by making available a fish *in vitro* biotransformation database covering intrinsic clearance data determined with *in vitro* methods (hepatocytes, S9, microsomes). The database is publicly available from the JRC Data Catalogue (Halder *et al.*, 2018b; see section 6.1.4) and may help users of the new OECD TG 319a and 319b to determine whether their test chemicals fall within the applicability domain of the methods.

The associated OECD Guidance Document 280 (OECD, 2018l) provides information on how to best perform the two *in vitro* methods. Moreover, it describes how the *in vitro* intrinsic clearance (indicating metabolism) can be extrapolated to a whole-body metabolism rate constant (see fig. 5.2). Inclusion of measured biotransformation rates enhances the reliability of models to estimate the Bioconcentration Factor (BCF), since the conventional log Kow-based QSARs or other models often neglect the contribution of biotransformation and potential reduction of bioaccumulation (Laue *et al.*, 2014; Nichols *et al.*, 2013).

The bioconcentration potential of a chemical is important information that is required in many pieces of chemical legislation

The bioconcentration potential of a chemical is important information that is required in many pieces of chemical legislation. It is used for hazard classification and for the assessment of persistent, bioaccumulative and toxic (PBT) substances. The BCF is either predicted or measured (typically in fish, but if necessary, also in invertebrates). Depending on the regulatory framework, predicted BCFs based on *in vitro* data may be applied to screen chemicals for their bioaccumulative properties and to decide whether a chemical is bioaccumulative at

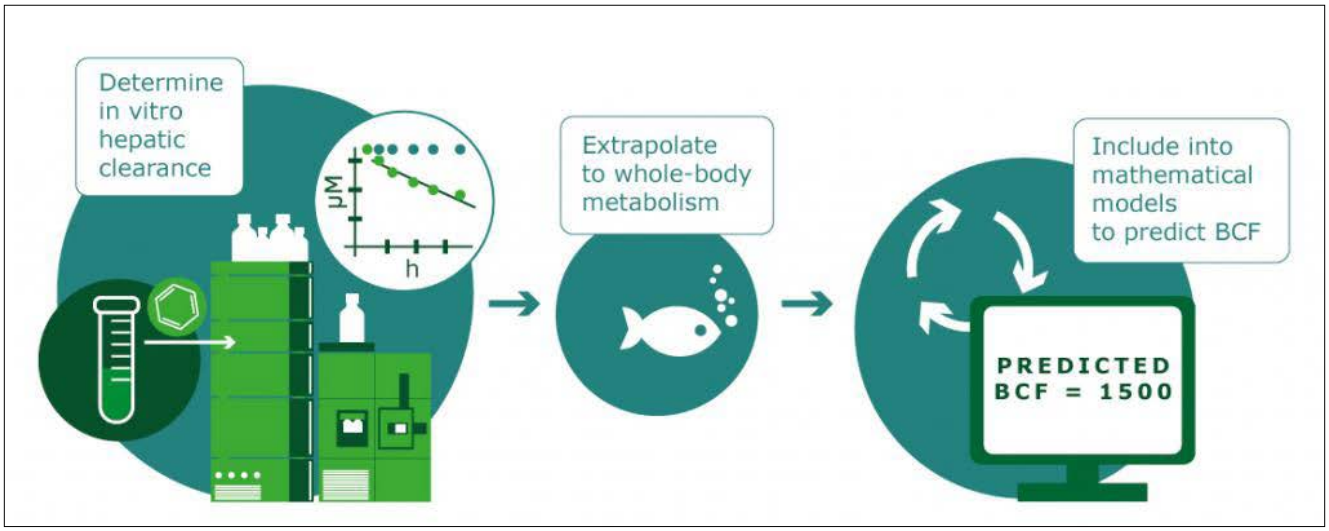


Figure 5.2 How to use *in vitro* determined hepatic clearance to predict BCF values.

the screening level; to assess the bioaccumulation potential as part of a weight of evidence approach or read across, or, to decide whether a full *in vivo* fish study is warranted. Predicted BCFs based on *in vitro* data may not replace *in vivo* fish bioaccumulation tests, however, they may be an alternative if *in vivo* testing is technically not feasible, or if the corresponding regulatory framework does not allow vertebrate testing.

READ MORE

- EURL ECVAM Fish *In Vitro* Intrinsic Clearance Database: data.jrc.ec.europa.eu/dataset/jrc-eurl-ecvam-fish-in-vitro-intr-clear-db

5.3.3 OECD Guidance Document 23 on Aqueous-phase Aquatic Toxicity Testing of Difficult Test Chemicals

This project initiated by the International Council on Animal Protection in OECD Programmes (ICAPO) and co-led by the EC (JRC-EURL ECVAM), USA and ICAPO is divided into two parts. Part 1, the update of GD 23, was successfully concluded in 2018 and the revised GD 23 is available on the OECD website (OECD, 2018n).

GD 23 supplements OECD test guidelines used for regulatory purposes. The revision reflects the experience gained in handling difficult-to-test chemicals in aquatic exposures as well as progress made in developing methods for testing these chemicals since GD 23's first publication in 2000. Specific focus was on the expansion of the guidance on testing of poorly water soluble test chemicals, *i.e.*, attention was paid to updating exposure methods that do not employ a solvent in order to eliminate the need for a solvent control, and thus, reducing the number of animals used in aquatic toxicity tests. Moreover, GD 23 now includes more detailed guidance for substances of unknown or variable composition, complex reaction products, and biological materials (UVCBs). The updated GD 23 will help government agencies, industry, and contract research organisations conduct valid and reliable aquatic toxicity studies on difficult-to-test chemicals while minimising both the number of animals used and the need to repeat studies. Part 2 of the project is ongoing and aims at determining whether it is possible to use only one control, the solvent control, when solvents are used in aquatic toxicity tests on fish. A retrospective review of existing data generated according to OECD test guidelines in the presence of a solvent will be used to determine if the use of only one control would impact the outcome of the study.

The updated GD 23 will help conduct valid and reliable aquatic toxicity studies on difficult-to-test chemicals while minimising both the number of animals used and the need to repeat studies

5.3.4 Guidance Document on IATA for Fish Acute Toxicity Testing

The project aims at developing an IATA for fish acute toxicity testing. As a first step the focus was on updating OECD GD 126, the threshold approach for acute fish toxicity (OECD, 2010), and integrate the fish embryo acute toxicity test (OECD, 2013b) into the step-wise approach for determining acute fish toxicity. The project started in 2015 under the lead of Austria and ICAPO and the development of the guidance document is ongoing.

5.3.5 Revision of OECD Test Guideline 203

OECD TG 203 fish acute toxicity test (OECD, 1992) determines the concentration of a chemical at which 50% of the fish die (LC_{50}) and is one of the few guidelines still using death as an endpoint. The project (led by Switzerland and UK) aims at including the use of non-lethal endpoints to reduce the suffering of the test fish. A new draft underwent WNT consultation during 2018 and is available on the OECD website.

READ MORE

- Draft Revised Test Guideline 203 - Fish Acute Toxicity Test: www.oecd.org/chemicalsafety/testing/Draft%20Update%20TG%20203_July%202018-for%20public%20comments.pdf

5.3.6 OECD Test Guideline on CYP induction

When *in vitro* methods are used for systemic toxicity testing, metabolically competent test systems need to be integrated, since toxicokinetic properties of most substances are dependent on metabolism. Elucidating metabolic competence allows better interpretation and comparability of *in vitro* methods. Cytochrome P450 (CYP) induction, requiring *de novo* protein synthesis, is a sensitive biomarker for evaluating phenotypic hepatic metabolic competence. Regarding induction, there are two EURL ECVAM validated methods for measuring cytochrome P450 induction of CYP1A2, CYP2B6 and CYP3A4 isoforms.

The CYP induction methods are important as the metabolism of a test item may (i) be increased by the test item itself (auto-induction) or by another concurrently administrated/exposed substance (*e.g.*, mixture), (ii)

perturb the endogenous metabolism by induction or inhibition of enzymes involved in the process, or (iii) lead to toxicity by reactive metabolite formation. Mechanistic information on CYP induction should be considered in the context of IATA for adverse effects.

EURL ECVAM is preparing a detailed manuscript that summarises the results of the validation study of two *in vitro* methods assessing the potential of a chemical to induce CYP1A2, CYP2B6, and CYP3A4 using cryopreserved primary human hepatocytes (PHH) and a cryopreserved human derived HepaRG cell line. The manuscript will recommend that:

a) CYP induction should be measured in human derived metabolically competent test systems,

b) HepaRG was comparable to PHH in predicting CYP induction, representing a substitute/complementary *in vitro* system for CYP induction studies,

c) CYP induction should be measured at phenotypic level (*i.e.*, activity),

d) 2-fold induction is an acceptable threshold for positive identification of *in vitro* CYP inducers,

e) a concentration-dependent response (*i.e.*, at least two consecutive concentrations generating 2-fold induction response) should be observed to reduce the risk of false positives, and to classify a compound as an *in vitro* inducer.

The manuscript will be shared with relevant OECD expert groups with a view to integrating the human CYP induction *in vitro* methods, and the generated metabolic activation and induction data, into testing strategies (Coecke *et al.*, 2006; Wilk-Zasadna *et al.*, 2015; Hakkola *et al.*, 2018).

READ MORE

- CYP induction assay using the cryopreserved human hepatocytes: tsar.jrc.ec.europa.eu/test-method/tm2009-13
- CYP induction assay using the human cryopreserved HepaRG™ cell line: tsar.jrc.ec.europa.eu/test-method/tm2009-14

5.3.7 OECD Guidance Document on Good In Vitro Practices for the Development and Implementation of In Vitro Methods for Regulatory Use in Human Safety Assessment (GIVIMP)

There is a strong belief that *in vitro* methods are fast becoming the key tool for a new way of doing toxicology. However, their potential will not be fully exploited if they are not developed and applied in a way that scientific integrity and quality is assured, as the data they produce will not be trusted by decision makers. New challenges arise with the development of high-throughput technologies, stem cells and new culture technologies (organotypical cell cultures, organ-on-chip technologies). Moreover, *in vitro* methods often require improvements in their design and implementation before they can be validated and evaluated regarding their reliability and relevance for a particular regulatory purpose.

The recently approved OECD Guidance Document on Good *In Vitro* Method Practices (GIVIMP; OECD, 2018a), led and coordinated by EURL ECVAM, aims to provide a framework of technical and quality practices for safeguarding that the *in vitro* method is of the highest possible scientific quality. Applying GIVIMP during the development of *in vitro* methods will help to improve the quality of submitted methods and ultimately the efficiency of validation studies. GIVIMP will not only support validation bodies but it is intended to support method developers and end-users working in academic, industry or government laboratories across all 36 OECD Member Countries and beyond. A large number of international experts, including members of a dedicated OECD expert group and EU-NETVAL, contributed to the state-of-the-art knowledge gathered within the guidance document. GIVIMP also benefitted from a number of written commenting rounds and two expert meetings before its final endorsement by the OECD WNT in April 2018.

GIVIMP is intended to support method developers and end-users working in academic, industry or government laboratories across all 36 OECD Member Countries and beyond

GIVIMP consists of ten chapters addressing key aspects throughout the *in vitro* method lifecycle from development to regulatory use: (1) Roles and responsibilities, (2) Quality considerations, (3) Facilities (4) Apparatus, material and reagents, (5) Test systems, (6) Test and reference/control items, (7) Standard operating procedures (SOPs), (8) Performance of the method, (9) Reporting of results, (10) Storage and retention of records and materials.

5.3.8 OECD Guideline on Defined Approaches relating to Skin Sensitisation

The need to replace regulatory animal assays for skin sensitisation with "Defined Approaches" (DAs) based on the integration of relevant information from a variety of non-animal sources (*in vitro* and *in chemico* methods, *in silico* predictions, physicochemical properties, read-across results, and other inputs relevant to the skin sensitisation adverse outcome pathway (AOP) initiated by covalent binding to skin proteins) is being considered by the OECD (Casati *et al.*, 2018). In 2017, the OECD included in its work program the proposal for a project on the development of a Guideline on DAs for skin sensitisation put forward by the EC (through JRC's EURL ECVAM), US (NICEATM/EPA/CPSC) and Canada (Health Canada) with the support of other ICATM partners. A special meeting of the OECD WNT took place from 13 to 15 December 2017 at the JRC (see fig. 5.3). On occasion of this meeting preliminary assessment criteria, as well as potential ways for analysing the *in vivo* (LLNA and human) data were proposed for discussion (see section 5.3). An expert group charged with assisting in the development of the guideline and composed by more than 50 members representing ten countries was set up by the OECD.

In 2018, activities focused on the consolidation of the evaluation framework for DAs (see section 5.2.10 in Zuang *et al.*, 2017) and application of the framework to initially review three of the simplest DAs (The Adverse Outcome Pathway-based "2 out of 3" integrated testing strategy from BASF, the sequential testing strategy (STS) and the integrated testing strategy (ITS) for sensitising potency classification both from Kao Corporation; see Annex I to OECD GD 256) (OECD, 2016h) that include validated and OECD adopted *in vitro* methods and have simple rule-based data interpretation procedures (DIP). The review process, finalised by the experts in June 2018, showed the adequateness of the proposed evaluation framework and highlighted the importance of a number of activities already initiated by the leaders of the project in order to progress the development of a guideline on these DAs.

The need to replace regulatory animal assays for skin sensitisation with "Defined Approaches" (DAs) based on the integration of relevant information from a variety of non-animal sources is being considered by the OECD

Figure 5.3 Special meeting of the OECD WNT on Defined Approaches for skin sensitisation at the JRC Ispra in December 2017.

Box 5.3

International guidance on good practice for the development and application of in vitro methods (GIVIMP)

The Organisation for Economic Cooperation and Development (OECD) has published guidance on Good *In Vitro* Method Practices (GIVIMP) to ensure the reliability and integrity of *in vitro* data used for the safety assessment of chemicals.

The guidance document is intended to support method developers and end-users working in academic, industry or government laboratories across all 36 OECD Member Countries and beyond.

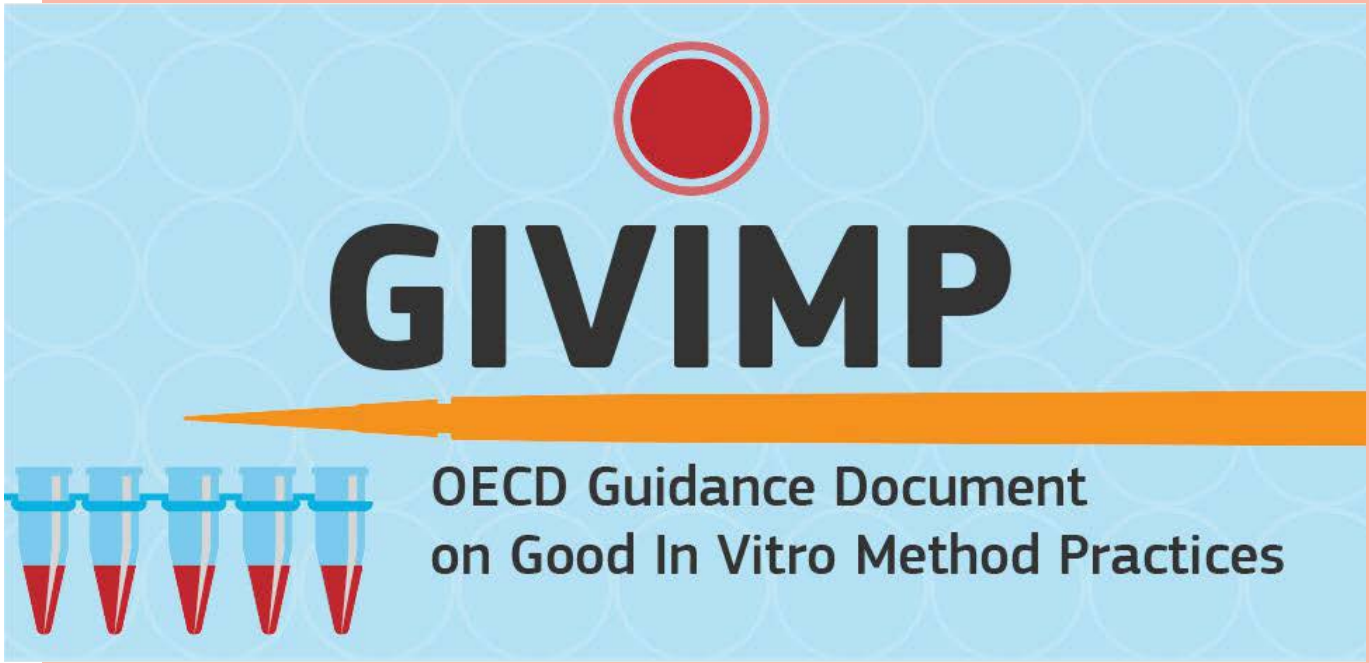
GIVIMP addresses key elements of good *in vitro* practice including:

- clear definition of roles and responsibilities,
- procedures for storing and handling cells and tissues,
- ways to prepare test items and avoid cross-contamination,
- defining and describing standard operating procedures and how to properly report results.

Modern *in vitro* methods are typically based on human cells or engineered tissues and are designed to identify potentially harmful effects of chemicals used in a variety of contexts including consumer goods, industrial processes and plant protection products. They are fast becoming key tools for a new way of doing toxicology without resorting to animal testing. Test data derived from *in vitro* methods are increasingly being used in combination with other information within Integrated Approaches to Testing and Assessment (IATA) to support safety decisions. However, good practices are essential to ensure that *in vitro* data can be trusted by industry end-users and regulatory authorities for the protection of workers, consumers and the environment.

Moreover, *in vitro* methods that undergo validation often require improvements in their design and implementation before they can be evaluated regarding their reliability and relevance for a particular regulatory purpose. Applying GIVIMP during the development of *in vitro* methods will help improve the quality of submitted methods and ultimately the efficiency of validation studies.

The development of GIVIMP was coordinated by EURL ECVAM within the context of a project of the OECD Test Guidelines Programme. A large number of international experts, including members of a dedicated OECD expert group and the European Union Network of Validation Laboratories (EU-NETVAL; see section 4.8) contributed to the state-of-the-art knowledge gathered within the guidance document. GIVIMP also benefitted from a number of written commenting rounds and two expert meetings before its final endorsement by the OECD's Working Party of National Coordinators of the test guidelines programme in April 2018.



The main activities include but are not restricted to:

- The analysis of the variability of the reference *in vivo* data (LLNA and human data),
- The analysis of the impact of the variability of the individual information sources composing the DAs on the DAs' predictions,
- A consistent definition of the DAs' applicability domain,
- The evaluation of how the DAs designed for potency categorisation predict GHS potency classes (cat 1A and cat 1B).

The results of these activities are informing the development of the draft guideline that was circulated for comments within the expert group and the WNT in Q4 of 2018.

Consideration is also given to the evaluation and inclusion in the draft guideline of DAs involving more sophisticated DIPs based for example on the use of neural or Bayesian networks. A meeting of the expert group is scheduled on 6 to 7 December 2018 at the OECD to discuss outstanding issues in view of incorporating the expert recommendations in the draft guideline. The aim is to reach consensus within the expert group on a final guideline to be submitted for approval by the WNT in 2019.

5.3.9 OECD Feasibility Study for Establishing Test Guidelines for In Vitro Human Hepatic Metabolic Clearance and Metabolite Formation

An OECD proposal was submitted by the Netherlands in November 2017 on "A feasibility study for establishing Test Guidelines for *in vitro* human hepatic metabolic clearance and metabolite formation". The project was approved in April 2018 and will be run under the lead of the Netherlands.

Although EURL ECVAM discontinued the OECD project on a guidance document for human hepatic metabolic clearance methods (see section 5.2.11 in Zuang *et al.*, 2017), it will contribute relevant background information to this new project, such as the components to be considered when characterising *in vitro* hepatic metabolic clearance methods, especially when employed for regulatory decision making (Gouliarmou *et al.*, 2018), as well as continue to participate in the OECD expert group on biotransformation assays.

5.3.10 OECD Developmental Neurotoxicity Project

Scientists in developmental neurotoxicity (DNT) from academia, regulatory bodies and industry, across the world, reached an overall consensus that current data requirements for *in vivo* DNT testing are not sufficient to adequately screen and characterise compounds potentially hazardous for the developing brain. They proposed to apply human *in vitro* DNT testing which together with *in silico* approaches will in the future allow development of predictive models for human DNT effects. This consensus statement (Fritsche *et al.*, 2018) and 18 other papers outlining novel concepts (*i.e.*, AOP, IATA, ontology-driven animal-free testing) as well as a range of alternative approaches (*i.e.*, *in vitro*, zebrafish, *C. elegans*, *in silico*, a systems biology approach), have recently been published in a DNT special issue of Toxicology and Applied Pharmacology entitled Alternative Approaches to Developmental Neurotoxicity Evaluation under an editorial lead of EURL ECVAM and the Leibniz Research Institute for Environmental Medicine in Düsseldorf (Germany; Fritsche & Bal-Price, 2018).

EURL ECVAM together with international partners (EFSA, OECD, US EPA, academia) is developing a strategy with a focus on a battery of in vitro methods, preferably derived from human induced pluripotent stem cells

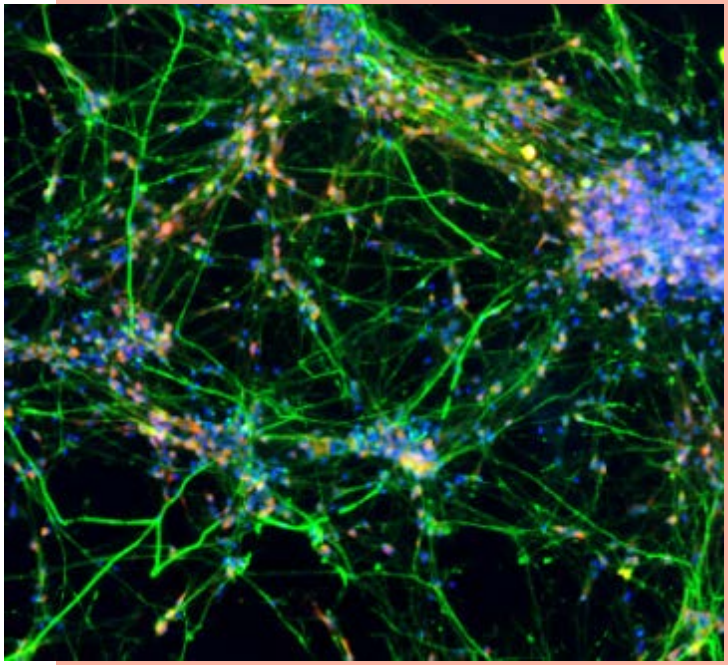
EURL ECVAM together with international partners (EFSA, OECD, US EPA, academia) is developing a strategy with a focus on a battery of *in vitro* methods, preferably derived from human induced pluripotent stem cells (Bal-Price *et al.*, 2018a). These methods permit evaluation of a chemical impact on critical neurodevelopmental processes, mimicking different windows of human brain development. Recently, readiness of these *in vitro* DNT assays for different regulatory purposes has been evaluated following thirteen criteria established by DNT experts, including JRC-EURL ECVAM scientists (Bal-Price *et al.*, 2018b).

Available *in vitro* assays should be incorporated into IATA together with other sources of information (*e.g.*, *in silico* methods, non-mammalian models as well as existing animal and human data). IATA development should be driven by a problem formulation and a battery of assays should be established based on 'fit-for-purpose' principles, delivering data for hazard identification/characterisation or risk assessment. Such approach has been taken on board by the OECD project that aims to develop an OECD Guidance Document on *in vitro* methods for DNT testing. This project, co-led by EFSA, EC (through JRC-EURL ECVAM) and US EPA, was included in the OECD work programme in 2017 and a GD will be developed.

Box 5.4

Improving chemical testing approaches for developmental neurotoxicity assessment

Currently there is only very limited information on potential developmental neurotoxicity (DNT) effects of the many thousands of chemicals that we come into contact with in our daily lives. Experts on DNT from the JRC, US EPA, OECD and academia reached a consensus that this issue cannot be overcome with current regulatory testing approaches which are based solely on conventional animal tests. This is because animal models for DNT poorly reflect key features of human biology and disease and are typically very expensive and time consuming to conduct. Children are particularly vulnerable to DNT related health effects. Recent studies have clearly shown an increase in the prevalence of conditions such as autism, learning disabilities, attention deficit and hyperactivity. While these disorders are due to a combination of multiple causes including inherited predisposition, environmental stressors and factors linked to socioeconomic status, both laboratory and human studies indicate that exposure to hazardous chemicals can contribute significantly to DNT related effects.



Recently, scientists from the EURL ECVAM and the Norwegian Institute for Public Health came together for a workshop to discuss the Children's health and exposure to environmental chemicals (see section 2.12). A particular focus was on the integration of multiple sources of information for DNT assessment derived from biomonitoring campaigns, epidemiological studies, computational models and *in vitro* methods. EURL ECVAM scientists propose to use mechanistic knowledge described in Adverse Outcome Pathways (AOPs) to rationally combine diverse but complementary data in the context of Integrated Approaches to Testing and Assessment (IATA).

Such IATA will address various regulatory needs including chemical screening and prioritisation, hazard identification and characterisation and the safety assessment of combined exposure to multiple chemicals (*i.e.*, the 'mixtures' challenge). It is strongly believed that an application of currently available human-relevant *in vitro* models derived from induced pluripotent stem cells combined with other proposed non-animal approaches will permit development of predictive models for DNT effects for better protection of pregnant women, infants and children exposed to environmental chemicals.

Thus EURL ECVAM has been working with the DNT community to define a set of criteria that can be applied to judge the readiness of currently available *in vitro* methods for regulatory application (see section 5.3.10 and annex 2).

5.3.11 OECD Guidance Document on IATA for Non-genotoxic Carcinogens

Non-genotoxic carcinogens contribute to an increased cancer risk through a variety of mechanisms that are not yet directly included in international regulatory approaches. To address this need, an OECD expert working group was set up to develop an IATA on non-genotoxic carcinogens and to identify mechanistic assays which can be used to cover specific cellular and molecular events involved in the development of tumours (Jacobs *et al.*, 2016). Among other activities, the working group has so far conducted an analysis of uncertainty in the rodent cancer bioassay (Paparella *et al.*, 2017) and is expanding it to novel approaches. The main objective of this latter analysis is to transparently illustrate the complexity associated with the *in vivo* approach currently used and its actual performance in order to support the development of acceptance criteria for future novel approaches and IATA.

One of the main achievements of this year was the agreement on a backbone structure of the IATA, which will serve to organise the available assays into levels of testing. So far, about 50 assays (mainly *in vitro*) have been collected. The level of readiness of these assays will be evaluated by the working group following

well defined and agreed assessment criteria. A preliminary analysis has identified assay gaps in the area of immunotoxicity/immunosuppression, however a further in-depth analysis will need to be conducted to identify further gaps in the database.

5.3.12 OECD Detailed Review Paper on the Miniaturised Ames Test

An OECD expert working group on the development of the miniaturised Ames test was established in November 2016 with the aim to conduct a retrospective analysis of data obtained from the different miniaturised versions of the Ames test, and a comparative evaluation with Ames results from the classic test method. The ultimate aim is to draft a Detailed Review Paper (DRP) for an eventual incorporation of these methods into an updated version of the Ames test in OECD TG 471. Several miniaturised versions of the Ames test are used routinely as early screening methods in product development. The added value for the miniaturised versions of the test is mainly based on significant reduction of test material, on reduction of costs, and on the possibility for simultaneous analyses of a large number of samples. The inclusion of these versions of the Ames test in an updated version of the TG would allow more regulatory acceptance and integration of the test in genotoxicity testing.

Based on a decision from the kick-off meeting held in February 2017, the expert working group first launched an exploratory survey (May 2017) in order to obtain better insights in the use of the miniaturised Ames tests, the amount of data available for the different tests, and the extent to which these data can ultimately be shared. The results of the exploratory survey have served the call for actual data sharing that was opened in March 2018. In this context, EURL ECVAM extended the exploratory survey to its EU-NETVAL and followed up the request of data with the 37 Members of EU-NETVAL.

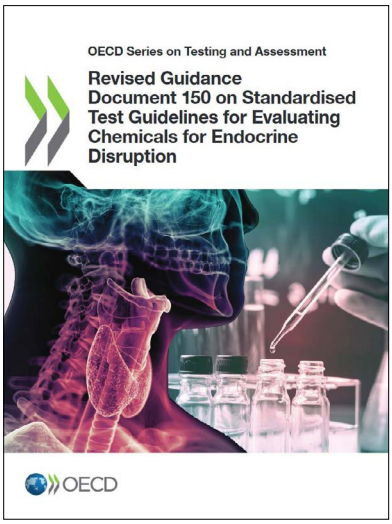
Several miniaturised versions of the Ames test are used routinely as early screening methods in product development

The expert working group worked on the definition of criteria for the evaluation of data (limit recommendations, reproducibility, correspondence with standard Ames results, use of historical data, etc.) and conducted a preliminary review of data received and a check for completeness. So far, 18 companies have provided data for more than 200 unique substances, plus 150 coded compounds tested through various versions of the miniaturised Ames tests. Data analysis is foreseen to be completed by first quarter of 2019 in view of the DRP drafting which is running in parallel and in preparation of the request (SPSF) for eventual OECD TG 471 revision.

5.3.13 OECD Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption

The OECD Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption was first published in 2012. It provides guidance on the interpretation of assays with respect to indications of endocrine disruption in order to support regulatory decision-making on the hazard of specific chemicals and toxicologically-relevant metabolites. In particular, it provides guidance on how to interpret the outcome of individual tests and how to strengthen the weight of the evidence on whether or not a substance may be an ED.

OECD GD 150 provides guidance on how to interpret the outcome of individual tests and how to strengthen the weight of the evidence on whether or not a substance may be an ED



Within this GD, the Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals lists the OECD TGs and standardised test methods available, under development or proposed, that can be used to evaluate chemicals for endocrine disruption. New methods that have been developed and validated or updated since 2012 have been added to the GD. Moreover it is now considering endocrine modalities beyond estrogens, androgens, thyroid and steroidogenesis, namely juvenile hormone, ecdysone

or retinoid modalities. Finally, the revised GD includes considerations on cross-species extrapolations, multiple modes of endocrine action, use of weight of evidence and adverse outcome approaches, and presents regulatory experience of assessment of endocrine disrupting chemicals from various regions of the world.

The revised version of the GD 150 has been adopted by the WNT in April 2018 and published (OECD, 2018d).

5.3.14 Detailed Review Paper on the Retinoid System

Under the co-lead of EC and Sweden, a DRP on the retinoid system is being drafted. The focus on the retinoid system responds to the need to extend our knowledge on endocrine disruption beyond the estrogen, androgen, and thyroid hormone signalling pathways. The document will describe the general background of the retinoid system, including cross-species comparisons, and focuses on the role of retinoids within various organs or systems. It will also include some information on tests and markers relevant to retinoid disruption with potential relevance for regulatory testing.

5.3.15 Update of OECD TG 408 on the Repeated Dose 90-day Oral Toxicity Study and TG 414 on the Prenatal Developmental Toxicity Study

In 2018, two OECD *in vivo* Test Guidelines have been updated to include endpoints relevant to endocrine disruption. This activity embraces the 3Rs since more information can be obtained from the same number of animals.

The repeated dose 90–day oral toxicity study in rodents (TG 408) has been updated to include: the measurement of thyroxine (T4), triiodothyronine (T3), thyroid stimulating hormone (TSH) and thyroid gland weight. In addition, the measurement of serum total cholesterol, low-density lipoproteins (LDL) and high-density lipoproteins (HDL) are also now required, as these parameters are directly controlled by thyroid hormone action and contribute (with other thyroid endpoints) to evidence of thyroid effects. Optional endpoints include other hormone measurements and assessments of sperm parameters.

The prenatal developmental toxicity study (TG 414) was updated by inclusion of the measurement of anogenital distance in all foetuses, and, in dams, the measurements of thyroid hormones levels (T3, T4 and TSH), thyroid weight and histopathology, and (optionally) measurements of other hormones if relevant. It should be noted that the newly added requirements are rat-specific (thus do not apply to rabbits).

5.3.16 Development of an OECD Guidance Document on Good Licensing Practices for Intellectual Property Elements in OECD Test Guidelines

A new project proposal for the development of a guidance document describing principles on good practices for the availability and distribution of protected elements in OECD TGs was approved at the 30th WNT meeting in 2018. It follows up on the recommendation of an OECD expert workshop on IPR held in September 2017 (OECD, 2018o), that more guidance, transparency and communication are needed for protected elements in new technologies included in OECD TGs.

The document explains the process and documentation needed by the TG Programme at various steps of TG development. Moreover, it includes the principles and conditions for accepting protected elements in TGs, and lays out the conditions to meet for the distribution and availability of the protected elements, and for the development of similar methods based on Performance Standards. The draft GD underwent a first commenting round by the WNT in October 2018.

More guidance, transparency and communication are needed for protected elements in new technologies included in OECD TGs

READ MORE

- Intellectual Property elements in OECD Test Guidelines: www.oecd.org/chemicalsafety/testing/intellectual-property-in-oecd-test-guidelines.htm

5.3.17 OECD Harmonised Templates

The OECD Harmonised Templates (OHTs) for reporting chemical test summaries are standard data formats which are publicly available. They are designed to report information used for the risk assessment of chemicals, mainly studies done to determine the chemical properties or effects on human health and the environment, and also (since 2016) to describe their use and related exposure to workers, consumers and the environment.

The 2017/2018 OECD revision of the OHTs included a series of updates, technical improvements and completions identified by OECD and template users over the past years. The changes contributed to harmonising the elements considered, the closed vocabulary and the terminology used in the OHTs. The template format

was simplified, and new templates made available including the one on intermediate effects (OHT 201), which was developed by the JRC-EURL ECVAM, by applying its in-house knowledge (predictive toxicology, toxicity pathways and assay validation), and involving ECHA as well as external stakeholders. This OHT on 'Intermediate effects' allows reporting of non-apical observations during *in vitro* testing, *e.g.*, intermediate effects at molecular, subcellular, cell, tissue or organ level which can be relevant when studying the hazard posed by a compound (and possibly inform the adverse outcome pathways).

The 2017/2018 OECD revision of the OHTs included a series of updates, technical improvements and completions identified by OECD and template users over the past years

The JRC-EURL ECVAM is currently collaborating with the OECD and ECHA to endorse the use of OHT 201 for all *in vitro* test results, in particular those that are currently stored in less suitable "apical" (*in vivo* test–oriented) templates. This will make access and retrieval of *in vitro* test results easier for the AOP community and is intended to bridge the gap between chemical-agnostic AOPs and real-life manifestations of the adversity described with them. Release of an updated OHT 201 is expected for 2019.

READ MORE

- OECD Harmonised Template 201: www.oecd.org/ehs/templates/harmonised-templates-intermediate-effects.htm

5.3.18 eChemPortal

The OECD eChemPortal provides free public access to information on chemical properties and direct links to collections of information prepared for governmental chemical review programmes at national, regional, and international levels. Access to information on existing chemicals, new industrial chemicals, pesticides and biocides is provided. eChemPortal also makes available national/regional classification results according to national/regional hazard classification schemes or according to the Globally Harmonised System of Classification and Labelling of chemicals (GHS). In addition, eChemPortal provides also exposure and use information on chemicals.

The JRC-EURL ECVAM is a member of the Steering Group for the management and further development of the eChemPortal, and as such provides expertise in both the subject matter (*e.g.*, chemical risk assessment and study results) and the Information and Communications Technology (ICT) aspect.

In 2018, a series of technological improvements behind the scenes were decided and implemented (most importantly, a better and faster search), and the establishment of a Joint IUCLID Dissemination Portal was initiated, which will make it easier for JRC IUCLID-based DBs to become eChemPortal participants. IUCLID stands for International Uniform Chemical Information Database and is a software application to capture, store, maintain and exchange data on intrinsic and hazard properties of chemical substances. Distributed free of charge, the software is useful in particular to chemical industry companies and to government authorities, *e.g.*, IUCLID is the key tool for chemical industry to fulfil data submission obligations under REACH.

READ MORE

- eChemPortal: www.echemportal.org
- IUCLID: iuclid6.echa.europa.eu

5.4 Activities in the Extended Advisory Group on Molecular Screening and Toxicogenomics

5.4.1 Update on the AOP-Knowledge Base

AOPs are the central element of a toxicological knowledge framework being built to support chemical risk assessment based on mechanistic reasoning. To enable the scientific community to share, develop and discuss their AOP-related knowledge in one central location, the OECD has, in parallel to the instigation of the overall AOP initiative, started the AOP-Knowledge Base (KB) project (see fig. 5.4). Within this project EURL ECVAM contributes to ICT design and analysis know-how, and co-manages the project together with the US EPA. AOP-KB consists of two modules, each tailored to specific needs; a third module titled e.AOP.Portal is the uniform search interface to retrieve AOPs from the other two; the data interchange format to be used between the AOP-KB modules, named AOP-XML, was developed by EURL ECVAM.

The first AOP-KB module available to the public was the AOP-KB Wiki, a system that organises, via

crowd-sourcing, the available knowledge and published research into a verbal description of individual pathways, via a user friendly wiki interface. Controlled-vocabulary drop-down lists from which to select methods, actions, biological objects, life stages, species, etc., related to the AOP had already simplified the entry of standardised information.

The introduction of ontologies to further harmonise the naming of AOP objects was implemented as a next step in the AOP-KB Wiki. While the AOP-Wiki had always combined both free text fields and closed vocabulary to describe mechanistically linked Key Events (KEs) and their Relationships (KERs), a new version of the Wiki (version 2.0) came with a full implementation of the ontology concept. The ontology-based annotations to AOP-KB objects are aimed to facilitate and improve the systematic re-use of KEs by minimising redundancy and also allowing for more flexibility in naming the KEs according to their AOP-specific context. Ontologies as computer readable descriptions of the biological components will drive the possibility to find connections and make inferences between KEs at different levels of granularity, thereby facilitating the automatic development of AOP networks. Overall, the introduction of a controlled terminology in the AOP-Wiki represents a significant advancement for the progress and optimisation of the AOPs and it will ultimately be beneficial also for other modules of the AOP-KB.

Overall, the introduction of a controlled terminology in the AOP-Wiki represents a significant advancement for the progress and optimisation of the AOPs

The introduction of the AOP concept into the area of chemical risk assessment is a major milestone towards the goal of identifying, assessing and ultimately accepting alternatives to animal tests for regulatory purposes. Without the AOP-KB tool, the AOP concept would remain a theoretical idea without any real-life impact. By facilitating the collection and also discussion of AOP-related information, the AOP-KB anchors this novel concept firmly in the scientific and regulatory environments, which is a prerequisite for a world with less animal testing. The new concept of IATA profits from the AOP

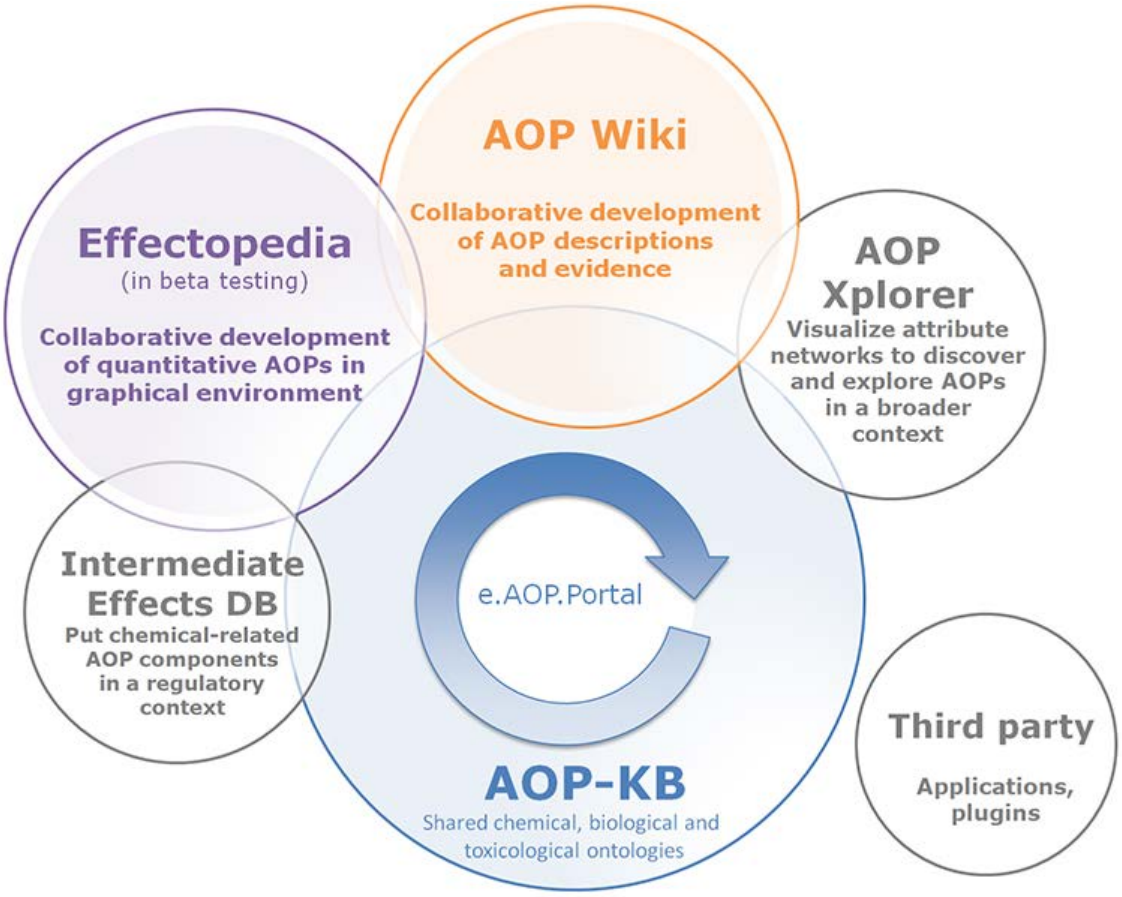


Figure 5.4 The Adverse Outcome Pathway Knowledge Base (AOP-KB).

concept, as it informs scientists and regulators about the biology behind a chemical's mode of action which needs to be modelled in *in vitro* and *in silico* methods.

While the current version of AOP-KB is fully functional and well maintained, in 2018, the decision was taken to redesign the knowledge base with a view to better accommodating knowledge users (as opposed to knowledge providers) needs and to thereby further promoting the AOP framework as such. Release of an updated AOP-KB 2.0 is expected in the coming years.

READ MORE

- e.AOP.Portal: aopkb.oecd.org
- AOP Wiki: aopwiki.org
- Adverse Outcome Pathway Knowledge Base (AOP-KB): aopkb.oecd.org/index.html

5.4.2 Development of Adverse Outcome Pathways Relevant to Neurotoxicity

EURL ECVAM was involved in the development of an AOP entitled "Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits" in collaboration with EFSA which has been endorsed by WNT/WPHA and published by OECD (Bal-Price *et al.*, 2018c).

An AOP related to the disruption of the synthesis of thyroid hormone entitled "Inhibition of Na+/I- symporter (NIS) leads to learning and memory impairment" passed an external OECD reviewing process and soon it will be forwarded to the OECD's WNT/WPHA for a final endorsement.

AOP 13 entitled "Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities" already endorsed by OECD in 2017 has been published in an open access format in Toxicology and Applied Pharmacology DNT special issue (Sachana *et al.*, 2018). This AOP has been used as the backbone of a project of the University of Lausanne (Department of Physiology) where quantitative measurements of Key Events Relationships identified in this AOP will be performed using brain aggregates derived from Human induced Pluripotent Stem Cells (hiPSCs, a project supported by Cefic). Most of the current AOPs are comprised of qualitative pathway descriptions, and further experimental work is required to develop the response-response relationships between key events in quantitative terms. These AOPs follow publicly available conventions adopted in the OECD AOP development programme to permit tailored application of AOPs for a range of different regulatory purposes.

READ MORE

- AOP 3 - Mitochondrial dysfunction and Neurotoxicity: aopwiki.org/aops/3
- AOP 54 - NIS inhibition and learning and memory impairment: aopwiki.org/aops/54
- AOP 13 - Binding of antagonist to NMDARs impairs cognition: aopwiki.org/wiki/index.php/Aop:13

5.4.3 Transcriptomics Reporting Framework

The lack of standardised reporting frameworks was recognised among the obstacles that limit the regulatory uptake of 'omics data (Buesen *et al.*, 2017). For this reason, under the auspices of the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST), a project was initiated, which aims to develop frameworks for the standardisation of reporting of 'omics data generation, and analysis, to ensure that all information required to understand, interpret and reproduce an 'omics experiment and its results are available. The project will initially focus on the development of a guidance document for reporting of transcriptomics data. Similar activities for metabolomics and proteomics will follow.

A working group is already working on the transcriptomics reporting framework (TRF) which will have a modular structure. It will include an introductory and an experimental module that will be independent of the 'omics platform used. These will be followed by 'omics' platform-dependent modules on the description of sample processing procedures, methods used to collect raw data and methods used to generate processed data and identify differentially expressed genes. Modules for more complex downstream analyses will also be drafted.

Under the auspices of the EAGMST, a project was initiated, which aims to develop frameworks for the standardisation of reporting of 'omics data generation, and analysis

For the experimental module, the working group, chaired by EURL ECVAM, will pay particular attention to align it with other existing OECD documents. Finally, a round-robin case study will be conducted to evaluate the utility of the TRF in fostering reproducibility of 'omics data analyses by different research groups.

5.5 VICH Guideline on Vaccines: Harmonisation of Criteria for Waiving of Laboratory Animal Batch Safety Testing of Vaccines for Veterinary Use

The requirements on batch safety testing differ between the various geographic regions. For example, general safety tests for batch release of human and veterinary vaccines are no longer required in Europe and were deleted from European Pharmacopoeia monographs several years ago (abnormal toxicity test; Schwanig *et al.*, 1997) or recently (target animal batch safety test; EDQM, 2012). Since these tests may still be required outside of Europe, European manufacturers may need to carry out these tests when exporting to third countries.

For many years, EURL ECVAM has been working on behalf of EMA with experts of the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) on the development of VICH guidelines on harmonisation of criteria to waive the target animal /laboratory batch safety testing for vaccines for veterinary use (see section 5.4 in Zuang *et al.*, 2017). Since May 2018, two VICH guidelines are in force that establish criteria for waiving the target animal batch safety test for inactivated (VICH GL50(R); VICH, 2017a) and live vaccines (VICH GL55; VICH, 2017b). A third guideline is under development aiming at the harmonisation of criteria to waive the general batch safety test in laboratory animals (*e.g.*, abnormal toxicity test) for veterinary vaccines.

Since May 2018, two VICH guidelines are in force that establish criteria for waiving the target animal batch safety test for inactivated and live vaccines

READ MORE

- Two new guidelines for the reduction of animal tests for quality control of veterinary vaccines – News item: europa.eu/!CN98kT

5.6 Promoting Regulatory Acceptance in the Frame of EMA: J3Rs Working Group

The Joint Committee for Medicinal Products for Veterinary Use (CVMP)/Committee for Medicinal Products for Human Use (CHMP) Working Group on the Application of the 3Rs in Regulatory Testing of Medicinal Products (J3Rs WG) provides advice to the CVMP and the CHMP on all matters concerning the use of animals in regulatory testing of medicines with particular focus on the application of the 3Rs principles. J3Rs WG, formerly established by EMA in 2016 as successor of the JEG 3Rs, consists of a core group of European experts from each of the existing CVMP and CHMP Working Parties for which animal testing is relevant, experts on 3Rs and representatives from EDQM and the European Commission (*e.g.*, EURL ECVAM). In this context, EMA has created a new section on its website on the ethical use of animals in the testing of medicines which describes the various activities and provides links to collaborators and stakeholders.

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The first biennial report of the J3Rs WG was published in 2018 (EMA, 2018a). It summarises the mandate of J3Rs, the workplan for 2018-2019 and refers to new publications, *e.g.*, a guidance aiming at vaccine quality control methods and use of data generated in collaborative trials for product-specific validation (EMA, 2017) or two reflection papers providing overviews of the current regulatory testing requirements for medicinal products for veterinary/human use and opportunities for implementation of the 3Rs (EMA, 2018b,c). In June 2018, J3Rs WG organised training on 3Rs for assessors of veterinary vaccines within the framework of the EU Network Training Centre (EU NTC), which is an initiative set up by the Heads of Medicines Agencies (HMA) in the EU Member States and EMA. The training was hosted by the Danish Medicines Agency.

READ MORE

- Ethical use of animals in medicine testing: www.ema.europa.eu/human-regulatory/research-development/ethical-use-animals-medicine-testing
- EU Network Training Centre (EU-NTC): www.hma.eu/otsg.html

5.7 Activities of EPAA to Promote the Regulatory Acceptance of Alternative Methods

The European Partnership for Alternative Approaches to Animal Testing (EPAA) is a public-private collaboration between the European Commission, European trade associations and companies from seven business sectors. The partners are committed to pooling knowledge and resources to accelerate the development, validation and acceptance of alternative approaches to animal use in

regulatory testing. The overall aim is the replacement, reduction and refinement (3Rs) of animal use in regulatory testing. JRC, represented by EURL ECVAM, is one of the Commission services that are members of the EPAA.

In recent years, EPAA focussed mainly on the promotion of regulatory issues and user acceptance. In this context, EPAA runs a number of projects that are described in the sections below. The partnership also engages in training and dissemination activities (see section 6.5). An overview of the EPAA activities in 2017 is given in the EPAA Annual Reports that are publicly available online, the most recent one covering the year 2017.

© EPAA

A former EPAA activity regarding the consistency approach for various vaccines set the ground for the current Horizon 2020 project "VAC2VAC - Vaccine batch to vaccine batch comparison by consistency testing" (see section 2.5).

READ MORE

- EPAA, European Partnership for Alternative Approaches to Animal Testing: europa.eu/!rq76qf
- EPAA Annual Report 2017: europa.eu/!tk44bD

5.7.1 Optimised Evaluation of Skin Sensitisation

3D skin tissue models provide a more realistic representation of the skin structure and organisation compared to 2D cell cultures and they have the potential to overcome some of the drawbacks of the aqueous medium-based cell culture models, for example those associated with the testing of hydrophobic and other "difficult to test" substances.

In December 2015, the EPAA skin sensitisation team launched a collaborative project aimed at gaining a better understanding on the performance of three methods based on 3D skin models (Reconstructed human Epidermis (RhE) IL-18 test method, SensCeeTox and SENS-IS) for predicting skin sensitisation hazard and potency categorisation by testing a set of 12 difficult substances, selected for their relevance to industry. In 2018, the testing was finalised and the results were presented at the EUROTOX 2018 congress. A peer-reviewed publication reporting the outcome of the study is in preparation. Moreover, the team aims at continuing the series of workshops in partnership with Cefic LRI and Cosmetics Europe to share experience on the latest available methods and approaches, and their applicability or limitations in meeting regulatory safety requirements for skin sensitisation. The organisation of a new workshop to be held in the first half of 2019 at the ECHA premises has started.

The EPAA skin sensitisation team launched a collaborative project aimed at gaining a better understanding on the performance of three methods based on 3D skin models by testing a set of 12 difficult substances

READ MORE

- 54th Congress of the European Societies of Toxicology (EUROTOX 2018): www.eurotox-congress.com/2018

5.7.2 Waiving of the Two-year Carcinogenicity Studies

A new project co-chaired by scientists from the Dutch National Institute for Public Health and the Environment (RIVM), industry and EURL ECVAM was initiated last year. It represents a follow-up of the previous carcinogenicity project for pharmaceuticals. The latter showed that taking into account the results of the three to six month rat studies and the pharmacological properties of a pharmaceutical substance may lead to a reliable prediction of a carcinogen without the need for two-year carcinogenicity studies (van der Laan *et al.*, 2016). The present project aims at assessing whether the same approach can also be applied to agrochemicals, namely to provide evidence that data from 3-month repeated dose toxicity studies together with mechanistic-based parameters can be leveraged to predict human relevant carcinogenic potential of agrochemicals with reduced or no need for a two-year carcinogenicity study. Literature search for more than 400 agrochemicals is being conducted which should result in an overview of the main Modes of Actions (MOAs) involved in carcinogenesis triggered by these chemicals. In collaboration with industrial partners the proposed novel approach will be evaluated with

a couple of case studies. The results will be discussed and shared with experts from industry, academia and regulatory agencies during a dedicated workshop.

5.7.3 Acute Toxicity

As described in the previous EURL ECVAM status report (see section 5.6.3 in Zuang *et al.*, 2017), a data mining exercise is carried out as part of the EPAA acute toxicity project, which aims to identify clinical signs predictive of mortality at higher dose levels. The ultimate goal is to propose an animal-free decision framework for acute systemic toxicity testing. Nevertheless, in those cases where animal usage cannot be avoided, the intention is to recommend the replacement of death as an endpoint by clinical signs predictive of mortality.

The project, initiated in 2015, is done in close collaboration with the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and the UK Chemicals Regulation Directorate (CRD). A large number of studies have been coded and analysed against the acceptability criteria. Only a few meet the criteria, making the final set of studies insufficient to support a robust statistical analysis. The team is currently discussing possible solutions to complete successfully the project.

5.7.4 Clostridial Vaccine Project

The EPAA clostridial vaccines group evaluated Vero cell based assays to replace the Minimum Lethal Dose and Total Combining Power assays required for in-process control of *Clostridium septicum* vaccines. The collaborative study was carried out in collaboration with the EDQM BSP and the outcome of study BSP130 was discussed with the study participants at a workshop in September 2015 (Sinitskaya *et al.*, 2016). The results show that the *in vitro* assays are repeatable and reproducible and that there is excellent overall concordance with the mouse tests. However, in order to fully exploit the advantage of the Vero cell assays, some further work is needed.

The results show that the in vitro assays are repeatable and reproducible and that there is excellent overall concordance with the mouse tests

A follow-up study (BSP130 III) with 14 participants was performed during 2016-2017 aiming at the further optimisation of the Vero cell assays to increase their sensitivity and accuracy. The final report of the study should become available in 2018 and a dissemination workshop is planned for 2020. Adaptations of the Vero cell assays to other clostridial vaccines is evaluated in the VAC2VAC project (see section 2.5).

READ MORE

- EDQM Biological Standardisation programme (BSP): www.edqm.eu/en/work-programme-bsp

5.7.5 Human Rabies Vaccine Project

The project aims at replacing the *in vivo* test carried out for batch potency testing of human rabies vaccines with an *in vitro* antigen quantitation test (*e.g.*, an enzyme-linked immunosorbent assay (ELISA) determing the glycoprotein-G content in rabies vaccines). Out of three different ELISAs used by manufacturers and control authorities, the most suitable ELISA has been identified (Morgeaux *et al.*, 2017) and is now validated under the umbrella of EDQM BSP. The BSP148 project "Validation of a rabies *in vitro* potency assay" was launched in 2017 and has attracted global interest during 2018.

READ MORE

- EDQM Biological Standardisation programme (BSP): www.edqm.eu/en/work-programme-bsp

5.7.6 Harmonisation on Biologicals

This EPAA project aims at progressing harmonisation of requirements for batch testing of vaccines and other biological products at a global level. The project team is following up the recommendations of a workshop held in 2015 (Schutte *et al.*, 2017) in collaboration with workshop participants and relevant stakeholders. For example, requests for revision on removal of the abnormal toxicity test from 49 monographs have been drafted by the Paul-Ehrlich Institute in Germany and presented to the European Pharmacopoeia Commission via the German Pharmacopoeia. In November 2017, the European Pharmacopoeia Commission endorsed the deletion of the abnormal toxicity test and agreed to the revision of the monographs (EDQM, 2017). Revised monographs will come into force in 2019. In collaboration with DG Health and Food Safety (DG SANTE), the group approached the World Organisation for Animal Health and references to the VICH GL50 and 55 (see section 5.5) have been included into the "Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2018".

At present, the group is evaluating the choice of methods for pyrogenicity testing, *e.g.*, test in rabbits, bacterial endotoxin test or monocyte activation test, and what the barriers to the use of the *in vitro* methods are.

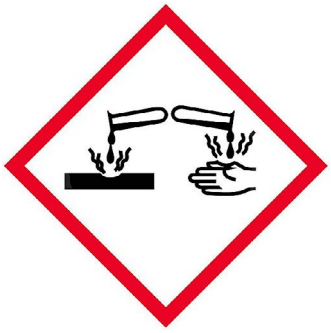
READ MORE

- Recommendations to delete or replace animal tests for quality control of biologicals - News item: europa.eu/!nR76yy
- Manual of diagnostic tests and vaccines for terrestrial animals 2018: www.oie.int/standard-setting/terrestrial-manual/access-online

5.8 UN subcommittee on Globally Harmonised System of Classification and Labelling of Chemicals (GHS)

5.8.1 Working Group on Introduction of Alternatives in the GHS Criteria

The UN sub-committee on GHS agreed to address the use of non-animal testing methods for classification of health hazards within its programme of work for the 2017-2018 biennium, and therefore the informal working group "Use of non-animal testing methods for classification of health hazards" was established, currently including about 50 members. Under the lead of NL and UK, the working group has re-drafted the current GHS chapter on skin corrosion/irritation to include non-animal methods, *i.e.*, internationally adopted *in vitro* OECD TG methods, as well as further elaborate the text on non-testing methods.



JRC through EURL ECVAM is contributing to the work of the group, and the aim is now to present a final revision of this chapter for adoption within this biennium. The plan is then to continue to look at serious eye damage/irritation and skin sensitisation, for which EURL ECVAM is preparing the discussions with support of NL and UK, respectively. Thereafter, it will be necessary to tackle the systemic health effects, which will be both interesting and challenging, as it might trigger the start to create a more efficient international system to evaluate chronic health hazards based on non-animal data and mechanistic knowledge.

5.8.2 United Nations Model Regulations for Transport of Dangerous Goods Subcommittee on Transport Packing Groups for Corrosion

At their summer 2018 session, the UN sub-committee on Transport of Dangerous Goods (TDG) discussed the proposal from the European Commission, presented by JRC EURL ECVAM, to update the text with regard to Test Guideline 431 "*In vitro* skin corrosion: Reconstructed Human Epidermis (RhE) Test Method" (2016), to allow for sub-classification in packing group 1, and recommended precautionary classification in packing group 2, if not possible to distinguish between packing group 2 and 3. TG 431 allows for separation into all three packing groups, but when revised at the OECD, there were not enough chemicals identified to belong to packing group 3 to enable validation of this sub-category.

The UN subcommittee is expected to adopt the revision related to TG 431 and thereby allow that the vast majority of corrosive chemicals can be classified based on in vitro data to ensure safe transport of chemicals worldwide

The UN subcommittee is expected to adopt the revision related to TG 431 at their December 2018 meeting, and thereby allow that the vast majority of corrosive chemicals can be classified based on *in vitro* data to ensure safe transport of chemicals worldwide.

5.9 Activities within the International Cooperation on Cosmetics Regulation (ICCR)

5.9.1 ICCR Joint Regulators-Industry Working Group on "Integrated Strategies for Safety Assessments of Cosmetic Ingredients"

Animal testing for the safety assessment of cosmetic ingredients and products has been completely banned in the EU. The safety of cosmetics therefore needs to be

ensured by new methods and strategies. This is in line with a general shift in toxicology from testing in whole organisms as a "black box" towards understanding the mechanisms behind adverse effects, allowing for a more informed and targeted evaluation of chemicals.

In Part 1 of a report (ICCR, 2017), the ad hoc ICCR Joint Regulators-Industry Working Group (JWG) on "Integrated Strategies for Safety Assessments of Cosmetic Ingredients" had outlined overarching principles that underpin the integration of novel methods and data for the safety assessment of cosmetic ingredients, or 'next generation' risk assessment, which have also been published in a journal Special Issue on "*In Silico* Approaches for the Safety Assessment of Cosmetic-Related Substances" (Dent *et al.*, 2018). The four main principles for an integrated strategy for risk assessment of cosmetics ingredients set human-relevant risk assessment and prevention of harm as overall goals, while basing the assessment on a mechanistic hypothesis and considering the exposure scenario. The other five principles describe how the assessments should be conducted and documented. EURL ECVAM has continued to contribute to the working group and writing of Part 2 of the report, which provides some additional guidance to safety assessors on the types of NAMs that may be used in a 'next generation' risk assessment. The report was adopted at the 12th ICCR Annual Meeting in Tokyo in July 2018 (ICCR, in preparation). The scope of NAMs considered in the report includes for example computational modelling approaches, read-across and exposure-based waiving, *in chemico* methods, *in vitro* testing in cell lines or 3D culture systems, organ-on-chip, 'omics or reporter gene assays.

Animal testing for the safety assessment of cosmetic ingredients and products has been completely banned in the EU

READ MORE

- International Cooperation on Cosmetics Regulation (ICCR): www.iccr-cosmetics.org
- Report from the Commission to the European Parliament and the Council on the development, validation and legal acceptance of methods alternative to animal testing in the field of cosmetics (2015-2017), COM/2018/531 final: europa.eu/!CG77Vc
- Computational Toxicology Special Issue "*In Silico* Approaches for the Safety Assessment of Cosmetic-Related Substances": www.sciencedirect.com/journal/computational-toxicology/special-issue/10P3BXBLJLK

5.9.2 ICCR Allergens II Joint Working Group

Both regulators and industry within ICCR agreed that the topic of allergens in the context of cosmetics remains of high importance. EURL ECVAM is supporting DG Internal Market, Industry, Entrepreneurship and SME (DG GROW) representation in the ICCR by contributing to the activities of the Allergens II Joint Working Group (JWG). The JWG is currently working on a report investigating how combination of non-animal methods recently adopted by OECD may be used within IATA to adequately substitute for animal tests in the evaluation of skin sensitisation potential. Once finalised and agreed by the ICCR, the report will be made publicly available on the ICCR website.

5.10 Activities with EC Directorate General Communications Networks, Content and Technology

In November 2017, EURL ECVAM participated at the DG Communications Networks, Content and Technology (DG CNECT) meeting on "Health, demographic change and well-being Workshop "*In-silico*" health new projects and regulatory needs", held in Brussels, Belgium. During this meeting seven projects (granted under H2020-SC1-2016-2017 personalised medicine, type of action Research and Innovation action (RIA): SC1-PM-16-2017 and SC1-PM-17-2017) were presented by the project coordinators. Out of these projects, EURL ECVAM was invited to two of the project kick-off meetings, and will follow these projects up to completion:

1. REPO-Trial: An *in silico*-based approach to improve the efficacy and precision of drug REPurpOsing TRIALs for a mechanism-based patient cohort with predominant cerebro-cardiovascular phenotypes. (21-23/02/2018).

2. StrituVad: *In Silico* Trial for Tuberculosis Vaccine Development (19-20/03/2018).

In connection to the topic of *in silico* medicine, EURL ECVAM was asked to contribute to a white paper on Computational Modelling and Simulation (CM&S) and medicinal products' lifecycle that the Avicenna Alliance Consortium presented at the European Parliament in

Box 5.5

International Cooperation on Cosmetics Regulation (ICCR)

The ICCR is a voluntary international group of cosmetics regulatory authorities from Brazil, Canada, the European Union, Japan, and the United States. This group of regulatory authorities meets on an annual basis to discuss common issues on cosmetics safety and regulation, and to enter into a constructive dialogue with their relevant cosmetics industry trade associations. The purpose of this multilateral framework is to maintain the highest level of global consumer protection, while minimizing barriers to international trade.

Brussels on 4 September 2018. This document aims at proposing cornerstones regulatory and ethical frameworks for CM&S in the life cycle of a new medicine.

READ MORE

- REPO-Trial: cordis.europa.eu/project/rcn/212939_it.html and repo-trial.eu
- StrituVad: cordis.europa.eu/project/rcn/212940_it.html
- International Avicenna Alliance Conference - 4 September 2018: avicenna-alliance.com/news/news/in-vivo-in-vitro-in-silico-why-computer-modelling-is-the-next-evolution-of-the-healthcare-sector

6 Dissemination of Information on Alternatives

6. Dissemination of Information on Alternatives

6.1 EURL ECVAM Databases

The dissemination of information about alternative approaches (*e.g.*, *in vitro* techniques and *in silico* models) and of chemical datasets contributes to the advancement and strengthening of the Three Rs knowledge. In this context, publicly accessible information systems not only can facilitate the engagement of the scientific community through the sharing and exploitation of existing data and information, but they can also inform regulators and assist educational and training activities. The EURL ECVAM coordinated information systems and services serve this purpose and are described in the following sections.

6.1.1 DB-ALM—EURL ECVAM's Database Service on Alternative Methods to Animal Experimentation

DB-ALM is an open-access public repository which provides information on the alternative (non-animal) methods in the field of biomedical sciences and toxicology. DB-ALM aims at facilitating the sharing of detailed and structured descriptions of alternative methods used in research and for regulatory purposes. Two different types of method descriptions are available:

- the method summary reviewing the objectives and applications of a method, the scientific rationale for its experimental design, its status of development and, when applicable, regulatory acceptance. The method summary format is compliant with the OECD Guidance Document No. 211 for Describing Non-Guideline *In Vitro* Test Methods (OECD, 2014d).
- the protocol providing a step-by-step documentation with technical details and supporting materials to enable the implementation of a method in a laboratory.

DB-ALM currently contains 180 method summaries and 167 protocols downloadable as PDF documents. The DB-ALM repository can be searched both via free text and by advanced search, which allows the construction of structured queries with keywords used for content annotation.

EURL ECVAM DATABASE SERVICE ON ALTERNATIVE METHODS TO ANIMAL EXPERIMENTATION

Initiatives to further facilitate access to the information about alternative methods are underway such as opening access to the DB-ALM content without the need to log in. In addition, an active link has been implemented to interlink DB-ALM with another EURL ECVAM hosted system, TSAR (see section 6.1.2).

READ MORE

- DB-ALM: ecvam-dbalm.jrc.ec.europa.eu

6.1.2 TSAR – Tracking System for Alternative Methods towards Regulatory Acceptance

TSAR allows to track in a transparent manner the progress of an alternative method, from the early phases of the validation process to its eventual inclusion into the regulatory framework (*e.g.*, EU legislation, OECD Test Guidelines or Guidance documents, European Pharmacopoeia, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), International Organisation for Standardisation (ISO) etc.).

TSAR, which is developed and hosted by EURL ECVAM, aims at disseminating information on methods that are considered by all the member organisations of the International Cooperation on Alternative Test Methods (ICATM) representing the EU, Canada, USA, South Korea, Brazil, China and Japan.

TSAR provides an overview of the status of a method, from being proposed for validation until the eventual regulatory acceptance. This overview is performed with the support of a colour-coding visualisation that shows whether the different steps of the process - submission, validation, peer-review, recommendations and regulatory acceptance - are ongoing or completed. For each TSAR record, relevant information, documents or links associated to each step are also provided. During the reporting period, a direct link to DB-ALM protocols has been introduced, where applicable, thus providing a more complete and easy-to-access documentation about the validated and regulatory accepted methods.

TSAR allows to track in a transparent manner the progress of an alternative method, from the early phases of the validation process to its eventual inclusion into the regulatory framework

READ MORE

- TSAR: tsar.jrc.ec.europa.eu

6.1.3 JRC QSAR Model Database

The JRC Quantitative Structure Activity Relationship (QSAR) Model database is freely available to users and developers and aims to provide users with scientifically valid QSARs that can be used to support the risk assessment of chemicals for regulatory purposes such as REACH. The database can be searched with free text, author names, chemical structures (including similarity to a specific structure), or title and QSARs can be freely downloaded. The developers of QSARs are welcome to submit their models to the JRC database using the QSAR Model Reporting Format (QMRF), which is an internationally agreed reporting format that facilitates the reporting of the five principles of the OECD for the validation of QSARs (OECD, 2007) and ensures harmonised reporting for all models. An editor for the compilation of QMRFs has been made available through the website.

The developers of QSARs are welcome to submit their models to the JRC database using the QSAR Model Reporting Format (QMRF)

During the reporting period of this Status Report, an entirely new JRC QSAR Model Database version has been released to enhance user interface and information display. The new database and QMRF also accept models for Nanomaterials. The database currently contains 147 QSAR models grouped according to OECD defined endpoints and covering physicochemical properties, environmental fate parameters, toxicokinetics, ecotoxicological and human health effects.

READ MORE

- QSAR Model Database: qsardb.jrc.ec.europa.eu/qmrf
- REACH Regulation: ec.europa.eu/environment/chemicals/reach/reach_en.htm

6.1.4 Open Access Datasets

In 2016, the JRC released its Data Catalogue which addresses, among others, the legal obligations from the European Commission's Reuse Decision (EC, 2011), which ensures that the European Commission as a producer and holder of public sector information sets an example by applying the same set of rules that it would like to be implemented at Member State level.

The JRC Data Catalogue therefore aims to complement the JRC Policy on Open Access to Scientific Publications and Supporting Guidance, and to promote open access to research data in the context of Horizon 2020.

The main purposes of the JRC Data Catalogue are to:

- Provide a central overview of data that is produced by the JRC (alone or in collaboration with third parties) and that can be shared with the public.
- Act as a single channel for automatically feeding the EU Open Data portal with relevant metadata to be published there.
- Contribute to the JRC's new role with respect to knowledge management.

In the reporting period, EURL ECVAM published five datasets/portals in the JRC Data Catalogue in its collection:

- ChemAgora (see section 6.1.4.1)
- EURL ECVAM Fish *In Vitro* Intrinsic Clearance Database (see section 2.7.4.1)
- EURL ECVAM Genotoxicity and Carcinogenicity Consolidated Database of Ames Positive Chemicals (see section section 6.1.4.2)
- Inventory of the 3Rs knowledge sources (see section 6.1.4.3)
- Supplementary materials of the Nanocomput project (Worth *et al.*, 2017b).

READ MORE

- EURL ECVAM collection in the JRC Data Catalogue: data.jrc.ec.europa.eu/collection/id-0088
- EU Open Data Portal: data.europa.eu/euodp/en/home
- ChemAgora: chemagora.jrc.ec.europa.eu/chemagora
- EURL ECVAM Fish *In Vitro* Intrinsic Clearance Database: data.jrc.ec.europa.eu/dataset/jrc-eurl-ecvam-fish-in-vitro-intr-clear-db
- EURL ECVAM Genotoxicity and Carcinogenicity Consolidated Database of Ames Positive Chemicals: data.jrc.ec.europa.eu/dataset/jrc-eurl-ecvam-genotoxicity-carcinogenicity-ames
- Inventory of the 3Rs knowledge sources: data.jrc.ec.europa.eu/dataset/jrc-eurl-ecvam-eurl-ecvam-3rs
- Computational models for the safety assessment of nanomaterials: data.jrc.ec.europa.eu/dataset/jrc-eurl-ecvam-nanocomput

6.1.4.1 ChemAgora

ChemAgora, the chemical information portal maintained by EURL ECVAM, facilitates the online retrieval of available information on a certain chemical substance. Chemicals can be searched by their name (or parts of it), CAS Registry Number, InChIKey or chemical structure in a series of public repositories. Making access to information on chemical substances easier across heterogeneous platforms raises the public awareness about chemical knowledge. Stakeholders in the chemical community can take more informed decisions when being fully aware of the information available about a certain substance, and people using ChemAgora have a head start when it comes to finding out many details about a chemical.

In 2017, a similarity search option based on the ChEMBL Data Web Services (with the choice among 90%, 80% and 70% Tanimoto similarity cut off) was added to the chemical structure editor page of the platform; in addition, following a request received from the National Center for Computational Toxicology of the US EPA, the EPA CompTox Chemistry Dashboard was added to the list of chemical resources searched by ChemAgora.

ChemAgora, the chemical information portal maintained by EURL ECVAM, facilitates the online retrieval of available information on a certain chemical substance

As a future improvement of the platform, the idea is under consideration to extend the search functionality adding the EC number used by ECHA. The ChemAgora search engine is currently used by the Information Platform for Chemical Monitoring (IPCHeM) exploiting the conversion done by ChemAgora from the CAS Registry Number – the chemical identifier used by the IPCHEM portal – to the InChIKey identifier.

In the reporting period, the Journal of Chemical Information and Modeling – a journal of the American Chemical Society – published a paper about ChemAgora (Zanzi & Wittwehr, 2017). The Chemical Abstract Service (CAS), which manages the *de facto* standard in chemical identification numbering, is a division of the American Chemical Society, and the publication there can be considered an acknowledgement of ChemAgora as a valuable resource (see box 6.1).

READ MORE

- ChemAgora: chemagora.jrc.ec.europa.eu/chemagora
- IPCHeM: ipchem.jrc.ec.europa.eu
- Chemical Abstracts Service (CAS): www.cas.org

Box 6.1

JRC portal ChemAgora simplifies access to chemical data

The JRC's ChemAgora web portal provides search capabilities to retrieve chemical data from a plethora of online resources enabling users to access both regulatory information on chemicals and public databases on chemical properties. ChemAgora is intended to support chemical risk assessment activities by assisting stakeholders to gain a quick overview of globally available data about chemicals they are interested in. This speeds up the process of data discovery and saves valuable resources.

ChemAgora, through an on-the-fly search, informs whether a chemical features in any of 17 external data sources or the OECD eChemPortal (featuring another 30 external sources), and provides clickable links leading to the third-party website pages containing the information. These third-party data sources contain regulatory and scientific chemical information and typically use the CAS Registry Number (CASRN, a registered trademark of the American Chemical Society) as the substance identifier. ChemAgora can also map InChIKeys to CASRNs, which bridges the gap between regulatory and scientific environments.

The ChemAgora portal has gained the recognition of the American Chemical Society (ACS) as highlighted in a recent ACS publication, "Journal of Chemical Modelling and Information" (Zanzi & Wittwehr, 2017).

6.1.4.2 EURL ECVAM Genotoxicity and Carcinogenicity Database of Ames Positive Chemicals

The EURL ECVAM Genotoxicity and Carcinogenicity Consolidated Database of Ames positive chemicals is a structured and highly curated database compiling available genotoxicity and carcinogenicity data for 726 Ames positive chemicals originating from different sources (Corvi & Madia, 2018; Kirkland *et al.*, 2014).

By using a harmonised format to gather the information, this database is representing a powerful resource for data analysis that is meant to be used to guide a thorough evaluation of genotoxicity and carcinogenicity: 1) as a resource for evaluating the predictivity of the Ames test for *in vivo* genotoxicity and carcinogenicity when considered alone or in association with *in vitro* mammalian cell assays (gene mutation and clastogenicity/ aneugenicity) and for a better characterisation of those cases where the Ames test leads to irrelevant ('false positive') results; but also, 2) as a platform for detailed structural characterisation of specific groups of compounds with or without carcinogenic or genotoxic activity. Inconsistencies (*e.g.*, contradictory data derived from different sources) and poor data quality have been addressed through rigorous curation which included expert peer review.

Since its launch, the EURL ECVAM Genotoxicity and Carcinogenicity Database represents a reference database for both the regulatory and scientific communities as demonstrated by its contribution to a number of activities (see section 6.1.5 in Zuang *et al.*, 2017). Recently, this dataset (9286 data points) was incorporated into the new version of the QSAR Toolbox that was released by OECD to support governments, chemical industry and other stakeholders in filling gaps in (eco)toxicity data needed for assessing the hazards of chemicals. It also contributed to the recently published EFSA Scientific Opinion on "Reflection on interpretation of some aspects related to genotoxicity assessment" where it has been the basis to conduct an analysis of the sensitivity of unscheduled DNA synthesis in transgenic and comet genotoxicity assays to detect carcinogens (EFSA, 2017).

The EURL ECVAM Genotoxicity and Carcinogenicity Consolidated Database of Ames positive chemicals is a database compiling available genotoxicity and carcinogenicity data for 726 Ames positive chemicals

The database is a living project with possibilities of continuous update as new genotoxicity and carcinogenicity data are made available. The database is currently being extended to include additional curated data from more than 200 new chemicals with Ames negative results.

READ MORE

- EURL ECVAM Genotoxicity and Carcinogenicity Consolidated Database of Ames Positive Chemicals: data.jrc.ec.europa.eu/dataset/jrc-eurl-ecvam-genotoxicity-carcinogenicity-ames
- QSAR Toolbox: www.oecd.org/chemicalsafety/risk-assessment/oecd-qsar-toolbox.htm

6.1.4.3 Inventory of Three Rs Knowledge Sources

In response to the European Citizens' Initiative (ECI) "Stop Vivisection", in 2015, the European Commission identified four actions to accelerate the development and uptake of non-animal approaches (EC, 2015).

Action 1 of the European Commission's response aimed to conduct an assessment of current technologies, information sources and networks from all relevant sectors with potential impact on the advancement of the Three Rs. To support this action the JRC's EURL ECVAM had undertaken a review to map Three Rs knowledge, determine how knowledge is shared and to identify opportunities to improve on the current situation. This review was performed by building an inventory of knowledge sources and carrying out a survey of the users of the knowledge sources. The outcome of the review was summarised in a study by Holley *et al.*, 2016 (see also section 5.2 in Zuang *et al.*, 2017), whilst in December 2017 the inventory comprising 800 knowledge sources relevant to the Three Rs was made publicly available (Holley *et al.*, 2017). More details on the inventory of 3Rs knowledge sources can be found in box 6.2.

The Three Rs knowledge sources inventory should be viewed as a snapshot of the status of knowledge sources available and as a starting point for further analysis of knowledge sharing strategies

The Three Rs knowledge sources inventory should be viewed as a snapshot of the status of knowledge sources available and as a starting point for further analysis of knowledge sharing strategies.

READ MORE

- ECI "Stop Vivisection": ec.europa.eu/citizens-initiative/public/initiatives/successful/details/2012/000007
- Inventory of the Thee Rs knowledge sources: data.jrc.ec.europa.eu/dataset/jrc-eurl-ecvam-eurl-ecvam-3rs

6.1.5 CheLIST

A key requirement for the development, characterisation and eventual validation of alternative (non-animal) methods for use in biomedical research and regulatory safety assessment is the availability of suitable reference or benchmark chemicals for which reliable structural, physicochemical and biological property data are available. However, the type of information needed to select such reference chemicals is typically scattered across a plethora of heterogeneous databases, project websites and peer-reviewed literature. To tackle this issue, EURL ECVAM manages and publishes the "Chemical Lists Information System" (CheLIST) that provides a means of identifying whether a chemical (or chemical group) has been tested in a major EU or international research project and whether the chemical appears on a specific regulatory inventory.

Information is provided on chemical identifiers (*e.g.*, name, CAS number) and chemical structure, and the database can be searched according to these types of information. The various datasets and inventories can also be compared in order to identify overlaps in chemical membership and to generate customised lists. All lists can be downloaded and the references provided for each list allow traceability back to the source. Using CheLIST, alternative methods can be developed faster as information about reference chemicals (for method validation) is available more easily.

In the reporting period CheLIST continued to grow, with EURL ECVAM monitoring the chemical programme landscape to identify more lists to add to CheLIST.

READ MORE

- CheLIST: chelist.jrc.ec.europa.eu

6.1.6 Endocrine Active Substances Information System (EASIS)

The revised 'Endocrine Active Substances Information System' (EASIS) uses the cloud based IUCLID 6 portal

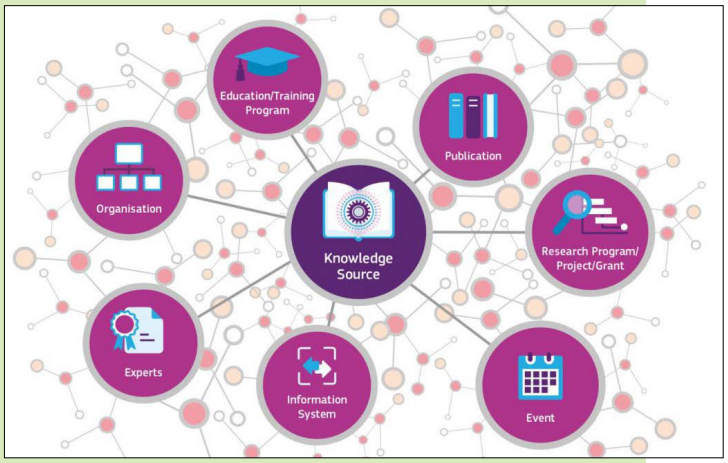
Box 6.2

Inventory of 3Rs Knowledge Sources published

A detailed inventory - compiled by JRC scientists - of 800 knowledge sources relevant to the Replacement, Reduction and Refinement (Three Rs) of animal procedures used for scientific purposes is now available online (Holley *et al.*, 2017). The JRC set out to assess the current situation regarding the sharing of knowledge relevant to the 3Rs with a mind to accelerate the development and uptake of non-animal approaches in research and testing. The findings of the report (Holley *et al.*, 2016) show that although much 3Rs knowledge exists, its sharing, particularly between sectors and communities can be improved through better coordination, communication and outreach, and by more emphasis on targeted education and training initiatives. As part of this review, these knowledge sources have been described in a way which facilitates their curation and the subsequent analysis of potential knowledge gaps and means of sharing. The inventory covers different types of knowledge sources (*e.g.*, organisations, events, expert groups, etc.) and identifies the ways in which these share information.

The knowledge sources considered for this inventory refer to any entity, tool or event that creates, collects, holds or disseminates knowledge with potential 3Rs relevance for the knowledge source types. The information captured in the inventory cover different knowledge sources with 3Rs relevance, including on-line information systems and specialised websites, experts groups, organisations, social media communities, professional associations and networks, conferences and workshops, industry initiatives and research programs.

Knowledge is captured in many different forms, so in addition to the more typical explicit knowledge sources (*e.g.*, on-line resources, publications and educational materials), other media used for knowledge dissemination and sharing, such as social media and scientific communities or 3Rs relevant research initiatives, which may contain more tacit knowledge, were also included. The data collection for the inventory took place over six months, from March to October 2016. Therefore, the inventory should be considered as a representative selection of the most visible 3Rs knowledge sources during the time of compilation. The methodology for establishing the inventory and the subsequent analysis can be found in the JRC Report 'Accelerating progress in the Replacement, Reduction and Refinement of animal testing through better knowledge sharing' (Holley *et al.*, 2016).



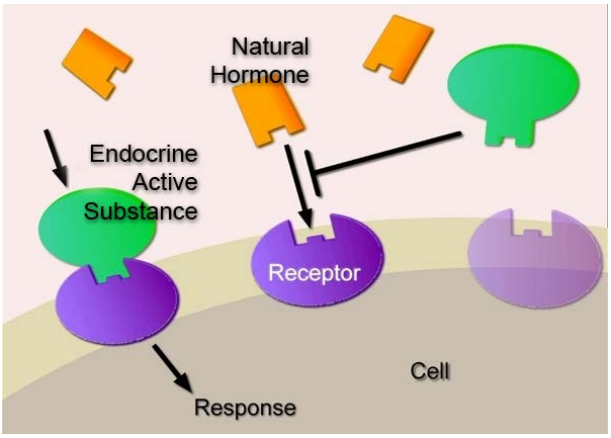
from ECHA. The term "Endocrine Active Substance" (EAS) describes any chemical that can interact directly or indirectly with the endocrine system, and subsequently result in an effect on the endocrine system, target organs and tissues. Whether the effect is adverse ("disruptive") or not, will depend on the type of effect, the dose and the background of the physiological situation (EFSA, 2010).

EASIS' web-based application is open to the public for query and review of results from scientific studies on chemicals related to endocrine activity or adverse effects (considered in relation to an endocrine disrupting mode of action). It deals generally with endocrine active substances, *i.e.*, not only with endocrine disruptors. The starting point was a database created by Directorate General Environment (DG ENV) in 2006. The new application, EASIS, was developed and the data from the DG ENV database were migrated into it. More data to cover the period after 2006 were added. There are currently data from 9246 studies across 541 compounds in EASIS, and the content is continuously increased. The focus of the current update is on pathways and chemicals that are not well covered by other databases/sources.

The data of the first web-based version of EASIS (launched in 2016) have been migrated from the phased-out IUCLID 5 to the new IUCLID 6 format

The data of the first web-based version of EASIS (launched in 2016) have been migrated from the

phased-out IUCLID 5 to the new IUCLID 6 format and new content is now being added directly in IUCLID format, which now also features a specific template for reporting *in vitro* observations. It is planned that the future evolution of EASIS will be in collaboration with ECHA to create an ECHA-JRC-OECD Joint Dissemination Portal (JDP) for non-REACH IUCLID data of which 'EASIS 2.0' will be an integral part.



READ MORE

- EASIS: ecs.echa.europa.eu/cloud/home.html

6.2 Knowledge Sharing Activities

6.2.1 Bridging Across Methods in the Biosciences (BeAMS)

The biosciences are at a critical point of development, with new innovative technologies constantly emerging, making possible new methods and techniques. Currently, we see many emerging technologies for *in vivo* experimental studies, *in vitro* and *in silico* studies. All of these technologies have their associated methods and techniques, which require high levels of specialisation and expertise on the part of researchers. Each innovation demands significant investment, both financial and in terms of researchers' time and effort, in order to ensure that it reaches its full potential and yields useful scientific results. However, there is also a risk that each will evolve within silos with little connection between them, and that opportunities to fully exploit the potential of methods will be lost.

BridgE Across Methods in the bioSciences (BEAMS) is a EURL ECVAM initiative that aims to support greater connectivity between methods

Bridging Across Methods in the bioSciences (BeAMS) is a JRC-EURL ECVAM initiative that aims to support

Figure 6.1 Participants of the first BeAMS (Bridging Across Methods in the bioSciences) workshop, which was organised by JRC's EURL ECVAM in June 2018.

greater connectivity between methods. The focus is on how knowledge sharing can play a role and what form it should take. The BeAMS initiative started in June 2018 with a workshop of renown actors in the field (see fig. 6.1) and will lead to first tangible results in 2019, by exploring and identifying a science and innovation strategy underpinning meaningful cross-disciplinarity.

6.2.2 Feasibility Study on Indicators of Alternative Methods or Approaches to Animal Experimentation

In December 2017, EURL ECVAM launched a feasibility study on indicators of alternative methods and approaches to animal experimentation. The study is being carried out with the support of external experts and is aimed at investigating indicators to monitor the level of development and use of alternative non-animal methods in basic and applied life-science research, as well as for educational and regulatory testing purposes.

This one-year project is organised in three main phases covering:
1) the identification of suitable indicators including their selection criteria.
2) the characterisation of a pool of indicators among those identified in phase one, covering all the relevant features and data to be collected.
3) a demonstration exercise based on the implementation of a subset of characterised indicators showing the feasibility of the methodological approach.

Overall, this feasibility study and its outcomes could help to identify impactful trends and further foster the development and uptake of alternative methods and approaches by ultimately supporting informed policy decisions related to the EU Directive on the protection of animals used for scientific purposes.

6.3 Education and Training Activities

6.3.1 Review of 3Rs Education and Training Resources

Following a study (Holley *et al.*, 2016) carried out by EURL ECVAM in 2016 to identify how 3Rs knowledge is shared (see section 6.1.4.3 and box 6.2), it was concluded that education and training is key to the successful uptake of the principle in the use of animals for science and the development of alternative methods. In 2018, EURL ECVAM started preparation for projects that will map available resources and scope ways to develop guidance and tools for introducing 3Rs into the curricula of high schools, universities and continuing education (professional development) programmes. Using external expertise both in the areas of education and training and 3Rs, EURL ECVAM is undertaking the following projects.

Firstly, a study to review available education and training resources to provide an overview of opportunities currently offered (see box 6.3). The objective is to identify courses, modules, teaching materials, guidance, and other resources to form a snapshot view of how, where and to whom the 3Rs principles and alternative to animal approaches are currently being taught. The resulting inventory and conclusions from the mapping exercise will provide a foundation for the other education and training projects.

With support from the European Parliament through a Pilot Project (where EP resources are allocated to test the feasibility of certain approaches in a policy area), EURL ECVAM will start work on developing strategies for successful introduction of 3Rs in curricula at high school, university and professional levels. Findings during this work will also help identify some optimal tools that will facilitate the introduction of 3Rs in the classroom or online. As part of this project, the tools will be built and distributed to educational bodies and course providers. Under the same EP Pilot Project, EURL ECVAM is collaborating closely with DG Environment to engage experts to design and produce further eLearning tools. These modules will be freely available and will provide interactive instruction to students and professionals on tasks required under Directive 2010/63/EU on the protection of animals used for scientific purposes, enabling animal users to fully apply the 3Rs in their day-to-day work and employ best practices when developing alternative methods or when searching for alternative methods and approaches.

EURL ECVAM will start work on developing strategies for successful introduction of 3Rs in curricula at high school, university and professional levels

Finally, using JRC in-house expertise a project will start this year to explore the possibilities of a 3Rs virtual reality training programme, bringing students as close as possible to real-life learning situations.

6.3.2 Training on Uncertainty Characterisation in 21st Century Toxicology

Understanding, describing, communicating and, where possible, quantifying and reducing uncertainties is an

Box 6.3

Mapping education and training on the 3Rs

EURL ECVAM has launched a study to review available education and training resources that support the 3Rs approach: Replacement, Reduction and Refinement of animal procedures used for scientific purposes. This ambitious global study aims to provide an initial overview of education and training opportunities being offered at high school, university and professional levels.

The objective is to identify courses, modules, teaching materials, guidance, and other resources to form a snapshot view of how, where and to whom the 3Rs principles and alternative-to-animal approaches are currently being taught keeping in mind that many such initiatives might not be "3R labelled".

The review is expected to be completed during 2019 and the information gathered will be made publicly available. It is expected that the results obtained will help to identify opportunities, to boost 3Rs education and training and to target initiatives and investment to accelerate progress. The study is an important step in efforts to advance the development and uptake of 3Rs approaches in research and safety testing and is also a follow-up to Action 1 of the Commission's Communication in response to the European Citizens' Initiative "Stop Vivisection".

In 2016 the JRC carried out a study to build an inventory of 3Rs knowledge sources and to identify how 3Rs knowledge is shared (Holley *et al.*, 2016; see also section 6.1.4.3 and box 6.2). Findings demonstrated that although much 3Rs knowledge exists, its sharing can be improved especially between different fields of expertise through better coordination, communication and outreach, and by more emphasis on targeted education and training initiatives. This, together with the current study and survey establishes the follow-up to Action 1 of the Commission's Communication in response to the European Citizens' Initiative "Stop Vivisection" (EC, 2015).

essential part of hazard and risk assessment, to allow informed decision-making and confidence in the results. Therefore, EURL ECVAM initiated and organised the Society of Toxicology (SOT) Continuing Education Course "Uncertainty Characterisation in 21st Century Toxicology: Current Practice and Practical Methods Supporting Regulatory Risk Assessment" together with a group of enthusiastic speakers at the 57th SOT Annual Meeting in March 2018. A focus of the course was the characterisation of uncertainties for new approaches, since the incorporation of new toxicological methodologies into safety assessment are still being hampered by a lack of knowledge on how to describe and assess the associated different uncertainties. The presentations are available from the SOT website.

READ MORE

- Society of Toxicology (SOT): www.toxicology.org/index.asp
- SOT Continuing Education (CE) Programme at the 57th SOT Annual Meeting 2018: www.toxicology.org/events/am/AM2018/continuing-education.asp
- CEd-Tox: SOT Continuing Education Courses Online: www.toxicology.org/education/ce/onlineCourses.asp

6.3.3 Training Activities related to Adverse Outcome Pathways

EURL ECVAM contributed with a lecture on the relationship between AOPs and chemistry-based *in silico* models to predict toxicity (Cronin & Richarz, 2017) to the in3 training workshop in Liverpool, UK in July 2018.

in3
integrated in vitro & in silico tools

The in3 project on integrated *in vitro* & *in silico* tools is a Marie Skłodowska-Curie Action Innovative Training Network, investigating an integrated interdisciplinary approach to animal-free nanomaterial and chemical safety assessment. Furthermore, the EURL ECVAM activities in the field of Modes of Action, AOPs and integration of different data sources were presented.

READ MORE

- in3: estiv.org/in3

6.3.4 Courses on Alternative Approaches in Modern Toxicology

In June 2018, EURL ECVAM gave a two-day course on "Alternative approaches to modern toxicology" for PhD students in life sciences at the University of Vienna upon invitation by the Head of the Department of Food Chemistry and Toxicology. This course aimed to create awareness on alternative methods for chemical risk assessment (toxicology/food safety) in theory and practice. The course was designed as a pilot project for the integration of alternative methods into the curriculum.

Presentations included toxicodynamics, AOPs and *in vitro* methods, introduction to risk and exposure assessment, biokinetics and exposure mathematical models. Exercises in break-out groups were related to AOP development to become familiar with the AOP Wiki and the principles of PBK models. A case study on 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and the Seveso Disaster underpinned the course and linked the various presentations and exercises. Students were very interested and interactive and their feedback was excellent.

Following the longstanding tradition, EURL ECVAM also invited in 2018 the students from the Karolinska Institute, Master programme in toxicology, to a course on modern toxicological sciences (see fig. 6.2). Twenty-two students and four tutors participated in a one-day course including various interactive presentations, which all aimed at increasing their understanding of non-animal methods and their use in life sciences.

READ MORE

- "Alternative approaches to modern toxicology" course: chemie.univie.ac.at/news-events/news/detail/news/27-28-juni-eu-jrc-lehrveranstaltung-alternative-approaches-to-modern-toxicology-jetzt-anmelde/?tx_news_pi1%5Bcontroller%5D=News&tx_news_pi1%5Baction%5D=detail&cHash=dd4b65bd6c0c51ad83b29bad54109862
- Master's Programme in Toxicology at the Karolinska Institute: ki.se/en/utbildning/4tx15-masters-programme-in-toxicology

Figure 6.2 Students of the Master's Programme in Toxicology of the Karolinska Institute (Sweden) visiting the JRC Ispra in May 2018.

6.3.5 Traineeships at EURL ECVAM

Since 2016, the new JRC Traineeship Scheme offers a stimulating, multi-cultural and multi-disciplinary research environment for trainees. Several positions were opened at the EURL ECVAM Laboratories with a high turnover of well-performing students over the last two years (see fig. 6.3). The EURL ECVAM Traineeship projects are part of EURL ECVAM's work program, allowing students to acquire or refine new techniques and publish their research results.

READ MORE

- JRC Traineeship scheme: europa.eu/!yn83Xx

6.3.6 Education and Training on Biokinetics and Physiologically-Based Kinetic Modeling

The US Society of Toxicology (SOT) offers several Continuing Education Course (CEC) Programs that cover established knowledge in toxicology (see also section 6.3.2). In 2018, EURL ECVAM co-chaired and participated to the course entitled "Physiologically-Based Pharmacokinetic Modeling to Support Modernised Chemical Safety Assessment" at the SOT2018.

PBK models have been applied to chemical risk assessment for more than three decades, extrapolation of animal toxicity findings to humans has been the major application. Under the proposed new toxicity testing paradigm, which relies on data from human-relevant *in vitro* toxicity assays interpreted through computational approaches, PBK models have been redefined as a critical translation tool for quantitative *in vitro* to *in vivo* extrapolation (IVIVE). The course was attended by 147 scientists and provided an opportunity to revisit the basic principles of PBK modeling with a special focus on supporting chemical risk assessment under the new toxicity testing paradigm. In addition, the basics of model construction, recent advances in model parametrization, including IVIVE, and evaluation of model performance and reliability along with use of available human data were presented and discussed with the participants. Finally, the focus was on the applications of the PBK models to support risk-based decisions in different tiers of risk assessment, and define and discuss challenges and future directions.

Under the proposed new toxicity testing paradigm PBK models have been redefined as a critical translation tool for quantitative in vitro to in vivo extrapolation (IVIVE)

At the end of the day course, a hands-on demonstration was provided using a free online simulation tool (PLETHEM) to demonstrate the workflow of building and parameterizing a PBK model, simulating different human populations, and applying the model to translate concentration-effect relationships from cell-based assays or *in vivo* studies to the dose-response relationship in target human populations to support chemical risk assessment.

In addition, EURL ECVAM was invited to participate to the SOT 2018 roundtable discussion "Can pharmacokinetic

Figure 6.3 Some trainees during summer 2018.

modelling keep up with risk assessment in the 21st Century?" Discussion on the impact of the changing regulatory environment on the utility of PBK modeling in chemical risk assessment, including:
1) Adequacy of currently available PBK modeling platforms for use in risk assessment.
2) Challenges associated with the movement toward high-throughput, animal-free toxicity testing.
3) Ways in which to foster increased acceptance of PBK modeling by regulatory scientists. The discussion was well perceived and attended by a large crowd of participants from the SOT 2018.

At the EUROTOX 2018, held in Brussels on 2 to 5 September 2018, EURL ECVAM staff chaired a CEC entitled: "Application of non-animal (toxico)kinetic data and tools in risk assessment from basic research to practice". With a similar aim as for the SOT 2018 CEC, a presentation was given to 56 students on the basics, how to construct, develop, evaluate and apply PBK models for risk assessment purposes, with insight in biokinetic data generated using alternatives and how to find and use TK databases.

READ MORE

- SOT 57th Annual Meeting: www.toxicology.org/events/am/AM2018/index.asp
- EUROTOX: www.eurotox.com

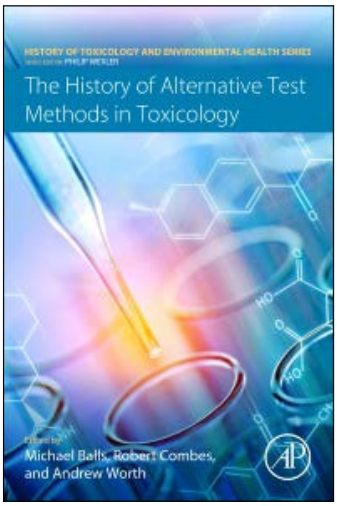
6.3.7 Course on Read-Across and In Silico Risk Assessment at Karolinska Institute

In November 2017, EURL ECVAM gave a two-day course on the use of read-across and *in silico* methodologies in risk assessment to second year students of the Master's programme in toxicology of the Karolinska Institute upon request of the director of the program. The first day of the course covered the theoretical part which included the basics of *in silico* tools and chemoinformatics, (Q)SARs, and grouping and read-across, quantification of uncertainty in read-across. The second day was dedicated to a hands-on exercise in which the students were introduced to the OECD QSAR Toolbox and given a series of read-across challenges to carry out.

6.4 History of Alternative Methods in Toxicology Book

EURL ECVAM staff have contributed to the editing and writing of a book entitled The History of Alternative Test Methods in Toxicology. The book was edited by Michael Balls (first Head of ECVAM; Nottingham University, UK), Robert Combes (Consultant, Norwich, UK) and Andrew Worth (EURL ECVAM) (Balls *et al.* (eds), 2018).

The book adopts a chronological approach to explore the history of alternative method development, validation, and use. Historical developments are described from a variety of perspectives – by country, region or organisation; technology type; and applications in the design or safety assessment of chemicals and products.



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Contributions by EURL ECVAM staff include the following chapters:

- Types of Toxicity and Applications of Toxicity Testing
- Contributions to Alternatives from Italy and Spain
- The Role of ECVAM
- Involvement of the Organisation for Economic Cooperation and Development
- Alternative Approaches for the Assessment of Chemicals in Food
- Alternative Approaches for Carcinogenicity and Reproductive Toxicity
- Dissemination of Information on Alternative Methods: Databases and Systems
- Integrated Approaches to Testing and Assessment
- The Validation of Alternative Test Methods
- Alternative Toxicity Test Methods: Lessons learned and yet to be learned

The *History of Alternative Test Methods in Toxicology* is published by Academic Press, Elsevier.

6.5 Dissemination Activities of EPAA

6.5.1 EPAA Partners Forum on Toxicokinetics and Read-Across

In November 2017, the EPAA convened a Partners' Forum on Toxicokinetics (TK) and Read-Across to provide an overview on research activities for developing *in vitro* TK methods and PBK models and for finding synergies to enhance use of TK data to strengthen read-across.

Specific activities were identified to facilitate the use of *in vitro* and *in silico* TK data to support read-across: The collation of available tools indicating the parameters and applicability domains covered; endpoint-specific guidance on TK parameters required for read-across; case studies exemplifying how TK data help support read-across. Activities to enhance the scientific robustness of read-across include the further user-friendly combination of read-across tools and, formal guidance by the authorities specifying the minimum information requirements to justify read-across for a given toxicity endpoint. The EPAA was invited to continue dissemination activities and to explore possibilities to create a database of TK tools that assist risk assessment. EURL ECVAM was invited to present the EURL ECVAM TK strategy document (Bessem *et al.*, 2015) including updates in the field of hepatic clearance, cyp induction and PBK modeling. The recommendations that were drawn from this meeting were published in the peer reviewed journal Regulatory Toxicology and Phamacology (Laroche *et al.*, 2018).

The EPAA convened a Partners' Forum on Toxicokinetics (TK) and Read-Across to provide an overview on research activities for developing in vitro TK methods and PBK models

6.5.2 EPAA Awards

The EPAA Awards are granted to young scientists (3Rs Science Prize) or laboratory technicians and animal caretakers (3Rs Refinement Prize), respectively, whose work has brought an outstanding contribution to the development and implementation of alternatives to animal testing. Both, the Science and the Laboratory technician prize are awarded alternating every other year. To this end, each year a jury of representatives from the EPAA partners is established, among them a staff member of EURL ECVAM.

The 2017 Refinement Prize was awarded to Camilla Bengtsson and Marie Eriksson of the Swedish Toxicology Sciences Research Center (Swetox) for their work entitled "Handling and training of mice and rats results in calmer animals during experimental procedures". They have developed routines for training and handling mice and rats during the acclimatisation phase to make them more comfortable with the experimental procedures. The trained and handled animals show a substantial reduction of stress and can be dosed and blood sampled without using devices to immobilise them during the experiment. This allows more accurate observations and an overall smoother execution of the testing.

Beginning of 2018, a new award programme was launched, the "EPAA 3Rs Student Grants", with the aim to foster participation of students and young scientists to high-profile scientific events in the area of 3Rs. Six awardees were financially supported to attend the 2018 conferences of EUROTOX, ESTIV or EUSAAT. A staff member of EURL ECVAM was member of the selection panel.

6.5.3 Other Dissemination Activities

As an outcome of EPAA-internal working sessions held in 2016, a manuscript (Dal Negro *et al.*, 2018) was published in 2018 reviewing the challenges that different industry sectors face in the implementation of alternative methods. It was concluded that for all sectors the main constraints to the application of non-animal alternatives are the gaps still existing in scientific knowledge and technological limitations.

The 13th EPAA Annual Conference, which took place on 22 November 2017, focused on "Building synergies to accelerate development and acceptance of alternatives". A recording of the Conference and the Conference Report (EPAA, 2017) were published on the EPAA website. Under the sponsorship of EPAA and in collaboration with the Institute for *In Vitro* Sciences (IIVS), two training videos have been produced in recent years (see section 6.7.2 in Zuang *et al.*, 2017). Such videos are now available with Spanish subtitles (besides the English original and Portuguese and Chinese subtitled versions).

READ MORE

- EPAA: europa.eu/!rq76qf
- EPAA Annual Conference 2017: webcast.ec.europa.eu/epaa-annual-conference-22-11-2017
- EPAA-IIVS Alternative methods video tutorials: europa.eu/!bc39gg

7 International Cooperation on Alternative Test Methods

7. International Cooperation on Alternative Test Methods

7.1 International Cooperation on Alternative Test Methods

Further to the successful ICATM workshop held at the JRC-EURL ECVAM on 4 to 6 October 2016, which led to concrete outcomes in the area of skin sensitisation (see section 7.1 in Zuang *et al.*, 2017; Casati *et al.*, 2018), EURL ECVAM, together with its ICATM partners, hosted another ICATM workshop on 23 to 24 October 2018, this time on the topic of "Validation of alternative methods towards internationally recognised standards for regulatory application" (see fig. 7.1). The workshop convened regulators from the EU, US, Canada, Japan, South Korea and Brazil.

The ICATM workshop on validation primarily aimed to:

- Identify and discuss the current issues concerning the validation of alternative methods/approaches and possible solutions.
- Discuss lessons learnt from past and ongoing validation studies.
- Reflect on the Principles and Practice of validation in the context of non-guideline methods, non-testing methods, IATA/DAs, and tools and technology (*e.g.*, microphysiological systems/organ-on-chip).
- Reflect on the elements necessary to establish credibility of a method.
- Discuss where validation as a process starts and where it ends.
- Discuss the possibility of and process for assessing (i) relevance without comparing to animal data (what does "biologically relevant" mean?) and (ii) reliability without conducting ring trials.
- Discuss the main reasons for conducting validation studies within ICATM.
- Discuss if OECD Guidance Document 34 is still adequate in the framework for validation in a regulatory context and what validation means in the context of an evolving Test Guidelines Programme at the OECD.
- Discuss how to evolve validation practice to increase efficiency, encourage more innovation and flexibility in study design and accommodate scientific progress.
- Explore how Standards could evolve as a tool to (i) facilitate the establishment of credibility of alternative methods/approaches and (ii) accelerate the acceptance of new approaches in a regulatory context.

An ICATM meeting, involving only ICATM partners, followed the workshop to discuss and agree on actions to be undertaken by ICATM in the near future in the area of validation.

Figure 7.1 Participants of the International Cooperation on Alternative Test Methods (ICATM) workshop on "Validation of alternative methods towards internationally recognised standards for regulatory application" held at the JRC Ispra in October 2018.

7.2 OECD - China Joint International Workshop on Genotoxicity

The OECD, the China National Institute for Food and Drug Control, the China National Centre for Food Safety Risk Assessment together with the Guangdong Provincial Centre for Disease Control and prevention are co-organising a Joint International Workshop on the "Progress of Genotoxicity Methods and Regulatory Acceptance" was held in November 2018 in Guangzhou. The workshop is meant to promote exchange on understanding and regulatory acceptance of genotoxicity methods with the ultimate aim of possibly adopting in China existing OECD test guidelines and other international guidance available in the area.

7.3 International Dialogue for the Evaluation of Allergens

The International Dialogue for the Evaluation of Allergens (IDEA) project is designed to provide a broadly agreed and transparent framework for assessing fragrance sensitisers globally. It provides the opportunity to build partnerships between the international fragrance industry and its stakeholders to improve the risk assessment of those fragrance ingredients identified as allergens for better consumer protection.

IDEA is committed to the integration of non-animal data to replace the LLNA for risk assessment

IDEA is committed to the integration of non-animal data to replace the LLNA for risk assessment. This is a goal shared by various stakeholders. EURL ECVAM is liaising with this IDEA project and participated in a dedicated workshop held on 16 to 17 May, 2018. Companies and stakeholders shared case studies explaining the application and current status of the approaches using only non-animal derived data for the determination of a non-expected sensitisation induction level (NESIL) to be used in quantitative risk assessment (QRA). The main key outcome from the workshop was that a number of non-animal approaches are available that can predict sensitisation potency of fragrance substances for use in risk assessment. The entire set of presentations and key conclusions from the workshop can be accessed on the IDEA website.

IDEA
International Dialogue for the Evaluation of Allergens

READ MORE

- IDEA project: ideaproject.info
- IDEA Workshop on the replacement of animal testing in QRA for skin sensitization: ideaproject.info/eventsmanager/27/16/IDEA-Workshop-on-the-replacement-of-animal-testing-in-QRA-for-skin-sensitization

7.4 Global Consortium on *In Silico* Toxicology Protocols

Computational ("*in silico*") methods include quantitative structure-activity relationship models (QSARs), structural alert profilers, read-across approaches inferring properties between similar chemicals, and receptor-ligand docking. They are widely used in applications such as drug development and screening of large lists of chemicals for potential toxicological properties. They provide valuable information for the evaluation of chemicals, in particular in terms of mechanistic understanding of the links between chemical structure and biological activity. However, the acceptance of *in silico* model results in regulatory toxicology decision making is still limited. One reason for lack of acceptance is thought to be the absence of defined and harmonised procedures on how to apply the methods and interpret the results.

The overall aim is to increase confidence in, and acceptance and uptake of, in silico methodologies for regulatory applications

The aim of the *In Silico* Toxicology Protocol initiative, led by Leadscope, Inc., is therefore to develop principles for the consistent use and assessment of computational model results in chemical hazard evaluations. In particular, it is devising a structured process to generate, interpret, document, assess and communicate computational method results in a reproducible and consistent manner, in order to increase confidence in their use. EURL ECVAM is part of the international consortium formed by over 55 members from industry, regulatory or governmental agencies, academics, model developers and consultants across different sectors and regions (US, Canada, Japan, Europe), in view to achieve a broad consensus. The overall aim is to increase confidence in, and acceptance and uptake of, *in silico* methodologies for regulatory applications.

The first publication of the consortium (Myatt *et al.*, 2018) describes the general overarching framework of the *In Silico* Toxicology Protocols, integrating computational predictions alongside available experimental data for a defined set of adverse effects, which are then combined in an overall assessment. It includes considerations on the assessment of reliability and relevance, using a novel scoring scheme. The necessity of expert review steps is recognised, at the same time as the importance of a guided and transparent process for this evaluation and its documentation. Checklists for the *In Silico* Toxicology Protocol components, expert review and reporting elements are provided.

Work is ongoing to adapt the structured framework to specific toxicological endpoints, such as genotoxicity, skin sensitisation or acute toxicity.

7.5 World Health Organisation Chemical Risk Assessment Network

The World Health Organisation (WHO) Chemical Risk Assessment Network (CRAN) within the International Programme on Chemical Safety (IPCS) is a collaborative initiative aiming at improving chemical risk assessment globally, through facilitating interaction and exchange of experience about risk assessment topics and activities between institutions engaged in chemical risk assessment activities around the world. It was established at the end of 2013 and comprises institutions from over 45 countries, including government departments, academia, WHO Collaborating Centres and professional societies.

The WHO Network promotes the objectives of the Strategic Approach to International Chemicals Management (SAICM). It is coordinating projects that can be international, regional, multilateral or bilateral in scope, resulting for example in guidance documentation, training sessions or materials, tools or databases. An important focus is developing countries' perspectives on risk assessment. EURL ECVAM is contributing to the Network, for example to the Network Coordination Groups on Mode of Action (MoA) and Combined Exposures and the development of guidance and tools on chemical risk assessment methodology.

During the IUTOX Congress of Toxicology in Developing Countries (CTDC10) in April 2018 in Belgrade, Serbia, 30 Network Participants from 15 Network institutions met for a WHO CRAN lunch time meeting with a focus on capacity building activities (see fig. 7.2). The WHO Chemicals Road Map to enhance health sector engagement in the SAICM

was introduced and the WHO CRAN Capacity Building Strategy for 2018-2020, linked to actions of the Road Map, was presented. The Strategy is aimed at increasing chemical risk assessment capacity in WHO Network participants and thereby strengthens environmental health decision-making. Specifically, it aims at enhancing national institutional capacities to address health threats from chemicals and enhancing knowledge and methodologies for risk assessment. The strategic areas of the plan focus on assessing the capacity needs, identifying technical resources and developing human resources, identifying priorities for future training as well as promoting best chemical risk assessment practice. Furthermore, different aspects and experience from Network institutions were discussed. The use of webinars for training was one specific topic. The WHO Secretariat will follow up on the ideas for capacity building activities to implement the different areas of the Network Strategy.

The Strategy is aimed at increasing chemical risk assessment capacity in WHO Network participants and thereby strengthens environmental health decision-making

7.6 Collaboration with the US Interagency Coordinating Committee on the Validation of Alternative Methods Working Groups

7.6.1 Read-Across Working Group

EURL ECVAM is participating as liaison of the International Cooperation on Alternative Test Methods (ICATM, see section 7.1) in the new ICCVAM Read-Across Working Group (RAWG) established in 2017. The RAWG aims at building capacity for ICCVAM members in the development and application of read-across approaches for chemical safety assessment as well as harmonising these approaches between the agencies. The Working Group thus contributes to implementing the goals of the ICCVAM "Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States" (ICCVAM, 2018).

READ MORE

- US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM): ntp.niehs.nih.gov/pubhealth/evalatm/iccvam/index.html

7.6.2 In Vitro to In Vivo Extrapolation Working Group

EURL ECVAM was invited to participate to the ICCVAM WG on *in vitro* to *in vivo* extrapolation (IVIVE). This WG is

Figure 7.2 Network meeting at the Congress of Toxicology in Developing Countries (CTDC10) in April 2018.

Box 7.1

Predictive Computational Toxicology Approaches

The term computational ("*in silico*") methods covers a wide range of approaches including:

- structural alert profilers
- quantitative structure-activity relationship models (QSARs)
- receptor-ligand docking
- read-across approaches inferring properties between similar chemicals.

Computational models provide valuable information for the evaluation of chemicals, in particular in terms of mechanistic understanding of the links between chemical structure and biological activity. *In silico* methods are already widely used in applications such as drug development and screening of large lists of chemicals to predict potential toxicological properties, and also contributing to regulatory chemical safety assessment submissions in different ways .

In silico approaches were part of several EURL ECVAM activities described in this report:

- Chemistry framework: generic description of chemicals (see section 2.9)
- OECD project on a guideline on DAs for skin sensitisation prediction which may include the use of QSARs (see section 5.3.8)
- Case study on grouping and read-across for nanomaterials: application of cheminformatics methods for grouping (see section 5.2.1 and Worth *et al.*, 2017a)
- Report of the International Cooperation on Cosmetics Regulation (ICCR) Joint Regulators-Industry Working Group on "Integrated Strategies for Safety Assessments of Cosmetic Ingredients": *in silico* methods as one of the NAM described to be used in the 'next generation' risk assessment (see section 5.9.1)
- Global consortium developing *In silico* Toxicology Protocols: structured process to support confidence in, and uptake of, *in silico* approaches for regulatory applications (see section 7.4 and Myatt *et al.*, 2018)
- Guidance for the implementation of the hazard-based criteria to identify endocrine-disrupting properties: *in silico* approaches included (see section 5.1.2)
- JRC QSAR Database and DB-ALM (see sections 6.1.3 and 6.1.1; and Tsakovska *et al.*, 2017 as examples for QSARs)
- ICCVAM Read-Across Working Group (see section 7.6.1)
- Training on AOPs and *in silico* models relationship (see section 6.3.3 and Cronin & Richarz, 2017)
- Course on read-across and *in silico* methods for risk assessment (see section 6.3.7)

also represented by agency representatives of ICCVAM and ICATM members experts from outside of the federal government. The goal of this WG is to identify the state of the science on IVIVE for use in prioritisation and risk assessment purposes. Several tasks were identified, from literature search on current IVIVE approaches up to determine best practise for IVIVE analysis. The WG is currently revising and harmonising the vocabulary and terminology with an effort to develop guidance on the application of IVIVE in risk assessment.

The goal of this WG is to identify the state of the science on IVIVE for use in prioritisation and risk assessment purposes

7.7 Health and Environmental Sciences Institute

7.7.1 Update on the Genetic Toxicity Technical Committee

The Health and Environmental Sciences Institute (HESI) Genetic Toxicology Technical Committee (GTTC) is focused on advancing the field of genetic toxicology and human risk assessment. Main objectives include the integration of genetic toxicology into risk assessment and decision-making for protection of human health; the improvement of new and existing test guidelines, strategies, and interpretation of results; and investigation of non-traditional modalities, including novel entities and technologies.

Health and Environmental Sciences Institute

During the annual meeting that took place in Arlington VA, US, in April 2018, the progress of various activities was presented by respective working groups. These were among others, an analysis of the most appropriate *in vivo* follow-up testing which include compared data for over 90 chemicals from the transgenic rodent assay, the *in vivo* comet assay, and cancer data; an evaluation of the

Box 7.2

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

The US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is an interagency committee of the US National Institute of Environmental Health Sciences (NIEHS), representing 16 US federal regulatory and research agencies that require, use, generate, or disseminate toxicological and safety testing information. ICCVAM is evaluating and promoting the development, regulatory acceptance, and use of new, revised, and alternative test methods, sharing experience among the US federal regulatory agencies.

current testing paradigm for genotoxicity assessment of nanomaterials and recommendations for a tailored test battery and modification of tests as needed (Elespuru *et al.*, 2018); a novel quantitative approach for analysing and interpreting genetic toxicity dose-response data with *in vivo* dose-response data being used to identify endpoint-specific critical effect size (CES) values that are suitable for routine determination of benchmark dose (BMD) values; an update of the clean sheet testing strategy activity (Dearfield *et al.*, 2017), where case studies are being prepared, under the different regulatory jurisdictions for publication to further illustrate the approach; an analysis of mode of action (MoA) and the development of AOPs related to specific genotoxic MoA pathways. In this context, methods to determine the mode of action of genotoxic agents are investigated, as well as the application of new technologies to establish the mode of action of genotoxicity for new chemical entities.

The HESI Genetic Toxicology Technical Committee (GTTC) is focused on advancing the field of genetic toxicology and human risk assessment

READ MORE

- Health and Environmental Sciences Institute (HESI) Genetic Toxicology Technical Committee (GTTC): hesiglobal.org/genetic-toxicology-gttc

7.7.2 Physiologically Based Pharmacokinetic Working Group

At the SOT 2017, the HESI Physiologically based Pharmacokinetic (PBPK) WG was established, including international experts in PBK modeling. The goal is to facilitate the use of PBK modeling approaches in various risk assessment applications by identifying specific needs. The first effort of this group was the establishment of a harmonised template to report information, and provide recommendations to model reviewers to facilitate the uptake of PBK modeling approaches in regulatory risk assessment. The next effort will be to develop a framework and decision tree on PBK applications based on different degrees of data availability.

7.8 Other International Activities in the Area of Toxicokinetics

In October 2017, EURL ECVAM participated at a Lorentz-Center international workshop on "Non-animal methods for Toxicokinetics". The goal of the workshop was to bring together experts and stakeholders from science, industry, and the regulatory area, including new generation toxicologists, to find ways to evolve the field of non-animal methods for toxicokinetics in toxicological research and regulatory safety evaluations. Based on pre-defined regulatory questions, a top four of essential human kinetic parameters were identified. These are (from high to low importance):

1) intrinsic hepatic clearance and identification of metabolites,
2) passive permeability (through intestine, lungs, other barriers),
3) tissue-partitioning and the fraction unbound in blood, and
4) transporter kinetics within different barriers (*e.g.*, intestinal, kidney, blood/brain, placenta). These parameters have also been identified previously (Bessems *et al.*, 2015; Paini *et al.*, 2017a,b).

Regarding transporter kinetics within different barriers, EURL ECVAM launched in early 2018 an EU survey entitled "Membrane Transporters in Chemical Safety Assessment" to gather the applicability of available data and knowledge on membrane transporters. The survey was designed with a specific interest to identify *in vitro* and *in silico* methods that are currently in use for transporter studies (Clerbaux

EURL ECVAM launched in early 2018 an EU survey to gather the applicability of available data and knowledge on membrane transporters

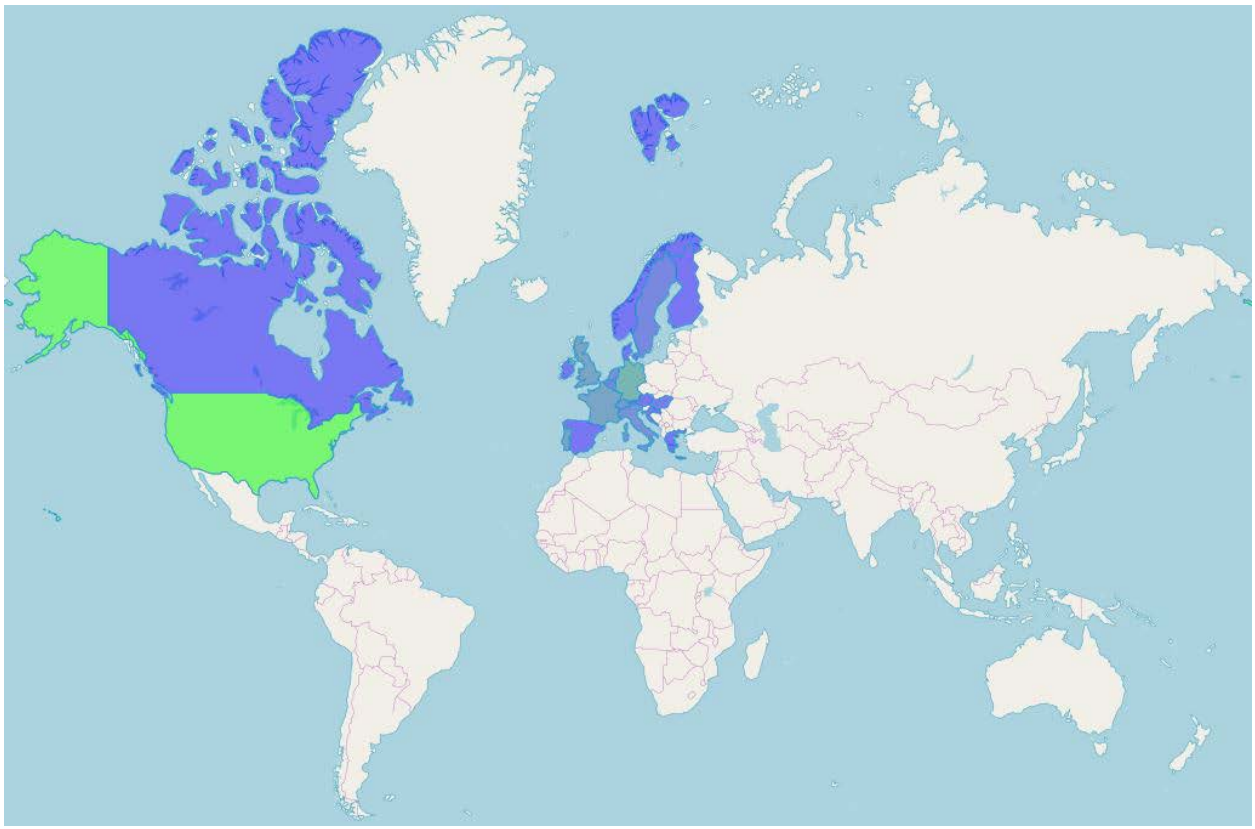

Figure 7.3 Geographical distribution of the Membrane Transporters in Chemical Safety Assessment questionnaire. The results are published in Clerbaux *et al.* (2018) and are available by individual country. They can be accessed at http://apps.klimeto.com/imt.

et al., 2018). Seventy-three respondents, from 21 countries, revealed that membrane transporters influencing chemical's absorption, distribution, metabolism and excretion, are investigated by applying *in vitro* and *in silico* approaches during drug development and chemical risk assessment in cosmetics and in food and feed safety. However, data from alternative approaches are not readily accepted by regulators. To gain trust and credibility, regulatory authorities require standardisation of methodologies and validation, against human data, when possible, as well as reproducibility and high quality in data generation (Clerbaux *et al.*, 2018).

Along these lines, EURL ECVAM's next steps will be to review the role of membrane transporters for environmental chemical risk assessment in collaboration with the US Food and Drug Administration (FDA) and with international experts in the field.

READ MORE

- Lorentz-Center: www.lorentzcenter.nl
- "Non-animal Methods for Toxicokinetics" Workshop: www.lorentzcenter.nl/lc/web/2017/943/info.php3?wsid=943&venue=Oort

8 Conclusions

Research and development activities continued during 2018 in areas for which 3Rs solutions are more difficult to find. For regulatory toxicity testing, research projects focus on repeated dose and reproductive toxicity testing, on chemical mixtures and endocrine disruptors. These projects are either based on read-across case studies or aim at developing new *in vitro* methods and integrating *in vitro* methods and *in silico* computational technologies in integrated assessment and testing strategies to translate mechanistic understanding of toxicity into risk assessments. In the area of carcinogenicity, EURL ECVAM is currently exploring how mechanistic data across toxicity endpoints (based primarily on existing *in vivo* and *in vitro* OECD TGs) could be best combined, instead of taking them in isolation, to either waive redundant testing or, ultimately, improve carcinogenicity testing.

The relationship between children's health and exposure to environmental chemicals has been investigated by the integration of multiple information sources including mechanistic knowledge, biomonitoring data, epidemiological studies, *in silico* as well as *in vitro* data. Combined effects of chemicals known to trigger DNT are also currently being studied using mechanistically relevant *in vitro* assays. For the quality control of vaccines, research projects aim to develop, optimise and evaluate non-animal methods for routine batch quality, safety and efficacy testing of vaccines.

Several R&D projects for fish toxicity and bioaccumulation testing are currently ongoing. For acute fish toxicity testing, a fish cell line-based cytotoxicity assay has been developed and validated, AOPs for chronic fish toxicity testing are being developed and the application of threshold of toxicological concern in aquatic toxicity assessment is being investigated. A tiered testing strategy based on *in vitro* approaches for fish bioaccumulation testing is under development as well as a toxicokinetic modelling framework for bioaccumulation assessment in mammals, combining *in vitro* and *in vivo* PBTK data, field collected bioaccumulation data, and quantitative structure-activity relationship models.

Maintenance of normal thyroid function is essential for the psychological and physiological well-being and it is thus essential to be able to identify chemicals with potential thyroid disrupting properties with appropriate test methods. Therefore, EURL ECVAM initiated a validation study, together with EU-NETVAL, on 17 *in vitro* methods for the identification of thyroid hormone disruptors. A multitude of *in vitro* tests are also currently being validated for DNT testing in the framework of an OECD Guidance Document on the use of an *in vitro* testing battery for DNT.

The well-advanced areas of topical toxicity, skin sensitisation and genotoxicity were complemented with additional *in vitro* methods or combined (*in silico* and *in vitro*) approaches which have either already been adopted or are currently being reviewed and discussed in international fora. As member of the EPAA, EURL ECVAM collaborates with the Biological Standardisation Programme on several validation studies which assess alternative methods for the safety and potency testing of human and veterinary vaccines.

The more recent developments of complex *in vitro* models such as 3D cell cultures, bioprinted tissues, bioreactor cultures and microphysiological systems (*e.g.*, multi-organ on chip models), as well as the increasing integration of multiple data sources for safety testing, triggered some reflection on the current principles and practice of validation, and on the elements that are needed to build user confidence in these new tools and approaches. EURL ECVAM engaged with its international partners (through ICATM), its network of regulators (PARERE) and its stakeholder forum (ESTAF) to discuss that topic.

A multitude of projects which promote the regulatory acceptance and use of alternative approaches, as well as their wider international uptake, were either successfully concluded or continued during 2018. For that purpose, EURL ECVAM is closely working with the other Commission services, the EU regulatory agencies and the EPAA at European level, as well as with international organisations such as the OECD, ICH, VICH, WHO, ICCR and the United Nations.

One major achievement was *e.g.* the publication of two VICH guidelines that establish criteria for waiving the target animal batch safety test for inactivated and live vaccines for veterinary use. These guidelines are in force since May 2018. The harmonisation of such criteria between different geographical regions in the world will avoid redundant and severe animal testing.

Regarding the activities at UN level, EURL ECVAM contributes to the drafting of the GHS chapter on skin corrosion/irritation to include non-animal methods, and leads the drafting of the chapters on serious eye damage/irritation and skin sensitisation within the informal working group "Use of non-animal testing methods for classification of health hazards" of the UN Subcommittee on GHS. This review of the GHS will include *in vitro*, *in silico* and *in chemico* methods, as well as grouping and read-across, as a basis for hazard assessment. The ultimate goal is not only to show how non-animal methods can be used to satisfy existing classification criteria (*e.g.,* for eye irritation and skin sensitisation), but ultimately, how the criteria themselves could be adapted to non-animal data.

Besides regulatory testing, EURL ECVAM intensified its activities in the area of basic, applied and translational research, as well as education and training, to efficiently support the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes also in those areas.

This included a study to review non-animal models and methods currently used to investigate respiratory tract diseases and neurodegenerative disorders; a knowledge sharing initiative on the testing procedures used within the different disciplines in biosciences; a feasibility study on indicators of alternative methods or approaches to animal experimentation; a review of current 3Rs education and training resources; the planning of a strategic introduction of 3Rs in curricula at high school, university and professional levels, and finally, the design of eLearning tools to be distributed to educational bodies and course providers. Information on alternatives continued to be disseminated through a variety of dedicated meetings, training efforts and specialised database services hosted by JRC-EURL ECVAM.

In conclusion therefore, considerable progress has been made on several fronts in the development, validation and acceptance of alternative approaches to animal testing. Moreover, the advancement of the Three Rs across multiple sectors is being expedited through efficient and effective collaboration between multiple stakeholders engaging at an international level in a variety of fora.

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List of abbreviations and definitions

ADHD	Attention Deficit and Hyperactivity Disorder
ADME	Absorption, distribution, metabolism and excretion
ANSES	Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (French Agency for Food, Environmental and Occupational Health & Safety)
AOP	Adverse Outcome Pathway
AOP-KB	Adverse Outcome Pathway-Knowledge Base
AR	Androgen Receptor
ARTA	Androgen Receptor Transactivation Assay
BCF	Bioconcentration Factor
BDS	BioDetection Systems (NL)
BEAMS	BridgE Across Methods in the bioSciences (EURL ECVAM)
BIAC	Business and Industry Advisory Committee (OECD)
BMD	Benchmark Dose
BrdU FCM	5-bromo-2-deoxyuridine Flow Cytometry
BSP	Biological Standardisation Programme
CALUX	Chemically Activated Luciferase Gene Expression
CARACAL	Competent Authorities for REACH and CLP
CEC	Continuing Education Course
Cefic	The European Chemical Industry Council
CES	Critical Effect Size
CF	Conceptual Framework
CheLIST	Chemical Lists Information System
CHMP	Committee for Medicinal Products for Human Use (EMA)
CLP	Classification, Labelling and Packaging
CM&S	Computational Modelling and Simulation
CPSC	The Consumer Product Safety Commission (USA)
CRAN	Chemical Risk Assessment Network (WHO)
CRD	Chemicals Regulation Directorate (UK)
CTDC	Congress of Toxicology in Developing Country (IUTOX)
CVMP	Committee for Medicinal Products for Veterinary Use (EMA)
CYP	Human cytochrome P450
DA	Defined Approach
DART	Developmental and Reproductive Toxicity
DB-ALM	EURL ECVAM DataBase on ALternative Methods
DG CNECT	Directorate-General for Communications Networks, Content and Technology (EU)
DG ENV	Directorate-General for Environment (EU)
DG GROW	Directorate-General for Internal Market, Industry, Entrepreneurship and SMEs (EU)
DG SANTE	Directorate-General for Health and Food Safety (EU)
DIP	Data Interpretation Procedure
DIS	Draft International Standard
DNA	Deoxyribonucleic Acid
DNT	Developmental Neurotoxicity
DPRA	Direct Peptide Reactivity Assay
DRP	Detailed Review Paper
EACR	European Association for Cancer Research
EAGMST	Extended Advisory Group for Molecular Screening and Toxicogenomics
EAS	Endocrine Active Substance
EASIS	Endocrine Active Substances Information System
EAWAG	Swiss Federal Institute of Aquatic Science and Technology
EC	European Commission

ECETOC	European Chemical Industry Ecology and Toxicology Centre
ECHA	European Chemicals Agency
ED(s)	Endocrine Disruptor(s)
EDQM	European Directorate for the Quality of Medicines & HealthCare (Council of Europe)
EEA	European Environmental Agency
EFPIA	European Federation of Pharmaceutical Industries and Associations
EFSA	European Food Safety Authority
EGME	Ethylene glycol methyl ether
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicines Agency
EP	European Parliament
EPAA	European Partnership for Alternatives to Animal Testing
ER	Estrogen Receptor
ESAC	ECVAM Scientific Advisory Committee
ESTAF	ECVAM Stakeholder Forum
ESTIV	European Society of Toxicology *In Vitro*
EU	European Union
EU-NETVAL	European Union Network of Laboratories for the Validation of Alternative Methods
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
Eurometaux	European Non-ferrous Metals Association
EUROTOX	Federation of European Toxicologists and European Societies of Toxicology
EUSAAT	European Society for Alternatives to Animal Testing
EWG	Expert Working Group (OECD)
Fab	Fragment antigen binding
FCM	Flow Cytometry
FDA	Food and Drug Administration (USA)
FELS	Fish Early Life-Stage
FRAND	Fair, Reasonable and Non-discriminatory
GARD	Genomic Allergen Detection Test
GD	Guidance Document (OECD)
GHS	Globally Harmonised System of Classification and Labelling of chemicals
GIVIMP	Good *In Vitro* Method Practices
GLP	Good Laboratory Practice
GTTC	Genetic Toxicology Technical Committee (HESI)
HBM4EU	European Human Biomonitoring Initiative
h-CLAT	human Cell Line Activation Test
HDL	high-density lipoproteins
HESI	Health and Environmental Sciences Institute (US)
HET-MN	Hen's Egg Test for Micronucleus Induction
HMA	Heads of Medicines Agencies
HTS	High-Throughput Screening
IATA	Integrated Approaches to Testing and Assessment
ICAPO	International Council on Animal Protection in OECD Programmes
ICATM	International Cooperation on Alternative Test Methods
ICCR	International Cooperation on Cosmetics Regulation
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods (NIEHS)
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ICT	Information and Communications Technology
IDEA	International Dialogue for the Evaluation of Allergens
IIVS	Institute for *In Vitro* Sciences (US)
IMI	Innovative Medicines Initiative
IP	Intellectual Property
IPCHeM	Information Platform for Chemical Monitoring

IPCS	International Programme on Chemical Safety
IPR	Intellectual Property Right
iPSC	induced Pluripotent Stem Cell
ISO	International Organization for Standardization
IUTOX	International Union of Toxicology
IVIVE	*In vitro* to *in vivo* extrapolation
IWGT	International Workshops on Genotoxicity Testing
J3Rs WG	Joint Committee for Medicinal Products for Veterinary Use/Committee for Medicinal Products for Human Use Working Group on the Application of the 3Rs in Regulatory Testing of Medicinal Products
JDP	Joint Dissemination Portal (ECHA-JRC-OECD)
JEG 3Rs	Joint Expert Group for Reduction, Replacement and Refinement - EMA (see also J3Rs WG)
JRC	Joint Research Centre (EC)
JWG	Joint Working Group (ICCR)
KE	Key Event
KER	Key Event Relationship
LD50	lethal dose, 50%
LDL	low-density lipoproteins
LLNA	Local Lymph Node Assay
LRI	Cefic's Long-Range Research Initiative
MN	Micronucleus
MoA	Mode of Action
NAM	New Approach Method
NC3Rs	National Centre for the Replacement, Refinement & Reduction of Animals in Research (UK)
NCC	Neuronal Crest Cell
NCT	Network Training Centre (EU)
NESIL	Non-expected Sensitisation Induction Level
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences (US)
NIS	Sodium Iodide Symporter
NL	the Netherlands
NTP	National Toxicology Programme (US)
OECD	Organisation for Economic Co-operation and Development
OHT	OECD Harmonised Template
PARERE	Preliminary Assessment of Regulatory Relevance network
PISC	PETA International Science Consortium
PBK	Physiologically Based Kinetic (also PBPK, PBBK, PBTK)
PBT	Persistent, Bioaccumulative and Toxic
PBTG	Performance-Based Test Guideline (OECD)
PNEC	Predicted No Effect Concentration
POP	Persistent Organic Pollutant
QMRF	QSAR Model Reporting Formats
QRA	Quantitative Risk Assessment
(Q)SAR	(Quantitative) Structure Activity Relationship
R&D	Research & Development
RAAF	Read Across Assessment Framework (ECHA)
RAWG	Read-Across Working Group (ICCVAM)
REACH	European Regulation (EC) no 1907/2006 Registration, Evaluation, Authorisation and Restriction of Chemicals
RhCE	Reconstructed human Cornea-like Epithelium
RhE	Reconstructed human Epidermis
RIVM	National Institute for Public Health and the Environment (the Netherlands)
RNA	Ribonucleic Acid
RSMN	Reconstructed Skin Micronucleus
SAICM	Strategic Approach to International Chemicals Management (WHO)

scFv	single-chain variable fragment
SME	Small and Medium-sized Enterprises
SOT	Society of Toxicology
SPSF	Standard Project Submission Form (OECD)
SVM	Support Vector Machine
TBG	Thyroxine Binding Globulin
TCDD	Tetrachlorodibenzo-p-dioxin
TDG	Transport of Dangerous Goods (United Nations)
TG	Test Guideline (OECD)
TGP	Test Guidelines Programme (OECD)
TK	Toxicokinetics
TRF	transcriptomics reporting framework
TRH	Thyroid Releasing Hormone
TSAR	EURL ECVAM Tracking System on Alternative Methods towards Regulatory acceptance
TSH	Thyroid Stimulating Hormone
TTC	Threshold of Toxicological Concern
TTR	Transthyretin
UK	United Kingdom
US	United States
US EPA	United States Environmental Protection Agency
UVBC	Unknown or Variable Composition, Complex Reaction Products and Biological Materials
VAC2VAC	"Vaccine batch to vaccine batch comparison by consistency testing"
VICH	International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products
WHO	World Health Organisation
WNT	Working Group of the National Coordinators of the Test Guidelines Programme (OECD)
WPHA	Working Party on Hazard Assessment (OECD)

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Annexes

Annex 1 — Summary status of the adoption of Test Guidelines based on *in vitro* methods in the OECD TG programme (2011-2018)

Table 1 summarises the status of adoption of OECD test guidelines on *in vitro* methods from 2011 to 2018. It should be noted that beside TGs, also Guidance Documents and new projects on alternative methods were respectively adopted and included in the OECD Work programme during that period. For additional information, please consult the OECD website of the Test Guideline Programme: http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicalsandrelateddocuments.htm

Table 1. Status of adoption of OECD Test Guidelines based on *in vitro* methods 2011-2018.

Nr.	Toxicity area	Test method description	Acceptance status
1	Skin corrosion	Reconstructed human Epidermis (RhE) test methods, as included in OECD TG 431/EU TM B.40 bis.	Adopted as a new TG in 2004; updated version (sub-categorisation, inclusion of performance standards, inclusion of SkinEthic™ RHE and epiCS®) adopted in 2013. Revised version including sub-categorisation with the epiCS® test method adopted in 2014. Updated in 2015 for the deletion of the performance standards (published separately in the Series on Testing and Assessment No. 219), inclusion of paragraphs referring to the IATA for Skin Corrosion and Irritation (OECD GD No. 203) and inclusion of the use of HPLC/UPLC-spectrophotometry as an alternative procedure to measure tissue viability (increasing the applicability domain of the test methods to coloured substances interfering with the measurement of MTT-formazan). Updated in 2016 for improving the predictive capacity of the three validated *in vitro* skin corrosion test methods (EpiDerm™, SkinEthic™ and EpiCS®) for the correct prediction of Sub-Cat.1A.
2	Skin corrosion	Transcutaneous Electrical Resistance (TER) test method, as included in OECD TG 430/EU TM B.40.	Adopted as a new TG in 2004; updated version (inclusion of performance standards) adopted in 2013. Updated in 2015 for the deletion of the performance standards (published separately in the Series on Testing and Assessment No. 218) and the inclusion of paragraphs referring to the IATA for Skin Corrosion and Irritation (OECD GD No. 203).

Nr.	Toxicity area	Test method description	Acceptance status
3	Skin corrosion	*In vitro* Membrane Barrier Test Method for Skin Corrosion, as included in OECD TG 435/EU TM B.40.	Adopted as a new TG in 2006; Updated in 2015 for the inclusion of the Corrositex® prediction model, the deletion of the performance standards (to be published separately in the Series on Testing and Assessment), the inclusion of paragraphs referring to the IATA for Skin Corrosion and Irritation and the updating of the list of proficiency substances (OECD GD No. 203).
4	Skin irritation	Reconstructed human Epidermis (RhE) test methods, as included in OECD TG 439/EU B.46.	Adopted as a new TG in 2010; updated version (inclusion of LabCyte EPI-model24 SIT) adopted in 2013. Updated in 2015 for the deletion of the performance standards (published separately in the Series on Testing and Assessment No. 220), inclusion of paragraphs referring to the IATA for Skin Corrosion and Irritation (OECD GD No. 203) and inclusion of the use of HPLC/ UPLC-spectrophotometry as an alternative procedure to measure tissue viability (increasing the applicability domain of the test methods to coloured substances interfering with the measurement of MTT-formazan).
5	Serious eye damage/ eye irritation	Fluorescein Leakage (FL) test method, as included in OECD TG 460.	Adopted as a new TG in 2012; updated version to include a new reference to OECD GD 236 on an IATA for Serious Eye Damage and Eye Irritation adopted in 2017.
6	Serious eye damage/ eye irritation	Bovine Corneal Opacity and Permeability (BCOP) test method, as included in OECD TG 437/EU TM B.47.	Adopted as a new TG in 2009; updated version (revision of positive controls, use to identify non-classified chemicals and several other revisions) adopted in 2013; updated version to include a new reference to OECD GD 236 on an IATA for Serious Eye Damage and Eye Irritation adopted in 2017.
7	Serious eye damage/ eye irritation	Isolated Chicken Eye (ICE) test method, as included in OECD TG 438/EU TM B.48.	Adopted as a new TG in 2009, updated version (use to identify non-classified chemicals and several other revisions) adopted in 2013; updated version to include a new reference to OECD GD 236 on an IATA for Serious Eye Damage and Eye Irritation adopted in 2017; updated versionto include histopathological examination as additional endpoint to identify UN GHS cat.1 non-extreme pH (2<pH<11.5) detergents and surfactants and modified decision criteria for chemicals requiring classification for eye hazard (taking into consideration the variability of the Draize eye test) adopted in 2018.

Nr.	Toxicity area	Test method description	Acceptance status
8	Serious eye damage/ eye irritation	Cytosensor Microphysiometer (CM) test method.	New draft TG first discussed at WNT in 2013 but not adopted, pending further clarification on its use to identify non-classified chemicals. The additional data requested by the WNT that should have been submitted by US to support the project were not received and thus the project has been discontinued because of lower priority for the EC (lead of project).
9	Serious eye damage/ eye irritation	Short Time Exposure (STE) test method for the detection of chemicals causing serious eye damage and chemicals not requiring classification for serious eye damage or eye irritation, as included in OECD TG 491.	Adopted as a new TG in 2015; updated version to include a new reference to OECD GD 236 on an IATA for Serious Eye Damage and Eye Irritation adopted in 2017.
10	Serious eye damage/ eye irritation	Reconstructed human Cornea-like Epithelium (RhCE) test methods for the detection of chemicals not requiring classification and labelling for eye irritation or serious eye damage, as included in OECD TG 492.	Adopted as a new TG in 2015; updated version to include SkinEthic™ HCE EIT and a new reference to OECD GD 236 on an IATA for Serious Eye Damage and Eye Irritation adopted in 2017; updated version to include the Labcyte CORNEA-MODEL 24 EIT adopted in 2018.
11	Skin sensitisation	*In chemico* skin sensitisation: Direct Peptide Reactivity Assay (DPRA), as included in OECD TG 442C.	Adopted as a new TG in 2015.
12	Skin sensitisation	Key-event based Test Guideline 442D: *In vitro* skin sensitisation assays addressing the AOP key event on keratinocyte activation.	Adopted as a new TG in 2015; updated version to include the ARE-Nrf2 Luciferase KeratinoSens™ test method using animal-free serum and the ARE-Nrf2 Luciferase LuSens test method adopted in 2018.
13	Skin sensitisation	*In Vitro* Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitisation, as included in OECD TG 442E.	Adopted as a new TG in 2016 as "*In Vitro* Skin Sensitisation: human Cell Line Activation Test (h-CLAT)"; updated version to include U-SENS™ and IL-8 Luc test methods adopted in 2017. The TG was revised to an "OECD Test Guideline for the Testing of Chemicals Based on Key events", grouping the three adopted test methods addressing key event 3 within the existing AOP into one single TG.

Nr.	Toxicity area	Test method description	Acceptance status
14	Carcinogenicity	*In vitro* Syrian Hamster Embryo (SHE) Cell Transformation Assay (CTA) as included in OECD GD No. 214[1].	Adopted as a new GD in 2015.
15	Carcinogenicity	*In vitro* Bhas 42 Cell Transformation Assay (CTA) as included in OECD GD no 231[1].	Adoption as a new GD in 2016.
16	Genotoxicity	*In vitro* Mammalian Chromosome Aberration Assay as included in OECD TG 473.	Updated OECD TG 473 (originally adopted in 1983) adopted in 2014. Updated in 2016 to reference the Guidance Document on genetic toxicology Test Guidelines.
17	Genotoxicity	*In vitro* Mammalian Cell Micronucleus Assay as included in OECD TG 487.	Updated OECD TG 487 (originally adopted in 2010) adopted in 2014. Updated in 2016 to reference the Guidance Document on genetic toxicology Test Guidelines.
18	Genotoxicity	*In vitro* Mammalian Cell Gene Mutation Test using *Hprt* and *xprt* genes as included in OECD TG 476.	OECD TG 476 (originally adopted in 1984) "*In vitro* Mammalian Cell Gene Mutation Test" has been split up into two TGs: 1. The updated TG 476 now using the *Hprt* and *xprt* genes was adopted in 2015; 2. OECD TG 490 using t*hymidine kinase* Gene was adopted in 2015. Both TGs were updated in 2016 to reference the Guidance Document on genetic toxicology Test Guidelines and TG 490 was also corrected (see below).
19	Genotoxicity	*In vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene as included in OECD TG 490.	Adopted as TG 490 in 2015 (see above). Updated in 2016 to reference the Guidance Document on genetic toxicology Test Guidelines and to correct a paragraph related to the maximum concentration that is based on cytotoxicity.
20	Endocrine disruption	H295R Steroidogenesis Assay.	Adopted as TG 456 in 2011.
21	Endocrine disruption	Estrogen receptor transactivation assay (BG1Luc ER TA; agonist and antagonist protocols) as included in OECD TG 457.	Adopted in 2012. OECD TG 457 was deleted in 2015. The method was included in OECD TG 455 in 2012 (agonist part) and 2015 (antagonist part) (see table entry below).

1 These test methods were initially proposed to be included in Test Guidelines. It was later decided to include them in Guidance Documents.

Nr.	Toxicity area	Test method description	Acceptance status
22	Endocrine disruption	Performance-Based Test Guideline for Stably Transfected Transactivation *In vitro* Assays to Detect Estrogen Receptor Agonists and Antagonists as included in OECD TG 455.	OECD 455 adopted in 2009 (STTA assay using the hERα-HeLa-9903 cell line); updated version (PBTG, inclusion of VM7Luc ER TA assay using the VM7Luc4E2 cell line) adopted in 2012; Second updated version, including the antagonist part of both methods was adopted in 2015. This update led to the deletion of OECD TG 457 in parallel as it is no longer needed (see above). Third updated version to include the ER-CALUX method (using a U2OS cell line) was approved in 2016.
23	Endocrine disruption	Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) *In vitro* Assays to Detect Chemicals with ER Binding Affinity as included in OECD TG 493.	Adopted as new TG in 2015. It includes two reference test methods: • *In Vitro* Estrogen Receptor (ER) Binding Assay Using a Full Length Human Recombinant ERα; • *In Vitro* Estrogen Receptor Binding Assay Using a Human Recombinant Ligand Binding Domain Protein
24	Endocrine disruption	Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity as included in OECD TG 458.	Adopted as new TG in 2016. The method uses the AR-EcoScreen™ cell line.
25	Acute fish toxicity	Fish Embryo Acute Toxicity (FET) Test[2] as included in OECD TG 236.	Adopted in 2013..
26	Fish bioaccumulation	Determination of *in vitro* intrinsic clearance using cryopreserved rainbow trout hepatocytes as included in OECD TG 319A.	Adopted in 2018. *In vitro* derived clearance data can be used to improve the prediction of *in silico* methods to derive a bioconcentration factor as a means for bioaccumulation.
27	Fish bioaccumulation	Determination of *in vitro* intrinsic clearance using rainbow trout liver S9 sub-cellular fraction as included in OECD TG 319B.	Adopted in 2018 *In vitro* derived clearance data can be used to improve the prediction of *in silico* methods to derive a bioconcentration factor as a means for bioaccumulation.

2 The Fish Embryo Acute Toxicity Test is, strictly speaking, not an *in vitro* test, as it uses fish embryos which are not covered by Directive 2010/63/EU.

Annex 2 — ICATM Alternative Test Methods Validation and Status of Regulatory Acceptance

Table 2. ICATM Alternative Test Methods Validation and Status of Regulatory Acceptance.

Method	Current Status	Lead Organisation	International Acceptance
		Dermal Corrosion Test Methods	
CORROSITEX Skin Corrosion Test	**Completed**		**OECD TG 435 (2006)** Updated version (including the Corrositex® prediction model, the deletion of the performance standards (to be published separately on the Series on Testing and Assessment), including paragraphs referring to the IATA for Skin Corrosion and Irritation in OECD GD No. 203 and the updating of the list of proficiency substances) adopted in 2015.
EpiSkin™, EpiDerm™ SCT, SkinEthic™ RHE, epiCS® Skin Corrosion Tests	**Completed**		**OECD TG 431 (2004)** Updated version (sub-categorisation, inclusion of performance standards, inclusion of SkinEthic™ RHE and epiCS™) adopted in 2013. Revised version including the sub-categorization with the epiCS™ test method adopted in 2014. Updated version [deleting the performance standards (published separately on the Series on Testing and Assessment No. 219), including paragraphs referring to the IATA for Skin Corrosion and Irritation in OECD GD No. 203 and including the use of HPLC/UPLC-spectrophotometry as an alternative procedure to measure tissue viability (increasing the applicability domain of the test methods to coloured substances interfering with the measurement of MTT-formazan)] adopted in 2015. Updated in 2016 for improving the predictive capacity of the three validated *in vitro* skin corrosion test methods (EpiDermTM SCT, SkinEthicTM RHE and epiCS®) for the correct prediction of Sub-Cat.1A.

Method	Current Status	Lead Organisation	International Acceptance
Rat TER Skin Corrosion Test	**Completed**		**OECD TG 430 (2004)** Updated version (inclusion of performance standards) adopted in 2013. Updated version [deleting the performance standards (published separately on the Series on Testing and Assessment No. 218) and including paragraphs referring to the IATA for Skin Corrosion and Irritation in OECD GD No. 203] adopted in 2015.
***In vitro* Reconstructed human Epidermis (RhE) test methods: LabCyte EPI-MODEL24 SCT**	JSAAE sponsored validation study was completed in early 2018. The peer review was completed in October 2018.	JaCVAM	Included in the OECD TGP work plan in 2018.
Dermal Irritation Test Methods			
***In vitro* Reconstructed human Epidermis (RhE) test methods: EpiSkin™, Epi-Derm™ SIT, SkinEthic™ RHE and LabCyte EPI-MODEL24 SIT**	**Completed**		**OECD TG 439 (2010)** Updated version (including the LabCyte EPI-MODEL24 SIT) adopted in 2013. Updated version [deleting the performance standards (published separately on the Series on Testing and Assessment No. 220), including paragraphs referring to the IATA for Skin Corrosion and Irritation in OECD GD No. 203 and including the use of HPLC/UPLC-spectrophotometry as an alternative procedure to measure tissue viability (increasing the applicability domain of the test methods to coloured substances interfering with the measurement of MTT-formazan)] adopted in 2015.
In vitro reconstructed human epidermis (RhE) test methods: Korean epidermis model	KoCVAM sponsored validation study is ongoing	KoCVAM	
In vitro reconstructed human full-thickness model test methods: LbL model	JSAAE sponsored validation study is ongoing	JaCVAM	

Method	Current Status	Lead Organisation	International Acceptance
		Phototoxicity Test Methods	
3T3 NRU Phototoxicity Test	**Completed**		**OECD TG 432 (2004).** Updated version is undergoing commenting. **ICH S10 (2014).**
Test method battery to predict phototoxicity (yeast growth inhibition phototoxicity assay and red blood cell photohemolysis assay)	Japanese Regulatory Acceptance Board recommended additional work be performed.	JaCVAM	
***In vitro* test method based on reactive oxygen species (ROS) and photostability**	**Completed**	JaCVAM	**ICH S10 (2014)** Included in the OECD TGP work plan in 2016. The draft TG is undergoing commenting..
		Ocular Toxicity Test Methods	
Bovine Corneal Opacity and Permeability (BCOP) Test Method	**Completed**		**OECD TG 437 (2009)** Updated version (positive control, use in a bottom-up approach to identify non-classified chemicals and several other revisions) adopted in 2013. Updated version to include a new reference to OECD GD 236 on an IATA for Serious Eye Damage and Eye Irritation adopted in 2017. Updated version under consideration to include a laser light-based opacitometer and histopathological examination to revise the Decision Criteria for classification of chemicals requiring classification for eye hazard.

Method	Current Status	Lead Organisation	International Acceptance
Isolated Chicken Eye (ICE) Test Method	**Completed**		**OECD TG 438 (2009)** Updated version (use in a bottom-up approach to identify non-classified chemicals and several other revisions) adopted in 2013. Updated version to include a new reference to OECD GD 236 on an IATA for Serious Eye Damage and Eye Irritation adopted in 2017**.** Updated version to include histopathological examination as additional endpoint to identify UN GHS cat.1 non-extreme pH (2<pH<11.5) detergents and surfactants and modified decision criteria for chemicals requiring classification for eye hazard (taking into consideration the variability of the Draize eye test) adopted in 2018.
Use of Histopathology as an additional endpoint in Ocular Safety Testing	**Completed**		**OECD GD 160 (2011)** Updated version [including: (i) the recommendation for having an internal peer-review process when evaluating histopathological effects, (ii) the use of semi-quantitative scoring systems for *e.g.* the ICE histopathology, and (iii) inclusion of an Atlas describing typical ICE histopathological effects] adopted in 2017. Updated version to provide more specific and detailed guidance on the preparation of corneas, the scoring of histopathological findings and the peer-review process of the histopathological evaluation, in particular for Test Guideline 438, where this is an additional endpoint in certain well-defined cases, adopted in 2018.
Cytotoxicity test: SIRC CVS	Peer review coordinated by JaCVAM is ongoing	JaCVAM	
Cytotoxicity test: three-dimensional dermal model (MATREX)	JaCVAM-sponsored validation study stopped	JaCVAM	
Cytotoxicity test: Short Time Exposure (STE) test	**Completed**		**OECD TG 491 (2015)** Updated version to include a new reference to OECD GD 236 on an IATA for Serious Eye Damage and Eye Irritation adopted in 2017.

Method	Current Status	Lead Organisation	International Acceptance
Use of anaesthetics, analgesics, and humane endpoints for routine use in TG 405	**Completed**		**OECD updated TG 405 (2012)** Updated version to delete the "Testing and Evaluation Strategy for Eye Irritation/ Corrosion" and include a new reference to OECD GD 236 on an IATA for Serious Eye Damage and Eye Irritation adopted in 2017.
In vitro approach for categorisation of anti-microbial cleaning products: recommendations for further studies	Completed. EPA/OPP[3] has concluded from submission and review of alternative eye irritation tests conducted on antimicrobial pesticide products with cleaning claims (AMCPs) that the proposed testing approach is acceptable for determining the appropriate eye hazard classification and labelling for AMCPs (see https://www.epa.gov/pesticides for the details of the scope of the policy).	ICCVAM	
In vitro approach for categorisation of agrochemical formulations	Phase 1 testing completed (n=6 formulations) to demonstrate proof-of-concept using six *in vitro* assays [BCOP, ICE, Neutral Red Release assay, PoCORA and EpiOcular (OECD TG 492 and time-to-toxicity protocols]. Phase 2A (n=10 formulations) currently being planned and will include all assays from Phase 1. Testing will continue into 2019.	NICEATM	

3 Environmental Protection Agency/Office of Pesticide Programme

Method	Current Status	Lead Organisation	International Acceptance
Cytosensor Microphysiometer® (CM) Test method	The draft TG was submitted to OECD for comments including a set of Performance Standards	EURL ECVAM	New draft TG discussed at WNT in 2013, 2015 and 2016 but not adopted. The additional data requested by the WNT that should have been submitted by US to support the project were not received and thus the project has been discontinued because of lower priority for the EC. The project is no longer on the OECD Work Plan.
Fluorescein Leakage (FL) test method	**Completed**		**OECD TG 460 (2012)** Updated version to include a new reference to OECD GD 236 on an IATA for Serious Eye Damage and Eye Irritation adopted in 2017
Reconstructed human Cornea-like Epithelium (RhCE) **EpiOcular™ EIT; SkinEthic™ HCE EIT, Labcyte CORNEA MODEL 24 EIT**	**Completed**		**OECD TG 492 (2015)** Updated version to include SkinEthic™ HCE EIT and a new reference to OECD GD 236 on an IATA for Serious Eye Damage and Eye Irritation adopted in 2017. Updated version to include Labcyte CORNEA MODEL 24 EIT adopted in 2018.
Vitrigel-EIT	Validation and peer review coordinated by JaCVAM completed	JaCVAM	Included in the OECD TGP work plan in 2017. The draft TG is undergoing commenting
OptiSafe	Validation Study coordinated by NICEATM. Phase I (qualification and training of naïve laboratories) completed. Phase II partially completed: inter-laboratory study with 30 chemicals completed; testing of 60 chemicals in the lead laboratory ongoing.	NICEATM	

Method	Current Status	Lead Organisation	International Acceptance
In vitro reconstructed human Cornea-epithelium model (RhCE) test method: Korean Cornea-model	Validation study and KoCVAM-coordinated peer review completed	KoCVAM	Included in the OECD TGP work plan in 2018.
Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) Test Method	Validation study sponsored by Brazilian Ministry of Science, Technology Innovation and Communication (MCTIC). Preliminary phase of validation study finalised. Substances selection for phase 2 validation started.	BraCVAM	
Immunotoxicity (Allergic Contact Dermatitis) Test Methods			
Murine local lymph node assay (LLNA) for skin sensitization	**Completed**		**OECD TG 429 (2002)** **ISO (2002)**
Updated Murine local lymph node assay (LLNA) for skin sensitization (20% reduction)	**Completed**		**Update to TG 429 OECD (2010)** **ISO (2010)**
Reduced LLNA (rLLNA)	**Completed**		**Update to TG 429 OECD (2010)**
Nonradioactive LLNA protocol using BrdU ELISA or BrdU FCM method	**Completed**		**OECD TG 442B OECD (2010).** Updated version to include the BrdU FCM method adopted in 2018.

Method	Current Status	Lead Organisation	International Acceptance
Nonradioactive LLNA protocol, LLNA:DA	**Completed**		**OECD TG 442A OECD (2010)**
Harmonized performance standards for the LLNA	**Completed**		**Update to TG 429 OECD (2010)**
***In vitro* skin sensitisation assay (DPRA)**	**Completed**		**OECD TG 442C (2015)**
***In vitro* skin sensitisation assay (h-CLAT)**	**Completed**		**OECD TG 442E (2016)**. Draft updated TG442E under consideration to include an adaptation to use human serum/antibodies.
***In vitro* skin sensitisation assay KeratinoSens™**	**Completed**		**OECD TG 442D (2015)**. Updated version to include an adaptation to animal-free conditions adopted in 2018
***In vitro* skin sensitisation assay IL-8 Luc assay**	**Completed**		**OECD TG 442E (2017)**
***In vitro* skin sensitisation assay U937 Cell Line Activation Test (U-SENS™)**	**Completed**		**OECD TG 442E (2017)**
***In vitro* skin sensitisation assay LuSens**	**Completed**	EURL ECVAM	**OECD TG 442D (2018)**

Method	Current Status	Lead Organisation	International Acceptance
In vitro skin sensitisation assay SENS-IS	External validation study finalised	EURL ECVAM evaluation finalised. Revised submission awaited	Included in the OECD TG work plan in 2016
In vitro skin sensitisation assay Genomic Allergen Rapid Detection (GARDskin)	External validation study finalised	EURL ECVAM evaluation stopped.	Included in the OECD TG work plan in 2016
In vitro skin sensitisation assay Vitrigel-SST	MAFF[4]-sponsored validation study is pending	JaCVAM	
In vitro skin sensitisation assay, Amino acid derivative reactivity assay (ARDA)	JCIA[5] and JSAAE[6] validation study and peer review completed	JaCVAM	Included in the OECD TGP work plan in 2018. The draft TG is undergoing commenting.
IL-2 Luc assay for the evaluation of the immunotoxic potential of chemicals	JaCVAM validation study is ongoing. The experimental part was completed in 2017	JaCVAM	
Electrophilic allergen screening assay (EASA)	Validation study coordinated by NICEATM is currently ongoing; conversion of assay to 96-well format in progress; testing will continue into 2019	NICEATM	

4 Ministry of Agriculture, Forestry and Fisheries
5 Japan Cosmetic Industry Association
6 Japanese Society for Alternatives to Animal Experiments

Method	Current Status	Lead Organisation	International Acceptance
Defined approaches for skin sensitisation	ICATM partners developed and/ or evaluated defined approaches for skin sensitisation to support the development of an OECD guideline on Defined Approaches		
IL-1β Luc assay for the evaluation of the immunotoxic potential of chemicals	JaCVAM validation study is ongoing	JaCVAM	
In vitro skin sensitisation assay: EpiSensA	JaCVAM validation study is ongoing	JaCVAM	
Acute Toxicity Test Methods			
Up and Down Procedure (UDP)	**Completed**		**OECD TG 425 (2008)**
***In vitro* cytotoxicity test methods for estimating starting doses for acute oral systemic toxicity tests**	**Completed**		**OECD GD 129 (2010)**
In vitro cytotoxicity test (3T3 Neutral Red Uptake) for identifying substances with acute oral LD50 > 2000 mg/kg b.w.	EURL ECVAM ESAC peer review completed, and EURL ECVAM Recommendation published in 2013	EURL ECVAM	

Method	Current Status	Lead Organisation	International Acceptance
EpiAirway human reconstructed lung epithelium for identifying acute inhalation toxicity	Validation study is ongoing. Testing in the lead laboratory underway and chemical selection for testing in two additional laboratories is in progress. ICCVAM agency representatives serving on the VMT	NICEATM/ ICCVAM	
The Collaborative Acute Toxicity Modeling Suite (CATMoS)	The ICCVAM Acute Toxicity Workgroup organised a global project to develop *in silico* models of acute oral systemic toxicity that predict five specific endpoints needed by regulatory agencies. Thirty-two international groups across government, industry, and academia built and submitted models. Outputs from the different models are now being combined to generate consensus predictions (via CATMoS) for the acute oral toxicity endpoints of interest.	NICEATM/ ICCVAM	
Toxicokinetic Test Methods			
In vitro hepatic biotransformation – CYP induction: Hepa RG and cryopreserved human hepatocytes	EURL ECVAM ESAC peer review completed, and EURL ECVAM manuscript in preparation	EURL ECVAM	SPSF for a PBTG approved in April 2013. Draft PBTG underwent a first commenting round in 2014. An OECD expert meeting was held in March 2015.

Method	Current Status	Lead Organisation	International Acceptance
		Endocrine Disruptor Test Methods	
Stably transfected human estrogen receptor-α transcriptional activation assay for detection of estrogenic agonist-activity of chemicals (STTA and BG1-Luc assays)	**Completed**		**OECD TG 455 (2009), updated 2012 and 2015**, inclusion of the antagonist protocols in addition to the agonist protocols, deletion of OECD TG 457 in parallel as it is no longer needed
H295R Steroidogenesis assay	**Completed**		**OECD TG 456 (2011)**
BG1Luc® human estrogen receptor transcriptional activation assay: agonist and antagonist protocols	**Completed**		**OECD TG 457 (2012)** TG 457 has been deleted in parallel to TG 455 updates (see previous table entry)
CertiChem MCF-7 cell proliferation assay for the detection of human estrogen receptor agonists and antagonists	International validation study completed. Protocol must be revised for adequate transferability.	NICEATM	Not in the OECD TGP work plan anymore
CertiChem MDA-Kb2 assay for the detection of human androgen receptor agonists and antagonists	NICEATM coordinated single lab validation study completed. Summary report in preparation.	NICEATM	

Method	Current Status	Lead Organisation	International Acceptance
Stably transfected CHO Androgen receptor-α transcriptional activation assay for detection of androgenic agonist and antagonist activity of chemicals (AR-STTA)	**Completed**		**OECD TG 458 (2016)**
MELN® human estrogen receptor transcriptional activation assay: agonist and antagonist protocols	Validation stopped		
Stably Transfected Transactivation *in vitro* Assay to detect Androgen Receptor Agonists and Antagonists	Validation study ongoing	EURL ECVAM	Included in the OECD TGP work plan in April 2013
AR-CALUX Stably Transfected Transactivation *in vitro* Assay to detect Androgen Receptor Agonists and Antagonists	EU-NETVAL validation study ongoing	EURL ECVAM	Included in the OECD TGP work plan in April 2013
Transactivation assay for the detection of compounds with (anti)androgenic potential using 22Rv1/MMTV cells	Validation study ongoing	Ministry of Food and Drug Safety (MFDS) South Korea	

Method	Current Status	Lead Organisation	International Acceptance
Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) In Vitro Assays to Detect Chemicals with ER Binding Affinity	**Completed**		**OECD TG 493 (2015)**
CHO-K1 cells thyrotropin-releasing hormone (TRH) receptor activation (beta-galactosidase) measuring agonist and antagonist activities	Preparation for EU-NETVAL Validation study ongoing	EURL ECVAM	In the context of the detection of chemicals with thyroid disrupting potential as called for by the OECD[7]
CHO-K1 cells thyrotropin-stimulating hormone (TSH) receptor activation based on cAMP measurement			
Thyroperoxidase (TPO) inhibition based on oxidation of Amplex UltraRed			
Thyroperoxidase (TPO) inhibition based on oxidation of Luminol			
Tyrosine iodination using liquid chromatography			

7 http://www.oecd.org/chemicalsafety/oecd-encourages-development-of-non-animal-test-methods-for-detection-of-thyroid-disrupters.htm

Method	Current Status	Lead Organisation	International Acceptance
Activation of the sodium iodide symporter (NIS) based on Sandell-Kolthoff reaction	Preparation for EU-NETVAL Validation study ongoing	EURL ECVAM	In the context of the detection of chemicals with thyroid disrupting potential as called for by the OECD
Thyroxine-binding prealbumin (TTR) / thyroxine-binding globulin (TBG) using fluorescence displacement (ANSA)			
Thyroxine-binding prealbumin (TTR) using fluorescence displacement (T4-FITC)			
Deiodinase 1 activity based on Sandell-Kolthoff reaction			
Glucuronidation of thyroid hormones (THs) using liquid chromatography/mass spectrometry (LC/MS)			
Inhibition of thyroid hormones (THs) sulfation using liquid chromatography			

Method	Current Status	Lead Organisation	International Acceptance
Inhibition of monocarboxylate transporter 8 (MCT8) based on Sandell-Kolthoff reaction	Preparation for EU-NETVAL Validation study ongoing	EURL ECVAM	In the context of the detection of chemicals with thyroid disrupting potential as called for by the OECD
Human thyroid hormone receptor alpha (TRα) and Human thyroid hormone receptor beta (TRβ) reporter gene transactivation measuring agonist and antagonist activities			
CALUX human thyroid hormone receptor beta (TRβ) reporter gene transactivation measuring agonist and antagonist activities			
Measurement of intrafollicular thyroxine (T4) using zebrafish eleutheroembryos			
Measurement of proliferation of rat pituitary-derived cell line GH3			
Proliferation, migration and oligodendrocyte differentiation of human neural progenitor cells			

Method	Current Status	Lead Organisation	International Acceptance
		Genetic Toxicity Test Methods	
***In vitro mammalian cell* micronucleus test**	**Completed**		**OECD TG 487 (2010), updated TG adopted in 2014**
***In vitro mammalian cell* chromosome aberration assay[8]**	**Completed**		**OECD TG 473 (1997), updated TG adopted in 2014**
***In vivo* comet assay**	**Completed**		**OECD TG 489 (2014)**
In vitro comet assay	Validation study for the *in vitro* comet assay stopped	JaCVAM	
Genotoxicity assays (micronucleus and comet) in 3D skin models	Validation study ongoing	Cosmetics Europe (lead); EURL ECVAM support	
		Carcinogenicity Test Methods	
In vitro Bhas 42 cell transformation assay (CTA)	**Completed**		**OECD GD 231 (2016)**
In vitro Syrian hamster embryonic cells (SHE) cell transformation assays (CTAs)	**Completed**		**OECD GD 214 (2015)**

8 The *in vitro* mammalian cell chromosome aberration assay has not been validated by any of the ICATM partners. It is added here for completeness as it has been adopted as an OECD TG.

Method	Current Status	Lead Organisation	International Acceptance
		Reproductive Test Methods	
Hand-1 Luc assay	METI[9] -sponsored validation is completed. The peer review is on-going	JaCVAM	Included in the OECD TGP work plan in 2017. The 1st step of the project consists in drafting a Detailed Review Paper of available methods and the evaluation of utility and application of the Hand-1 Luc assay; The 2nd step consists in a feasibility study on the development of a Test Guideline.
Mouse Embryoid Bodies (mEBT) assay	KoCVAM sponsored validation study is ongoing	KoCVAM	
		Developmental Neurotoxicity Test Methods	
Pluripotent Stem Cells differ-entiation into neural precursor cells/neural stem cells NPC/NSC (embryonic phase)	Retrospective validation according to defined readiness criteria. Prospective validation ongoing	EFSA/EC (through EURL ECVAM)/OECD	Development of an OECD Guidance Document (GD) on the use of an *in vitro* testing battery for DNT. Included in the OECD TGP work plan in 2017. Validation of *in vitro* test methods based on experimental testing ongoing. Drafting GD based on existing and newly produced data ongoing
Human neural precursor cells (hNPC) proliferation	Retrospective validation according to defined readiness criteria. Prospective validation ongoing		
hNPC migration	Retrospective validation according to defined readiness criteria. Prospective validation ongoing		
hNPC neuronal differentiation	Retrospective validation according to defined readiness criteria.		

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Method	Current Status	Lead Organisation	International Acceptance
hNPC differentiated neurons	Retrospective validation according to defined readiness criteria	EFSA/EC (through EURL ECVAM)/OECD	Development of an OECD Guidance Document (GD) on the use of an *in vitro* testing battery for DNT. Included in the OECD TGP work plan in 2017. Validation of *in vitro* test methods based on experimental testing ongoing. Drafting GD based on existing and newly produced data ongoing
hNPC oligodendrocyte different.	Retrospective validation according to defined readiness criteria. Prospective validation ongoing		
hNPC oligodendrocyte maturation and thyrod hormone disruption	Retrospective validation according to defined readiness criteria.		
Neural Crest Cells (NCC) proliferation and migration (cMINC)	Retrospective validation according to defined readiness criteria. Prospective validation ongoing		
Morphological differentiation of embryonic stem cells (ESC to neurons)	Retrospective validation according to defined readiness criteria		
Differentiation towards astrocytes, oligodendrocytes, myelination, microglia in 3D rat model	Retrospective validation according to defined readiness criteria		
Differentiation towards astrocytes, oligodendrocytes, myelination, microglia in 3D human foetal phase model	Retrospective validation according to defined readiness criteria		

Method	Current Status	Lead Organisation	International Acceptance
Neurite outgrowth of central neurons,	Retrospective validation according to defined readiness criteria. Prospective validation ongoing	EFSA/EC (through EURL ECVAM)/OECD	Development of an OECD Guidance Document (GD) on the use of an *in vitro* testing battery for DNT. Included in the OECD TGP work plan in 2017. Validation of *in vitro* test methods based on experimental testing ongoing. Drafting GD based on existing and newly produced data ongoing
Neurite outgrowth of peripheral neurons	Retrospective validation according to defined readiness criteria. Prospective validation ongoing		
Neuronal sub-type ratio, neuronal maturation	Retrospective validation according to defined readiness criteria		
Synaptogenesis	Retrospective validation according to defined readiness criteria		
Neuronal network formation and function	Retrospective validation according to defined readiness criteria. Prospective validation ongoing		
Zebrafish assays	Retrospective validation according to defined readiness criteria		
Acute Aquatic Toxicity Test Methods			
Zebrafish Embryo Acute Toxicity test (ZFET)	**Completed**		**OECD TG 236 (2013)**

Method	Current Status	Lead Organisation	International Acceptance
		Fish Bioaccumulation Test Methods	
Determination of *in vitro* intrinsic clearance using cryopreserved rainbow trout hepatocytes as included in OECD TG 319A **Determination of *in vitro* intrinsic clearance using rainbow trout liver S9 sub-cellular fraction as included in OECD TG319B**	**Completed**	EURL ECVAM and US EPA	**OECD TG 391A and 391B (2018)**
		Various	
Zebrafish assays	NTP SEAZIT: the Systematic Evaluation of the Application of Zebrafish in Toxicology (SEAZIT) program, jointly led by NTP and NICEATM scientists. Summarized below are four key SEAZIT program activities: • Zebrafish information gathering • A webinar series focused on the use of informatics to improve data analysis for zebrafish screening studies (2017) • A n interlaboratory zebrafish study (2018) • A zebrafish best practices workshop	NICEATM	

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