EURL ECVAM Status Report

on the Development, Validation and Regulatory Acceptance of Alternative Methods and Approaches (2017)
Status of Alternative Approaches to Animal Testing

Abstract

EU legislation encourages a shift away from animal testing and the use of new advanced methodologies without reducing human and environment protection.

Cell and tissue-based methods are integrated with computational modelling in testing and assessment strategies for a more mechanistic-based safety assessment of chemicals and products.

Alternative methods and approaches are also promoted and disseminated in areas of basic and applied research in life sciences.
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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). Every year, EURL ECVAM produces a Status Report to inform its stakeholders about the progress being made in the development, validation and regulatory acceptance of alternative methods and approaches. The report describes primarily, but not exclusively, all the activities that EURL ECVAM has undertaken or has been involved in since the publication of the previous report (November 2016).

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 Community Strategy on combined exposures ‘Mixtures’ (Bopp et al., 2015; Kienzler et al., 2016a) and the Community Strategy on Endocrine Disrupters (Bremer et al., 2012; Munn et al., 2016) 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.

Research and development activities continue to be funded within large European collaborative programmes such as the H2020 flagship project EU-ToxRisk which is tackling complex health effects (endpoints) such as repeated-dose and reproductive toxicity. Research being undertaken aims to integrate scientific and technical advances in experimental and computational biology/toxicology to probe the complex chains of events that link chemical exposure to potential adverse outcomes in humans and environmental species. At international level, there is a growing impetus to explicitly use mechanistic reasoning to devise hypothesis-driven Integrated Approaches to Testing and Assessment (IATA) that incorporate multiple data streams derived from in vitro methods, ‘omics techniques and computational modelling. In support of this, the Adverse Outcome Pathway (AOP) development programme at the OECD continues to play a key role in providing a framework to structure and synthesise scientific knowledge on toxicological processes to make it suitable and freely accessible for chemical risk assessment purposes.

At the level of validation and standardisation of non-animal approaches, there is growing interest to evolve the definition and utility of performance standards for assessing classes of methods that provide similar (mechanistic) information, and to move towards evaluation frameworks for so-called Defined Approaches (DA), that combine non-animal methods together with a rule-based data interpretation procedure to arrive at a conclusion in support of a regulatory decision. A related initiative at the OECD is exploring how scientifically valid DAs can be put on an equal footing with conventional in vivo Test Guidelines in order to satisfy requirements for the Mutual Acceptance of Data between OECD Member Countries. Mutual Acceptance of Data is the main instrument for ensuring global harmonisation of chemical testing to minimise the use of animals and reduce costs.
For health effects such as skin irritation, skin corrosion, eye irritation, serious eye damage and skin sensitisation, IATA frameworks have already been adopted at international level. In addition, an informal working group was set up under the United Nations Sub-Committee of experts on the Globally Harmonised System of Classification and Labelling of Chemicals (GHS) to explore the use of non-animal methods for toxicological hazard classification. For other areas including non-genotoxic carcinogenicity, developmental neurotoxicity, endocrine disruption, fish bioaccumulation testing, repeated dose toxicity and reproductive toxicology, steady progress is being made and the work is at different stages of development or validation.

The EU flagship project VAC2VAC is progressing the development, optimisation and evaluation of non-animal methods for routine batch testing of vaccines in order to avoid severe animal testing.

Finally, EURL ECVAM is working with its stakeholders to pursue opportunities to enhance knowledge sharing across sectors and communities to accelerate overall progress in the Three Rs in various domains including regulatory testing, basic, applied and translational research, and education and training.
Every year, EURL ECVAM prepares a Status Report with the primary purpose to inform its stakeholders and all interested parties (public, press, etc.) on updates on the status of alternative methods and approaches.

The EURL ECVAM status report provides updates on activities since the last report published in November 2016. It reports on research, development and validation activities, as well as on activities which promote the regulatory and international adoption and use of alternative approaches and their dissemination.

It describes primarily, but not exclusively, all the activities that EURL ECVAM has undertaken or has been involved in since the publication of the last report.
2 Research and Development Activities on Alternative Methods

Under Horizon 2020, EURL ECVAM is collaborating on projects which develop animal-free strategies for the risk assessments of chemicals and chemical mixtures from different perspectives, evaluate the progress achieved in the area and identify further gaps to be still addressed.

The work of the EURL ECVAM laboratory directly supports the mandate given by Directive 2010/63/EU on the protection of animals used for scientific purposes to coordinate and promote the development of alternative methods.

In this chapter current ongoing studies are described including collaborative work on projects for example with the consortium of the H2020 project EU-ToxRisk and the US Environmental Protection Agency (US EPA). Several Research & Development projects related to fish toxicity and bioaccumulation, which are of specific interest to EURL ECVAM, are also described in this chapter.

The project “Vaccine batch to vaccine batch comparison by consistency testing” (VAC2VAC) focuses on the development and validation of non-animal methods for vaccine quality control.
2 Research and Development Activities on Alternative Methods

2.1 SEURAT-1 Chemical Safety Assessment Workflow

In the framework of the European Commission and Cosmetics Europe 50 million euro-funded research initiative SEURAT-1, a workflow was designed to be applicable to cosmetic ingredients as well as to other types of chemicals, e.g., active ingredients in plant protection products, biocides or pharmaceuticals.

The intention was to develop an exposure driven workflow to assess chemical safety without relying on any animal testing, but instead constructing a hypothesis based on existing data, in silico modelling, biokinetic considerations and prove it through targeted non-animal testing. The outcome was recently published and is freely accessible on-line (Berggren et al., 2017).

The workflow is divided into tiers including a series of possible exit (decision) points, e.g., application of the Threshold of Toxicological Concern (TTC) approach might be used for low concentrations or read-across, in case the target chemical is "similar enough" to (an) already tested and assessed substance(s).

An evaluation and update of the TTC concept for application to cosmetics-related substances has also been carried out within one of the SEURAT-1 projects, COSMOS (Yang et al., 2017; Williams et al., 2016). If none of these ‘short cuts’ (based on structural information and existing animal data) can be applied, the chemical safety is determined entirely by new approach methods and in vitro to in vivo extrapolation by means of mathematical modelling.

Figure 2.1: Workflow for the safety assessment of chemicals without animal testing (From Berggren et al., 2017, Computational Toxicology, 4, p. 33, under CC BY-SA 4.0).
The workflow (see Figure 2.1) is a tool to inform targeted and toxicologically relevant in vitro testing, by structuring knowledge and data in a logical sequence for an integrated chemical safety assessment relying specifically on alternative methods and based on exposure considerations. Thus it is a means to gain confidence in safety decision making without the need for animal testing.

This "ab initio" workflow was also discussed from the perspective of ‘Integrated Approaches for Testing and Assessment (IATA)’ in the OECD IATA Case Studies Project (see section 5.1.1). The chemical safety assessment workflow is thus an example of a living legacy from the SEURAT-1 Cluster, which has been taken up for further development by other ongoing research initiatives.

2.2 EU-ToxRisk
EU-ToxRisk, the Integrated European ‘Flagship’ Programme Driving Mechanism-based Toxicity Testing and Risk Assessment for the 21st century, is a European collaborative project funded by the EU Framework Programme for Research and Innovation, Horizon 2020.

The project started on 1 January 2016 and will stay active for six years, with a budget of over 30 million €. The vision of EU-ToxRisk is to progress towards an animal-free toxicological assessment based on human cell responses and a comprehensive mechanistic understanding of cause-consequence relationships of chemical adverse effects.

EU-ToxRisk integrates advancements in cell biology, ‘omics technologies, systems biology and computational modelling to define the complex chains of events that link chemical exposure to toxic outcome, and is a successful continuation of the prior FP7 research initiative SEURAT-1.

In fact, EURL ECVAM hosted in November 2016 a workshop where SEURAT-1 got the opportunity to hand-over to EU-ToxRisk, communicating their main achievements and lessons learned (see section 4.11 and Box 2.1). EU-ToxRisk presented their case studies and made a reality check of their regulatory relevance, by discussing with the EURL ECVAM regulatory network, PARERE, and its stakeholder forum, ESTAF, invited to the workshop.

The EU-ToxRisk first eight case studies have thereafter progressed considerably. Strong collaborations have been established with the US Tox21 programme and the European Commission, through the Joint Research Centre and its EURL ECVAM. There is a common belief that progress will be faster working together, and as much as possible ensure complementary and cross-fertilisation between different initiatives.

EURL ECVAM’s PARERE network has officially been consulted on the four more advanced case studies within EU-ToxRisk and the outcome of that consultation will be discussed at the PARERE meeting on 27 to 28 November 2017.
EU-ToxRisk also organised its first winter school in February 2017.

### 2.3 European Research Projects on Chemical Mixtures

Several large research projects in the area of chemical mixtures have started in recent years in which EURL ECVAM is participating.

**EuroMix** is a Horizon 2020 project that started in 2015. EuroMix aims to develop an experimentally verified, tiered strategy for the risk assessment of mixtures of multiple chemicals derived from multiple sources. Important concepts for this new strategy are prioritisation criteria for chemicals based on their exposure and hazard characteristics and evaluation of the role of mode of action in grouping chemicals into cumulative assessment groups.

**EDC-MixRisk**, which started in 2015, focuses on integrating epidemiology and experimental biology to improve risk assessment of exposure to mixtures of endocrine disruptive compounds (EDCs). EDC-MixRisk investigates risks for multiple adverse health outcomes based on molecular mechanisms involved after early life exposure to EDC mixtures.

**HBM4EU** was launched in December 2016. It is a joint effort of 26 countries and the European Commission, co-funded by Horizon 2020. The interdisciplinary project contributes to: i) Identification of mixtures of EDCs that are associated with multiple adverse health outcomes; ii) Identification of molecular mechanisms and pathways underlying these associations; and iii) Development of methods for risk assessment of EDC mixtures to increase societal impact.

The workshop was organised together with the recently concluded FP7 research initiative SEURAT-1 and the new Horizon 2020 research project EU-ToxRisk. It brought together over 70 participants from across the EU including experts from EURL ECVAM, the two research consortia, the EURL ECVAM regulatory network, PARERE, and the EURL ECVAM stakeholder forum, ESTAF.

The scope of the meeting was to address strengths and limitations of new approach methods (NAMs) emerging from scientific research and to understand how NAMs can be translated more efficiently and effectively into regulatory use. A selection of case studies exploring the utility of NAMs for different predictive toxicology and safety assessment scenarios, which have been developed within the SEURAT-1 and EU-ToxRisk consortia, were used to fuel the discussions and trigger debate. There was general agreement that going forward, such case studies can serve as a very useful vehicle for continued engagement between developers, end-users and regulatory safety assessors.
main aim of the initiative is to coordinate and advance human biomonitoring in Europe in order to provide better evidence of the actual exposure of citizens to chemicals and the possible health effects to support policy making. One of the work packages is specifically on mixtures. This focuses on defining priority mixtures and real-life exposure patterns, and further developing practical approaches to identify and assess potential health risks and impacts of mixtures.

These projects, together with EU-ToxRisk (described in section 2.2), linked up to discuss risk assessments of mixtures from the different perspectives, to evaluate the progress achieved and identify further gaps to be still addressed. A workshop on these projects involving several Commission services and EU agencies is currently being prepared with EURL ECVAM and will be hosted at JRC in May 2018.

2.4 The VAC2VAC Project
The VAC2VAC project - ‘Vaccine batch to vaccine batch comparison by consistency testing’ is funded under the Innovative Medicines Initiative 2 (IMI 2), a joint undertaking of the European Union’s Horizon 2020 research and innovation programme and the European Federation of Pharmaceutical Industries and Associations (EFPIA).

It was officially launched on 1 March 2016 and brings together 21 public and private partners including the JRC represented by EURL ECVAM. The project is focused on the use of the consistency approach for quality control of established vaccines for human and veterinary use. The consistency approach moves away from the current focus on final product control often relying on animal testing. In the light of this, VAC2VAC partners are developing, optimising and evaluating non-animal methods, e.g., physicochemical and immunochemical methods, cell-based and other assays 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 this role, EURL ECVAM organised with VAC2VAC partners a workshop to discuss ways of improving multi-centre validation studies and making use of the data generated for product-specific validation purposes.

The workshop was held at JRC (Ispra, Italy) on 30 January to 1 February 2017 and involved 30 experts from veterinary and human vaccine manufacturers, official medicines control laboratories, academia, translational research organisations, and vaccinology alliances. The summary of the discussions and recommendations will be published in 2018.

2.5 In-house and Collaborative Experimental Studies in support of the EURL ECVAM Mandate
The EURL ECVAM Laboratory is designed to carry out typical cell biology/toxicology procedures which include cell banking (cryopreservation), cell culturing, cell characterisation (e.g., morphological or functional assessment), cell treatment (e.g., with reference toxicants), and cell-response analysis (e.g., a variety of technologies including high content cellular imaging, electrophysiological measurements, analytical (bio-) chemistry including Liquid Chromatography-Mass Spectrometry (LC-MS), and flow cytometry).

The Laboratory includes a number of automated (robotic) platforms for liquid handling and cell culturing which enable higher throughput and better reliability when testing larger sets of chemicals. The laboratory directly supports EURL ECVAM mandate as stated in Directive 2010/63/EU (EU, 2010) and also serves in collaboration projects such as those with the US Environmental Protection Agency (EPA) and the H2020 consortium EU-ToxRisk. Some examples of experimental studies are given below.

An in vitro metabolic hepatic clearance method (Lostia et al., 2016) was implemented to respond to the increasing demand to integrate kinetics information in toxicity testing. The results and experience gained in the project will support the drafting of an OECD Guidance Document with the objective to characterise and describe in vitro hepatic metabolic clearance methods (see section 5.2.11).
In the frame of the detection of chemicals with thyroid disrupting potential, EURL ECVAM is coordinating a validation study using a set of selected, mechanistically informative in vitro methods in collaboration with EU-NETVAL (see section 4.10 and Box 4.1). Some of these methods (e.g., the ANSA (8-anilino naphthalene sulfonic acid ammonium salt) displacement assay) have already been implemented in house and assessed.

A study on oxidative stress, one of the cellular responses determined at early stages of toxicity, was performed (Pistollato et al., 2016; Pistollato et al., 2017a,b). Nrf2 signalling activation upon induction of oxidative stress was assessed in neuronal and glial cells derived from hiPSCs treated with rotenone, an inhibitor of complex I of the mitochondrial respiratory chain. These studies indicate that analysis of Nrf2 signalling activation could serve as a basis for the establishment of a horizontal assay to assess oxidative stress induction (see Box 2.3).

2.6 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.

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

As a follow-up to the CELISens project (Tanneberger et al., 2013) a ring trial evaluating the transferability and within-laboratory reproducibility of the RTgill-W1 (rainbow trout gill cell line) cytotoxicity assay has been organised by the Swiss Federal Institute of Aquatic Science and Technology (EAWAG; K. Schirmer; Cefic Long-Range Research Initiative [LRI] project ECOB.3-NC3Rs-EAWAG). A paper on the outcome of the ring trial is in preparation.

An ISO guideline (ISO/CD 21115) “Water quality - Determination of acute toxicity of chemicals and water samples to a fish gill cell-line (RTgill-W1)” is in preparation.

2.6.2 Development of Adverse Outcome Pathways for Chronic Fish Toxicity Testing

Several research groups are working on the identification and description of potential Adverse Outcome Pathways (AOPs) relevant to...
chronic fish toxicity, which is currently assessed with a fish early life-stage (FELS) test (OECD TG 210, 2013a). As outcome of the Cefic LRI-funded project (LRI-ECO20-UA) 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.

The overall objective of the recently started follow-up project LRI-ECO20.2 is the validation of the assays developed for the two most promising AOPs, the thyroid AOP and the narcosis AOP. It is planned to test around 25 chemicals in vitro and to predict, based on the assay results, acute and chronic toxicity. The consortium further plans to perform, for a subset of the chemicals tested, acute (fish embryo toxicity test) and chronic fish toxicity tests (OECD TG 210, 2013a) to validate the predictions derived with the in vitro assays.

2.6.3 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 ILSI Health and Environmental Sciences Institute has been established who addressed over the last two years challenges relating to data collection, quality control, data characterisation and analysis as well as the development and application of useful eco-TTC concepts.

The working group developed a database of approximately 110,000 unique ecotoxicological records, 6,200 chemicals and 1,900 species from three trophic levels (fish, invertebrates, algae/plants) and an on-line analytical tool that aims to calculate, based on those data, eco-TTC value according to particular research criteria.

A workshop held in September 2017 brought together participants representing academia, industry, national competent authorities, and regulatory bodies to discuss and evaluate the eco-TTC database and web-based tools through a series of presentations and case studies on potential use of the eco-TTC concept for environmental risk assessment. During this workshop, some potential improvements were identified to make the tool more practical.

**Box 2.3**

**Human relevant in vitro models for developmental neurotoxicity testing**

Exposure of developing organisms to chemicals may affect the development of the nervous system. In recent years, neurodevelopmental disorders in children have increased significantly. Therefore, the JRC’s EUR ECVAMis working with international partners to address the pressing need to implement reliable models to test potentially harmful effects of environmental chemicals and investigate mechanisms of developmental neurotoxicity (DNT) underlying chemical exposure.

Modern toxicity screening approaches are focused on reducing and replacing animal testing with alternative and human relevant models. In this context, human induced pluripotent stem cells (hiPSCs) represent important tools for toxicity screening because of their unique characteristics.

In a recent JRC study (Zagoura et al., 2017), hiPSC neuronal derivatives were used to assess the neurotoxic effects of rotenone - a broad-spectrum pesticide - after short-term exposure (24 hours). Rotenone induced the activation of the Nrf2 signalling pathway, which regulates cellular response to oxidative stress. Oxidative stress is an important hallmark of various neurodegenerative diseases, including Parkinson’s disease.

Treatment with rotenone induced a progressive activation of the Nrf2 signalling, together with an induction of astrocyte reactivity and neuronal cell death, in particular of dopaminergic neurons. Altogether these data indicate that hiPSC-neural models are relevant test systems for the evaluation of Nrf2 pathway activation upon induction of oxidative stress, allowing further understanding of the molecular mechanisms underlying exposure to developmental neurotoxicants.

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CHAPTER 2

user friendly and fit for purpose. Those changes will be implemented shortly and the tool should be made publically available in 2018.

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., 2017a).

2.6.4 Scientific Options for Avoiding Chronic Fish Testing on the Basis of Existing Data and Extrapolation Approaches

In the light of the EU Directive 2010/63/EU (EU, 2010) on the protection of animals used for scientific purposes and the EURL ECVAM strategy to replace, reduce and refine the use of fish in aquatic toxicity and bioaccumulation testing (Halder et al., 2014), EURL ECVAM explored whether interspecies extrapolations and acute-to-chronic relationships could be used to scientifically support the waiving of chronic fish tests.

For this purpose, acute and chronic toxicity data for Daphnia and fish were extracted from various databases and analysed to identify possible relationships taking into consideration different mode of actions. The results of this analysis indicate that several types of aquatic toxicity data can be used to assess the potential for chronic fish toxicity.

In particular, interspecies extrapolations based on invertebrate (Daphnia) data, and acute-to-chronic extrapolations from existing acute fish toxicity data, are recommended as a means of deriving information on chronic fish toxicity without the need to perform additional fish tests (see Box 2.4).

More information is available in the JRC Technical Report (Kienzler et al., 2016b) and scientific paper (Kienzler et al., 2017b).

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

This new Cefic LRI-funded project (LRI-ECO34) 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.

A modelling approach to avoid chronic fish toxicity tests

The assessment of aquatic toxicity is an important component of the environmental hazard and risk assessment of all types of chemicals. Aquatic toxicity is usually determined by testing on organisms representing the three trophic levels: plants (or algae), invertebrates (crustaceans such as Daphnia spp.) and vertebrates (fish).

Whereas acute aquatic toxicity testing is a basic requirement in most pieces of EU chemicals legislation, chronic aquatic toxicity testing may be required on a case by case basis, for example when the outcome of the acute testing indicates a risk, or when long-term exposure to the chemical is expected. However, in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes, all available information should be considered before testing on vertebrates, including fish, is carried out.

In this context, the JRC has explored the utility of simple extrapolation approaches, based on existing aquatic toxicity data, for avoiding chronic fish testing (Kienzler et al., 2016b; Kienzler et al., 2017b). Acute and chronic toxicity data for Daphnia and fish were extracted from various databases and analysed to identify possible relationships taking into consideration the chemical mode of action.

The results of this analysis indicate that interspecies extrapolations based on invertebrate (Daphnia) data could support the waiving of chronic fish toxicity test in low-tier risk assessments, especially for chemicals acting by unspecific reactivity and non-polar narcosis. Acute-to-chronic extrapolations from existing acute fish toxicity data are also recommended as a means of deriving information on chronic fish toxicity without the need to perform additional fish tests, irrespective of the chemical’s mode of action.
2.6.6 Development of a Biotransformation Database

The biological fate of a substance in an organism plays an important role in exposure and risk assessment of chemicals in general. Four underlying processes: absorption, distribution, metabolism and excretion (ADME), determine the time-course of internal or systemic exposure, e.g., chemical half-life. For hydrophobic, low volatility chemicals, the chemical half-life is largely determined by the biotransformation (metabolism) rate constant. Biotransformation is usually determined with animal models or in vitro using intact cells or subcellular fractions from the liver.

The availability of a curated and quality assured database containing in vivo and in vitro biotransformation data will contribute to several EURL ECVAM in-house activities (e.g., comparative predictive toxicity, development of adverse outcome pathways) and support the work carried out in collaboration with external partners, e.g., prediction of in vivo fish bioconcentration factor (BCF) using information on metabolism derived with in vitro methods (see section 5.2.4).

EURL ECVAM launched this project in 2016 involving an external contractor and plans to make the database publicly available in 2018. It will provide 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) and chemical assessors.

2.7 Activities in Basic, Applied and Translational Research

In the seventh Commission report on the number of animals used in the EU in 2011 for experimental and other scientific purposes, it was highlighted that more than 60% of the animals were used for research and development in the fields of human medicine, veterinary medicine, dentistry and in biological studies of fundamental nature. Ethical concerns, as well as the cost, maintenance and relative inefficiency of animal research, have encouraged the development of alternative methods for the study of diseases.

Major efforts are needed to identify and promote the use of non-animal tools in biomedical research, particularly in developing ambitious education and training programmes, as well as improving communication and dissemination. Furthermore, Directive 2010/63/EU (EU, 2010) reinforced and extended EURL ECVAM’s central role in promoting the use, validation and acceptance of alternative approaches that originally focused on regulatory testing of chemicals or biological agents.

At the Joint PARERE-ESTAF meeting in November 2016, some selected ESTAF members (e.g., those involved in research, education and training activities) had been asked to give 10-minutes flash presentations on their activities related to alternatives used for basic, applied and/or translational research purposes and/or education and training (see also section 4.11).

ESTAF and PARERE members were also asked to contribute their ideas and initiatives to reduce animal use in biomedical research taking the priorities proposed at an EURL ECVAM - European 3Rs Centres meeting (held in April 2015, see Zuang et al., 2015, section 5.13) as a basis to establish common goals and strategic aims for the network to collaborate on.

These priorities were i) more systematic and effective critical assessment of animal-based studies, ii) improve communication about all aspects of the Three Rs including the development of ambitious education and training programmes, iii) understand and describe organisational and institutional obstacles to the Three Rs and iv) facilitate more extensive input from Three Rs centres in the project evaluation process.

The adoption of methods using human tissue has the potential to replace some of the animal models, improving predictivity and reducing animal use. In this context, EURL ECVAM participated in a two-day workshop organised by NC3Rs that brought together cancer researchers working with human tissue from across academia and industry.

New activities which have the potential to be translated to compound testing and mechanistic studies currently carried out by academia and industry could be identified at the workshop. EURL ECVAM will continue to participate in this type of meetings where the research focus is mainly on the use of human in vitro models in biomedical research.
Moreover, JRC’s EURL ECVAM launched a call for tender on 11 August 2017 on a ‘Review of Non-animal Methods in Use for Biomedical Research’. The outcome of the project will contribute to the uptake, implementation and promotion of non-animal methodologies in biomedical sciences by performing reviews in selected human disease areas.

The review should cover in vitro methods and in silico approaches, and in particular, human-specific (sub)cell and tissue-based models including two or three dimensional cultures/organotypic models; stem-cell technologies, such as human-induced pluripotent stem cells (iPSC), lab-on-a-chip devices with microfluidic systems, (ex) vivo approaches and computational modelling, indicating their applications and development status.

2.8 EURL ECVAM/ESTIV Workshop on Moving Forward in Carcinogenicity Assessment

A joint ESTIV-EURL ECVAM workshop on ‘Moving forward in carcinogenicity assessment’ took place in October 2016 in conjunction with the European Society of Toxicology In vitro (ESTIV) congress in Juan Les Pins, France.

The workshop addressed current progress in the area of carcinogenicity and how this, together with technological advances, can be exploited to move away from the two-year rodent bioassay (Corvi et al., 2017, see Figure 2.2).

The workshop was opened with an introduction on a recent analysis of carcinogenicity testing for regulatory purposes in the EU (Madia et al., 2016). This was followed with presentations of a number of international initiatives ranging from the ICH approach for pharmaceuticals to waive the cancer study, the recent revised approach for the systematic classification of carcinogens by the International Agency for Research on Cancer (IARC), to integrating different ‘omics and high throughput technologies together with disease knowledge into more traditional testing.

Despite a seemingly diverse range of strategic developments, commonalities are emerging. First, providing insight into carcinogenicity mechanisms is an increasingly essential aspect of hazard assessment applicable to all types of substances. Second, there is a need to direct efforts towards the integration of all available information on relevant endpoints, including from epidemiology, traditional and alternative toxicology test systems, and from novel data streams (e.g., ‘omics, high through technologies).

2.9 Analysis of Mechanistic Information in the Context of Acute Systemic Toxicity

The development of Integrated Approaches to Testing and Assessment (IATA) for acute systemic toxicity is hampered by our incomplete knowledge of the numerous toxicity pathways and/or modes-of-action that lead to acute systemic toxicity. In fact, the importance of improving the understanding of toxicological mechanisms in this area is well recognised.

Figure 2.2: Integration of data, application of new technologies and approaches, regulatory relevance and acceptance across sectors can warrant a better carcinogenicity safety assessment (From Corvi et al., 2017, Toxicology in Vitro, under CC BY-SA 4.0).
Chemical substances may have negative effects on human health at sufficiently high doses. Therefore, regulatory authorities require the assessment of their toxicity to categorise such substances and indicate on product labels precautions to be taken while handling them. The current animal tests included in regulatory test guidelines generate an LD50, which is the dose that produces death in 50% of the animals tested. However, recent efforts have been directed at identifying non-animal alternatives for acute toxicity testing with the aim of reducing and refining the use of animals.

Briefly, the nervous system and the cardiovascular system appear as the most frequent targets. For chemicals acting on the nervous system, interference with neurotransmitters and/or neurotransmission and impairment of propagation of electrical activity are among the main reported mechanisms.

Chemicals that target the cardiovascular system often interfere with ion balance/signalling/membrane potential of the cell and with intracellular signalling mechanisms.

General cytotoxicity is confirmed as an important determinant of acute systemic toxicity. The false negative prediction obtained with the cytotoxicity assay can, in some cases, be explained by specific target organ effects.

Nevertheless, it is difficult to explain in vitro misclassifications only on the basis of mechanistic information. Other factors, such as the variability of the in vivo and in vitro data and in vivo kinetics may have an impact, which deserves further in depth investigation.

Hamm et al., 2017; NRC, 2015; Prieto et al., 2014) (see Box 2.5).

Therefore, in line with the objectives outlined in the EURL ECVAM strategy to replace, reduce and refine the use of animals in the assessment of acute mammalian systemic toxicity (Prieto et al., 2014), EURL ECVAM has centered its in-house activities in this area on the collection and evaluation of mechanistic information on eight organs identified as relevant for acute systemic toxicity (central and peripheral nervous system, cardiovascular system, liver, kidney, lung, blood, gastrointestinal system and immune system) (Gennari et al., 2004).

Information was derived from published literature, toxicology handbooks, short descriptions of reference compounds used in the ACuteTox project and internet databases (e.g., HSDB). The aim was to identify properties that are required for in vitro test methods for target organ toxicity testing which are not covered by cytotoxicity assays. The outcome of the analysis of the information collected is under finalisation and a manuscript is under preparation.
Since the last EURL ECVAM status report published in October 2016 (Zuang et al., 2016), five test submissions have been evaluated by EURL ECVAM.

The results of the PARERE consultation on a test method for bioaccessibility (bioelution) testing were evaluated and the test submitter was asked to complete a full submission (see section 3.1).

Similarly, the test submitter of the Toxtracker® assay was invited to provide a full submission further to the EURL ECVAM assessment and the PARERE consultation on the pre-submission (see section 3.2).

Additional data were submitted to EURL ECVAM at the end of 2016 on the SENS-IS assay and the evaluation process is currently under finalisation (see section 3.3).

Based on the feedback received from the PARERE members, the assessment of the EDITOX pre-submission was completed in June 2017 and the test submitter was invited to complete a full submission (see section 3.4).

Finally, a pre-submission for the assessment of a method based on γH2AX/pH3 biomarkers for the prediction of genotoxic potential was submitted to EURL ECVAM in August 2017 (see section 3.5). The assessment is on-going.

The EURL ECVAM database (TSAR\(^1\)) that tracks the status of a test method from its submission through its final adoption into a regulatory framework (EU, OECD and related standards) will be updated by the end of the year 2017.

\(1\) https://eurl-ecvam.jrc.ec.europa.eu/test-submission
3 Test Method Submissions

3.1 Bioelution
The bioelution test method is not a toxicity test. It provides the fraction of a substance that dissolves under surrogate physiological conditions (e.g., simulated gastric fluid) and is potentially available for absorption into systemic circulation (bioaccessible concentration).

In the pre-submission received in summer 2016 from the European non-ferrous metal association (Eurometaux) on the bioelution in vitro method (see section 3.2 in Zuang et al., 2016), the test method was proposed 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. Alloys are defined as special preparations under REACH in recognition of the fact that they do not behave as simple mixtures (EC, 2006).

Thus, the rationale provided in the pre-submission was that using the gastric bioaccessible concentration of a metal in an alloy appears more informative and relevant for predicting the toxicity than simply using the concentration of the metal ingredient. This assumes that the dissolved metal ion is the cause of toxicity.

The issue on how to classify alloys under CLP for human health endpoints was triggered at the REACH Committee in relation to the inclusion of a new harmonised classification of lead in Annex VI of the CLP Regulation. Member States raised concerns and questions around the applicability and interpretation of Article 12(b) (specific cases requiring further evaluation) in the case of mixtures/alloys.

An Expert Group established by the European Chemicals Agency (ECHA) in September 2016 further debated the regulatory application of the bioelution test method. This group limited the discussions to alloys and their systemic effects after oral exposure in the context of hazard assessment related to CLP.

EURL ECVAM actively participated in the meetings and teleconferences organised by ECHA. The group drafted a document that was shared and discussed at the Competent Authorities for REACH and CLP (CARACAL) meeting in June 2017.

Following the pre-submission of the bioelution in vitro method, EURL ECVAM launched a consultation within the PARERE network in November 2016. The purpose was to get their views with regard to 1) the interpretation of the CLP legal text, 2) the regulatory applicability of the bioelution test method, 3) its limitations and scientific relevance, 4) the potential further validation of the test method, and 5) the impact on animal testing.

Overall, there was a general opinion among PARERE members that the bioelution test method is interesting and might be of potential use in a regulatory context. However, no clear picture emerged of how the test method could be used.

On the basis of the evaluation of the information retrieved from the test method pre-submission and the feedback received from PARERE members, the test submitter has been invited to submit a full submission and to provide clarifications and more detailed information on a number of questions that emerged from the assessment, the PARERE consultation and the ECHA expert group discussions.
3.2 ToxTracker® Assay

A test pre-submission was received in late November 2016 on an \textit{in vitro} method intended for testing genotoxicity called the ToxTracker® Assay.

The ToxTracker® was presented as a genotoxicity assay that also includes a number of non-genotoxic endpoints associated with human carcinogenicity hazards. It was proposed by the test submitter as an additional \textit{in vitro} test able to allow an accurate genotoxicity assessment, providing insight into the mechanisms of (geno)toxicity and reducing the frequency of misleading positive \textit{in vitro} test results that would trigger \textit{in vivo} follow-up testing in the current genotoxicity strategy.

The test method consists in a microplate format including a set of six different mouse Embryonic Stem (mESC) green fluorescent protein (GFP) reporter cell lines for four distinct biological responses: i) DNA damage; ii) Cellular stress; iii) Oxidative stress and iv) Protein damage as unfolded protein response.

Cells are simultaneously treated in a 96-well plate and detection of GFP reporter genes induction and cytotoxicity by flow cytometry is performed after 24 hours exposure to at least four different concentrations. The relative GFP induction of the reporter cell lines is measured in function of each chemical concentration as a proportional assessment of genome damage and other stress responses.

The test method underwent prevalidation and the information and data, compiled in the EURL ECVAM pre-submission template together with a well standardised protocol, were submitted to EURL ECVAM for a preliminary assessment.

In November 2016, the test submitter also submitted to the OECD a new project proposal on a Detailed Review Paper on the “ToxTracker assay: a stem cell-based reporter assay for mechanistic carcinogenicity hazard assessment” which will include the results of the validation study.

Based on the information received, EURL ECVAM has considered that the possibility to provide insight into the mechanism of genotoxicity by the combination of various endpoints could make the ToxTracker® a promising method potentially suited to cover human genotoxic effects, and judged the test as biologically and mechanistically relevant.

The above consideration has been confirmed by the positive feedback from OECD WNT members and positive comments received following consultation (March, 2017) with PARERE.

In June 2017, EURL ECVAM asked the test submitter to complete a full submission when the data from the validation study becomes available.

3.3 SENS-IS

As reported in the EURL ECVAM status report 2016 (Zuang \textit{et al.}, 2016, Section 3.6), the SENS-IS assay is a reconstructed human skin model-based method for identifying potential skin sensitising substances and for classifying them into four potency classes (weak, moderate, strong and extreme) on the basis of the analysis of the expression of three groups of genes.
Following the evaluation of the full submission of July 2016, reporting the validation of the SENS-IS coordinated by ImmunoSearch (Grasse, France), EURL ECVAM requested the test developer to provide additional information on the method and recommended to analyse the ability of the method for classifying substances according to the GHS classification scheme. Additional data were submitted to EURL ECVAM at the end of 2016 and in 2017 and the evaluation process is currently under finalisation.

3.4 EDITOX
In June 2016, EURL ECVAM assessed the pre-submission of an in vitro cell based method, EDITOX, to assess the risk for chemical compounds to induce psychiatric adverse side effects, (e.g., depression and/or suicide).

This test method quantitatively analyses variations of the relative proportion of the 32 mRNA editing isoforms of the serotonin receptor (5-HT2cR) using a next generation sequencing approach in SH-SY5Y cells (human neuroblastoma cell line).

Figure 3.1: SH-SYSY neuroblastoma cell line.

The information provided in the test pre-submission form was considered sufficient enough to understand the principle of the test method, and its biological and general mechanistic relevance.

In October 2016, PARERE members were asked to evaluate the potential use of EDITOX for regulatory purposes. Based on the feedback received from the PARERE members, in June 2017 EURL ECVAM completed the assessment of the EDITOX pre-submission and invited the test submitters to progress the test method to a full submission, taking into account a list of questions raised by EURL ECVAM.

In particular, since this assay is entirely based upon one mode of action (e.g., editing of 5-HT2cR), this method, as a standalone, is not expected to fully replace in vivo testing for depression.

Therefore, EURL ECVAM asked the submitter to provide with their full Test Submission a review of other possible modes of action of drugs (and potentially environmental chemicals) with the risk of causing depression and/or pro-suicidal side effects, and of in vitro test methods (e.g., battery of tests) that may be available to potentially cover these other modes of action, highlighting their relation to currently available in vivo methods.

3.5 γH2AX/pH3 Biomarkers
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 different substances.

The combination of the two 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) serves as an indicator of mitotic index and cell proliferation status. Specifically, aneugenic chemicals often elevate the mitotic index, thus pH3 tends to be reduced by cytotoxic conditions, including cytotoxicity associated with many DNA-reactive chemicals.

The submitter declared the integration of information derived by the two biomarkers detection to be more predictive of genotoxicity than common Micronucleus in vitro and Ames tests. For this reason, the test method is intended to be used as i) screening and early selection test of candidate molecules before entry into drug development or ii) 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 assessment of the γH2AX/pH3 test method is ongoing and will be finalised by the end of 2017.
4 Validation of Alternative Methods

This section refers to validation studies which are either carried out by EURL ECVAM or for which EURL ECVAM has been consulted. It is a non-exhaustive list of all possible validation studies carried out around the world.

Nonetheless, EURL ECVAM is aware of external (to EURL ECVAM) validation studies that are carried out by companies/industry on methods which are intended to be submitted to EURL ECVAM for evaluation and peer review.

These studies are mentioned under section 3 (Test Method Submissions) and section 4.8 (EURL ECVAM Scientific Advisory Committee Peer Reviews).

The validation studies undertaken with EURL ECVAM’s international partners in the framework of the International Cooperation on Alternative Methods (ICATM) are described in Annex 2.
4 Validation of Alternative Methods

4.1 Endocrine Disruption - AR-CALUX Test Method

EURL ECVAM has the lead in developing an OECD Performance Based Test Guideline (PBTG) for Androgen Receptor Transactivation Assays (ARTAs) and related Performance Standards. This PBTG 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) or in the process of a validation (the ARTA using the 22Rv1/MMTV cell line, led by Korea) and the EURL ECVAM ongoing validation study of the in vitro method AR-CALUX.

The latter 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 BioDetection Systems (BDS). The validation study is carried out with three participating laboratories of EURL ECVAM’s network of specialised laboratories, the European Union Network for the Validation of Alternative Methods (EU-NETVAL).

So far, EURL ECVAM has carried out an experimental assessment of the method; conducted a GLP study in order to refine the assay and established transfer criteria; provided a training for the three partner facilities at the JRC laboratories; and coordinated all the phases of the validation study.

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**Validation study to assess in vitro methods for the detection of chemicals which disrupt thyroid function**

There is global concern about substances, natural and man-made, which have the potential to interfere with the endocrine system. The thyroid’s main role in the endocrine system is to regulate metabolism through the action of thyroid hormone, by extracting iodine from the blood and incorporating it into thyroid hormones.

Cells and human body systems depend on the thyroid to manage their metabolism and for regulating vital body functions, including breathing, heart rate, central and peripheral nervous systems, body weight, muscle strength, menstrual cycles, body temperature and cholesterol levels. Some man-made chemicals have the potential to interfere with the functioning of the thyroid and related hormone signalling processes, which can result in adverse health effects in humans and other organisms that have been sufficiently exposed.

A total of 17 in vitro methods have been identified by EURL ECVAM as candidates for a validation study which will be carried out in collaboration with the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) (see section 4.10). Those methods which perform well may be selected for further assessment with a view to their eventual use in a regulatory context, in support of EU initiatives to address the potential risks to human health and the environment posed by endocrine disruptors.
The transfer phase, where the three participating laboratories have applied the method in their own laboratories, has been successfully finalised. The phases to assess between-laboratory reproducibility and predictive capacity are ongoing.

4.2 Thyroid Hormone Disruption
EURL ECVAM launched a call to EU-NETVAL members for participation in the validation study of in vitro methods for the detection of thyroid disruptors (see section 4.10 and Box 4.1).

Due to concerns about the potential of chemicals to interfere with the endocrine system in humans and animals, a number of OECD Test Guidelines (TGs) have been developed for the screening and testing of potential endocrine disrupting chemicals, as listed in the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disruptors. TGs for the screening of chemicals that disturb the thyroid hormone signalling are however lacking, largely due to the complexity of the thyroid system.

In 2014, OECD published a scoping document on in vitro and ex vivo assays for the identification of modulators of thyroid hormone signalling (OECD, 2014a). Several key biological mechanisms of thyroid system disruption were reviewed and the corresponding methods evaluated for their state of readiness as candidates to enter the validation process. Relevant in vitro and ex vivo methods were identified and recommendations were given for their development/use.

EURL ECVAM collected information on 17 methods, taking primarily into account the information reported in the OECD review (OECD, 2014a) but also the OECD Detailed Review Paper (OECD, 2006), and feedback received at various meetings (e.g., the EU NETVAL meeting of 2016 (see Box 4.4), OECD Validation Management Group-Non-Animal meeting 2016 and the DG ENV/ANSES Thyroid Disruptor workshop in 2017 (DG ENV, 2017)).

Further information has been retrieved for each of these in vitro methods, and on the basis of this information, a set of in vitro methods, covering all of the eight blocks identified as known targets of thyroid disruption (except epigenetic changes) and described in the OECD review (OECD, 2014a), have been selected for the EURL ECVAM coordinated validation study.

This validation study will consist of two parts: part one will define the methods and assess their transferability and reliability, and part two will assess the overall relevance based on the underlying mechanisms of the selected in vitro methods using the same set of reference chemicals (test items) for all test methods.

4.3 Developmental Neurotoxicity
Deficiencies in the testing methodology for developmental neurotoxicants represent a significant gap in the regulatory testing and assessment of chemicals and increases the uncertainties in the establishment of safe levels of exposure to developing humans (Bal-Price et al., 2015a).
The existing OECD TG 426 on Developmental Neurotoxicity (DNT) Study is entirely based on in vivo studies and is very resource intensive in terms of animals, time and overall cost (Tsuji and Crofton, 2012). It is therefore rarely used, resulting in a small amount of chemicals being tested for their DNT potential.

Based on the conclusions of two recent workshops on developmental neurotoxicity (October 2016 and January 2017) organised by EFSA/OECD and CAAT Europe/OECD, respectively, it was decided to develop a testing battery of alternative DNT methods that could be applied right now in a fit-for-purpose manner for different regulatory purposes.

EFSA, OECD and US EPA support the development of an integrated in vitro neurotoxicity testing strategy which could serve as complementary to the rodent in vivo method (OECD TG 426) in order to speed up screening of the high number of chemicals for their DNT potential and to prioritise chemicals for further testing.

One way to fulfil this goal is through more targeted data production, focusing on DNT-specific endpoints using in vitro methods and non-mammalian models (e.g., zebrafish). Therefore, development of an Integrated Approach to Testing and Assessment (IATA) has been proposed as a practical solution, composed of in vitro assays anchored to key neurodevelopmental processes and key events identified in the existing DNT AOPs (Bal-Price and Meek, 2017).

In parallel, further development of Adverse Outcome Pathways (AOPs) relevant to DNT will take place, providing mechanistic information on the causal links between molecular initiating events (MIEs), key events (KEs) and adverse outcomes (AOs) of regulatory concern. Mechanistic knowledge built during AOP development will provide the biological context for the in vitro assays anchored to AOP(s) key events, facilitating development of AOP-informed IATA for various regulatory decision-making (Bal-Price and Meek, 2017).

4.4 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 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 EPAA, BSP130 Phase III Validation of in vitro methods for the testing of Clostridium septicum vaccine (see see section 5.6.4) and BSP148 Validation of a rabies in vitro potency assay (see section 5.6.5).

4.5 Skin Sensitisation - Genomic Allergen Detection Test Method

As described in the EUR. ECVAM status report of 2015, the Genomic Allergen Detection (GARD) test method (Johansson et al., 2011; Johansson et al., 2013; Johansson et al., 2014) for the binary classification of chemicals into skin sensitisers and non-sensitisers is based on global transcriptomic analysis of differential expression in a human myeloid cell line, induced by sensitising chemicals in comparison to non-sensitising controls.

The resulting biomarker signature, the GARD prediction signature (GPS), consists of 200 transcripts, which are used as input into a support vector machine (SVM) model trained on a set of reference chemicals (Johansson et al., 2011).

The GARD is currently undergoing a validation study under the umbrella of the OECD. The method was recently further developed to predict three sensitiser potency classes according to the European Classification, Labelling and Packaging (CLP) Regulation, targeting categories 1A (strong), 1B (weak) and no category (non-sensitiser). Using a random forest approach and 70 training samples, a potential biomarker signature of 52 transcripts was identified for this purpose (Zeller et al., 2017).

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

The experimental phases of the validation studies of methods for genotoxicity testing in reconstructed human 3D skin models, coordinated by Cosmetics Europe
VALIDATION OF ALTERNATIVE METHODS

...have been finalised (manuscripts in preparation). In April 2017, CosEU organised a workshop aimed at informing different stakeholders on the validation status of 3D-skin based genotoxicity assays (micronucleus test and comet assay).

The presentations of the results of the two studies were followed by a more strategic discussion on how to use the tests within the testing battery. For the time being the assays are considered for the assessment of genotoxicity caused by substances applied topically.

There was agreement that both the micronucleus and the comet assays in reconstructed skin models are promising assays. However, they are not to be considered as stand-alone assays, but are foreseen to be used within a testing strategy for genotoxicity without animals to follow-up the positive results in the *in vitro* testing battery.

Further discussion on the specific use of these assays and how to proceed in order to accelerate their implementation took place at the International Workshops on Genotoxicity Testing (IWGT) held in Tokyo on 8 to 10 November 2017.

4.7 Genotoxicity Testing - Hen’s Egg test for Micronucleus Induction (HET-MN)

The hen’s egg test for micronucleus induction (HET-MN) has also been proposed as a follow-up test method for *in vitro* positives in a testing strategy for genotoxicity. The HET-MN combines the use of the commonly accepted genetic endpoint “formation of micronuclei” with the well-characterised and complex model of the incubated hen’s egg, which mimics systemic exposure, thus not needing the addition of S9. A validation study is currently under finalisation by a German consortium (Greywe *et al.*, 2012).

4.8 EURL ECVAM Scientific Advisory Committee Peer Reviews

Between April and June 2016, the EURL ECVAM Scientific Advisory Committee (ESAC, see Box 4.2) reviewed five test methods and their respective validation studies that had been submitted to EURL ECVAM for evaluation and peer-review.

These were:
(i) the validation of the Ocular Irritation® test method for assessing serious eye damage/eye irritation potential of chemicals submitted by Secam Services & Consultation on Alternative Methods Sagl,
(ii) the validation study of the SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT) submitted by L’Oréal,
(iii) the validation study of the U-SENS™ test method for skin sensitisation testing submitted by L’Oréal,
(iv) the Performance Standards-based validation study of the LuSens test method for skin sensitisation testing submitted by BASF, and
(v) the Performance Standards-based validation study of the epiCS® Skin Irritation Test (SIT) submitted by CellSystems GmbH.

Two ESAC Rapporteurs coordinated the peer-review of the epiCS® SIT, whereas the peer-reviews of the other four methods were coordinated by two newly appointed ESAC Working Groups composed of ESAC members and experts on eye irritation or skin sensitisation nominated by EURL ECVAM and ICATM partners.

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**Box 4.2**

**EURL ECVAM’s Scientific Advisory Committee (ESAC)**

The EURL ECVAM’s Scientific Advisory Committee (ESAC) advises EURL ECVAM on scientific issues. Its main role is to conduct independent peer review of validation studies of alternative test methods and to assess their scientific validity for a given purpose. The peer review is normally prepared by specialised ESAC Working Groups and, at the end of the peer review, ESAC’s advice to EURL ECVAM is formally provided as ‘Working Group Reports’ and ‘ESAC Opinions’. More information is available [here](http://europa.eu/nd68pq).
The ESAC Working Group on eye irritation met at EURLECVAM on 11-13 May 2016 to conduct the review of the SkinEthic™ HCE EIT and of the Ocular Irrition assay.

The ESAC Working Group on skin sensitisation met at EURLECVAM on 17-19 May 2016 to conduct the review of the U-SENS™ and of the LuSens test methods. Working Group/ Rapporteur reports and ESAC Opinions on each individual method were discussed and endorsed by ESAC at its 42nd meeting (ESAC42) on 9-10 June 2016.

A sixth ESAC Opinion on the use of Performance Standards to evaluate test methods similar to a Validated Reference Method was also issued at ESAC42. The six ESAC Opinions were published by the JRC in November 2016.

They are:
- Ocular Irrition® (ESAC, 2016a)
- SkinEthic™ HCE EIT (ESAC, 2016b)
- U-SENS™ (ESAC, 2016c)
- LuSens (ESAC, 2016d)
- epiCS® SIT (ESAC, 2016e)
- Performance Standards (ESAC, 2016f)

Also available is an earlier related ESAC Opinion on the EURLECVAM/Cosmetics Europe Eye Irritation Validation Study (EIVS) on EpiOcular™ EIT and SkinEthic™ HCE and a Cosmetics Europe study on HPLC/UPLC-spectrophotometry as an alternative endpoint detection system for MTT-formazan (ESAC, 2014).

4.8.1 Renewal of the ECVAM Scientific Advisory Committee

EURLECVAM launched on 14 December 2016 an open call for the renewal of the core membership of its Scientific Advisory Committee, ESAC, with closing date for submitting an application on 31 January 2017.

Twenty-five candidates submitted an application by the deadline for nine available positions. A Selection Committee, comprising EURLECVAM staff and representatives from other Commission services (external members) with experience in managing expert groups, was established to assess the candidates, as described in the "Information on the Call for Applications 2016".

All 25 applicants were deemed eligible for selection by the Selection Committee on the basis of the eligibility criteria published in the "Information on the Call for Applications 2016", and were therefore evaluated against the selection criteria described in the same document.

In its selection process, the Selection Committee also considered the candidates’ independence (potential conflicts of interest), representation from different geographic regions and gender balance. The official appointment of the nine selected candidates is expected to occur before the end of 2017.

4.9 EURLECVAM Recommendations

4.9.1 Non-animal Approaches for Skin Sensitisation

The EURLECVAM Recommendation (EURLECVAM, 2017) for skin sensitisation (see Box 4.3) builds on the progress made in the area since the publication of the EURLECVAM strategy document for this endpoint in 2013 (Casati et al., 2013).

The document illustrates EURLECVAM views on the regulatory use of individual OECD adopted test methods, including two in vitro methods, the LuSens and the U-SENS™, that underwent ESAC peer review in 2016 (ESAC, 2016c,d) and on Defined Approaches (DAs) integrating data from various non-animal sources \textit{e.g.}, \textit{in silico}, \textit{in chemico} and \textit{in vitro} methods.

In relation to the individual methods, EURLECVAM recommends using the qualitative and quantitative mechanistic information generated by the \textit{in chemico} and \textit{in vitro} methods adopted by the OECD, together with other relevant information, within DAs and Integrated Approaches to Testing and Assessment (IATA) for assessing skin sensitisation hazard and for hazard classification purposes.

In addition EURLECVAM supports using the LuSens and U-SENS™ as valid scientific methods for generating information respectively on KE2 and KE3 of the skin sensitisation AOP and to be considered together with other relevant information in the context of DAs and IATA. EURLECVAM also fully supports the inclusion of the LuSens as similar method to the KeratinoSens™ in OECD TG 442D and the development of an OECD TG on the U-SENS™.

As concerns predictions generated with valid DAs\textsuperscript{2}, EURLECVAM recommends using them,

\textsuperscript{2} Examples of DAs which are considered for further evaluation are reported in Annex I to OECD GD 256.
where applicable and adequate, instead of LLNA data or in conjunction with such data if they already exist, in the context of IATA for assessing skin sensitisation hazard and for hazard classification purposes and to properly document new DAs applied for regulatory purposes using the templates provided in OECD GD 255.

EURL ECVAM also recommends that in view of promoting international applicability and acceptance of alternative non-animal approaches to skin sensitisation, future work should focus on the definition of internationally agreed standards (e.g., OECD TGs) for DAs and individual test methods that provide equivalent or better level of information than the current animal tests for skin sensitisation.

### 4.9.2 Cytochrome P450 Induction

EURL ECVAM is preparing an EURL ECVAM Recommendation on the human cytochrome P450 (CYP) activity n-fold induction in vitro test methods. The CYP induction method is 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. Metabolism is thus increasingly recognised as a key element in chemical hazard and risk assessment. Mechanistic information on CYP induction should be considered in the context of IATA for adverse effects. This information is relevant when the induction of the specific CYP isoform is directly related to a (downstream) adverse outcome or it serves as a surrogate for the upstream transcription factor activation leading to adverse effects through other parallel (non-CYP activity-dependent) pathways, such as Aryl Hydrocarbon Receptor (AhR), Constitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXr) response pathways.

### 4.10 Update on 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 ensure the availability of alternative methods for regulatory purposes. The EURL ECVAM recommendation on the use of non-animal approaches for skin sensitisation (allergy) testing This recommendation builds on the progress made in the area since the publication of the EURL ECVAM strategy in 2013 (Casati et al., 2013) and provides EURL ECVAM views on the latest two methods for skin sensitisation, the LuSens and the U-SENS™, peer-reviewed by the EURL ECVAM Scientific Advisory Committee (ESAC), and on Defined Approaches which are based on the integration of different kinds of non-animal data.

Three recommendations were issued by EURL ECVAM on non-animal methods for skin sensitisation testing, the DPRa, the KeratinoSensTM and the h-CLAT. A major achievement was the OECD adoption of these first three in vitro methods (OECD Test Guidelines 442C, 442D and 442E) based on key chemical and biological mechanisms of the process that leads to development of skin allergies. Following independent peer review by ESAC, EURL ECVAM now supports the use of two additional methods, the LuSens and the U-SENS™ when used in combination with other relevant information.

Since none of the regulatory adopted methods provides the same level of information as the traditional animal tests, a number of defined approaches, i.e., approaches based on the use of different type of non-animal data, have been proposed for the identification of chemicals that may cause skin allergy. On behalf of the European Commission, the JRC’s EURL ECVAM led the development of international guidance on the harmonised reporting of these approaches. The defined approaches for skin sensitisation have comparable performance to the standard animal test, the Local Lymph Node Assay (LLNA), for identifying potential skin allergens. In addition some of them provide useful information to distinguish between strong and weak sensitisers. In the light of this evidence EURL ECVAM recommends these defined approaches be used where applicable and adequate instead of LLNA data or together with the animal data if these are already available.
States assist the European Commission in the validation of alternative methods.

Currently, there are 37 members of EU-NETVAL representing fifteen countries in the network, selected against pre-defined eligibility criteria (including the European Commission’s own in vitro GLP test facility) and endorsed by the National Contact Points.

EU-NETVAL is coordinated by EURL ECVAM, in close collaboration with the EC Directorate-General for Environment. EU-NETVAL’s mission is to provide support for EURL ECVAM validation studies to assess the reliability and relevance of alternative methods that have a potential to replace, reduce or refine the use of animals for scientific purposes and the tasks are outlined in the terms of reference.

The most recent call for membership of EU-NETVAL closed in September 2015. The current network holds a range of expertise and competences and includes laboratories experienced in advanced in vitro procedures, test systems and measurement techniques which are considered important to address specific aims and objectives identified in EURL ECVAM’s strategies or in accepted conceptual frameworks to achieve Three 3Rs impact in different areas of regulatory safety testing.

The network contributes to the development of guidance documents and training materials supporting good in vitro method development. Furthermore, it promotes practices that ensure scientific integrity and quality of the data generated with in vitro methods in order to stimulate trust by decision makers and industrial end-users.

EU-NETVAL has provided input in the drafting of an OECD technical guidance document (led by EURL ECVAM) on Good In Vitro Method Practices for the development and implementation of in vitro methods for regulatory use in human safety assessment (see section 5.2.9) which is currently undergoing a second commenting round.

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 the confidence that the method(s) are fit for a particular purpose.

The first pilot validation project involves three selected EU-NETVAL test facilities from Sweden, UK and France for the generation of experimental data using the in vitro AR-CALUX method to support the development of an OECD Performance-Based Test Guideline (PBTG) and associated performance standards for Androgen Receptor Transactivation Assays (ARTA) for the detection of compounds with (anti)androgenic potential (see section 4.1).

During this validation, mechanisms were developed to ensure efficient communication and transfer from the test developer of the method to the validation partner EU-NETVAL facilities and to run the in vitro method under GLP conditions since the method would be part of an OECD PBTG.

In June 2017, EURL ECVAM launched a validation study (see Box 4.1) within the EU-NETVAL network to assess 17 in vitro methods for the detection of thyroid disruptors. EU-NETVAL test facilities interact during the validation study with the in vitro method.

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**Figure 4.1:** Knowledge sharing and training session during the EU-NETVAL meeting in 2016.
VALIDATION OF ALTERNATIVE METHODS

developers to define the methods and establish SOPs that can run in a GLP environment. They will also assess within-laboratory reproducibility followed by relevance assessment of the methods using a set of reference compounds. The ultimate goal is to combine several methods in a testing strategy.

The 17 methods were selected based on the OECD review paper (OECD, 2014a) and with input from previous meetings and workshops (see section 4.2 and section 5.2.17). Those methods which perform well may be selected for further relevance assessment using a set of selected reference items with a view to their eventual use in a regulatory context. In vitro method developers and experts that have shown interest or have in-depth knowledge on in vitro method best scientific and quality practices are involved.

Upon request by the OECD, an EU-NETVAL survey was carried out to embrace advances in technologies applied to existing in vitro OECD TG methods, like e.g., the OECD TG 471 on the Ames bacterial gene mutation (see section 5.2.14). The survey on the uptake by EU-NETVAL members of the throughput miniaturised Ames bacterial gene mutation test collected information to support the decisions of the OECD Expert Working Group for the development of a Detailed Review Paper (DRP).

Thirty-four EU-NETVAL facilities replied to the survey including the current nine EU-NETVAL test facilities using already the miniaturised Ames. Fifty-four percent of the responders showed interest for future testing purposes while 61% showed interest in training opportunities, put into practice at the EU-NETVAL meeting on 10 and 11 October 2017.

At the EU-NETVAL meeting in October 2016, a training and knowledge-sharing session on new in vitro skin sensitisation OECD TGs (including DPRA, h-CLAT, KeratinoSens™ and LuSens) took place. In addition, a follow-up to assess the impact of the training in terms of practical implementation in more EU-NETVAL facilities has been assessed by a dedicated survey discussed at the EU-NETVAL meeting in October 2017.

4.11 Meeting of the Preliminary Assessment of Regulatory Relevance (PARERE) network and the EURL 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 the EU-NETVAL: Sharing practical expertise and knowledge on in vitro methods

The JRC’s European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) hosted a two-day meeting with the members of the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) on 10-11 October 2016.

In 2016 EURL ECVAM welcomed 13 new test facilities to the network bringing the total to 37 facilities from 15 different countries across Europe.

The network holds 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 Replacement, Reduction, and Refinement of animal testing (the ‘three Rs’) in different areas of regulatory safety assessment.

Box 4.4

Meeting of the EU-NETVAL: Sharing practical expertise and knowledge on in vitro methods

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In 2016 EURL ECVAM welcomed 13 new test facilities to the network bringing the total to 37 facilities from 15 different countries across Europe.

Representatives from test facilities engaged in a workshop related to the development of Good In vitro Method Practice (GIVIMP) (see Box 5.2), an international project being led by JRC (EURL ECVAM) on behalf of the EU at the OECD. The purpose of this guidance is to contribute to increased standardisation, harmonisation and overall quality of in vitro studies that inform chemical and product safety assessment in a regulatory context.

The second day of the meeting was dedicated to knowledge sharing and training sessions organised in the EURL ECVAM laboratory. These practical sessions covered in vitro methods for assessing the skin sensitisation potential of chemicals and included the DPRA, KeratinoSens™/LuSens and h-CLAT OECD TG methods.
CHAPTER 4

meeting of PARERE and the ECVAM Stakeholder Forum (ESTAF) Networks on 8 November 2016, which includes stakeholder organisations from academia, industry and civil society/animal welfare (see Box 4.6).

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 (see Box 4.5).

During the PARERE meeting, members provided feedback on their experiences in carrying out their roles and highlighted the work they have done within their Member States to engage the relevant people to support the implementation of Directive 2010/63/EU (EU, 2010). New test method submissions were also presented to PARERE members, who gave valuable input to the consultation preparation.

The joint meeting with both PARERE and ESTAF focused on the promotion of non-animal approaches in basic, applied and translational research and education and training as well as updates from several of the stakeholder organisations.

The meeting was followed by a workshop which brought together experts from the Horizon2020 project EU-ToxRisk (see section 2.2), FP7 research initiative SEURAT-1 (see section 2.1) with PARERE and ESTAF members. The scope of the meeting was to address strengths and limitations of new approach methodologies (NAMs)\(^5\) and to explore the possible regulatory use of alternative (non-animal) approaches to systemic toxicity that are intended for the hazard and safety assessment of chemicals used in a variety of sectors.

Further details can be found in the summary records of the PARERE meeting and the joint PARERE ESTAF meeting.

The PARERE meeting which will take place at the JRC on 27 to 28 November 2017 will provide updates on test submissions related to the Bioelution (see section 3.1), the ToxTracker (see section 3.2) and the EDITOX (see section 3.4) methods on which PARERE had been consulted.

It will also include a session on toxicokinetic data in regulatory frameworks where EFSA, EMA and ECHA will provide their perspectives on the use and integration of toxicokinetic data in regulatory safety decisions. EURL ECVAM will present its current activities in the toxicokinetics area which will be followed by a Q&A session on Absorption, Distribution, Metabolism and Excretion (ADME).

In the morning of 28 November, the more advanced case studies of the EU ToxRisk

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\(^5\) In general, new approach methodologies (NAMs) means the same as alternative methods/approaches and more recently in certain fora, people tend to use it interchangeably. The only difference between the terms NAMs and alternative methods is that in the term NAM emphasis is given to the fact that they are newer and better technologies (when compared to traditional animal tests) rather than replacement (alternative) methods of the traditional animal tests. However in areas where animal tests do not exist (e.g., in the area of hazard/risk assessment of mixtures) the term NAM (instead of alternative method) may be more appropriate.
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Box 4.6

EUROPEAN COMMUNITY HEALTH INSTITUTE (ECVIAM) meets with its networks of regulators and stakeholders

On 8 November, the JRC’s EURL ECVAM hosted a meeting of its network for the Preliminary Assessment of Regulatory Relevance (PARERE) of alternative methods, followed by a joint meeting of PARERE and the EURL ECVAM Stakeholder Forum (ESTAF).

During the PARERE meeting, members highlighted the work they have done within their Member States to engage relevant experts to support its role and the implementation of Directive 2010/63/EU. Discussions also focused on two consultations recently launched by EURL ECVAM concerning in vitro methods for detecting potential neuro-toxicants and for measuring the potential release of toxic substances from solid materials such as metal alloys.

This year the joint PARERE-ESTAF meeting focused primarily on the promotion of non-animal approaches in education and training and in basic, applied, and translational research. Several stakeholder organizations reported on their own activities and initiatives in this area. An overview of EURL ECVAM’s activities was also presented, which have also been described in the 2016 EURL ECVAM Status Report (Zuang et al., 2016).

During the PARERE meeting, members highlighted the work they have done within their Member States to engage relevant experts to support its role and the implementation of Directive 2010/63/EU. Discussions also focused on two consultations recently launched by EURL ECVAM concerning in vitro methods for detecting potential neuro-toxicants and for measuring the potential release of toxic substances from solid materials such as metal alloys.

This year the joint PARERE-ESTAF meeting focused primarily on the promotion of non-animal approaches in education and training and in basic, applied, and translational research. Several stakeholder organizations reported on their own activities and initiatives in this area. An overview of EURL ECVAM’s activities was also presented, which have also been described in the 2016 EURL ECVAM Status Report (Zuang et al., 2016).

The joint PARERE-ESTAF meeting (28 to 29 November 2017) will build on the conclusions of the study on 3Rs knowledge sharing (Holley et al., 2016) by exploring the status of 3Rs relevant knowledge sources and sharing practices in three specific areas, namely research, education, and training, and regulatory testing. Participants will take part in a workshop to identify good practices, explore opportunities and propose solutions for better knowledge sharing.
5 Promoting the Regulatory Acceptance and International Adoption of Alternative Methods and Approaches

As well as promoting the development and dissemination of alternative methods and approaches, EURL ECVAM also promotes their international recognition and acceptance by regulators through numerous activities, many of which are detailed in this chapter.

Much of this international work is undertaken together with OECD partners where essential experience and knowledge is exchanged through expert groups and guidance and harmonised approaches are agreed, ensuring the 3Rs are always taken into account where possible.
5 Promoting the Regulatory Acceptance and International Adoption of Alternative Methods and Approaches

5.1 Activities in the OECD Working Party on Hazard Assessment

In the OECD Working Party on Hazard Assessment (WPHA), Member Countries work together to improve and harmonise chemical assessment methods using the most current science.

These include integrated approaches to testing and assessment (IATA) and (quantitative) structure activity relationships, or (Q)SARs. This results in the publication of OECD monographs that illustrate general considerations drawing from example cases, with the overall goal of moving towards technical convergence.

5.1.1 OECD Integrated Approaches to Testing and Assessment Case Studies Project

The IATA Case Studies Project under the OECD 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 (based on 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.

The first cycle in 2015 comprised four case studies focused on grouping and read-across for different hazard endpoints (OECD, 2016a-e). Five case studies were reviewed in the second cycle in 2016, including three read-across case studies (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 (OECD, 2017e), based on the SEURAT-1 cross-cluster ab initio case study (Berggren et al., 2017). This workflow aims to structure knowledge and data in a logical sequence for an integrated chemical safety assessment considering multiple data streams for safety assessment decisions.

The five case studies of the 2016 cycle and its considerations document (OECD, 2017f), updating the learnings with new experience from the second cycle, were endorsed at the WPHA meeting in June 2017 and published on the OECD IATA website in September 2017.

The third cycle, that started in 2017, is reviewing a case study on read-across with metabolism playing a key role in toxicity6 (led by ICAPO), an IATA workflow using inter-chemical comparison (read-across) and intra-chemical comparison of data from traditional and alternative approaches7 (led by Canada/US), the prioritisation of chemicals using an IATA-based Ecological Risk Classification (led by Canada) and the JRC case study on grouping and read-across for nanomaterials - genotoxicity of nano-TiO₂, based on the Nanocomput project carried out at EURL ECVAM (see section 5.9).

The aim of this case study was to determine the genotoxic hazard potential of two nano-TiO₂ target substances by reading across in vitro comet assay results. It is illustrating the applicability of the workflow for grouping and

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6 Use of Integrated Approaches for Testing and Assessment for sub-chronic repeated-dose toxicity of simple aryl alcohol alkyl carboxylic esters

7 Estrogenicity of substituted phenols
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 computational methods in grouping of nanoforms was explored.

5.1.2 Chemical Mixtures and Combined Exposure

Humans and the environment are continuously exposed to a multitude of substances via different routes of exposure.

The toxicological risk of chemical mixtures, relates both to intentional mixtures (e.g., known compositions, such as personal care products, food additives and pesticides) and unintentional ones (e.g., the combination of dozens to hundreds of substances in surface water, drinking water or air). For the latter, the assessment is much more challenging because the compositions are various and complex, many of the substances are unidentified and toxicity data are lacking.

The current risk assessment approach of chemicals, for regulatory purposes, does not generally take into account this complex situation of exposure to multiple substances and mainly relies on the assessment of individual substances. Moreover, although the current EU regulations identify different types of mixtures, there is no harmonised methodological approach to their assessment. In follow-up to the Commission Communication on the Combined Effects of Chemicals (EC, 2012), EURL ECVAM started its activities in the area of mixture risk assessment with a review of regulatory requirements, available guidance and approaches (Kienzler et al., 2014; Kienzler et al., 2016a). Furthermore, EURL ECVAM investigated the applicability of novel, non-animal tools in the assessment of combined effects of chemicals on humans and the environment.

New approaches and methods, such as the AOP concept, *in vitro* methods, ‘omics techniques, quantitative structure activity relationships (QSARs), read-across, toxicokinetic and dynamic energy budget (DEB) modelling, and IATA can help achieve a more effective regulatory assessment and at the same time reduce the reliance on animal testing (Bopp et al., 2015).

Their main strengths lie in their integrated use in smart combination, finally allowing a better, mechanistically based prediction of mixture effects.

In order to gain further insight into the issues linked to the risk assessment of chemical mixtures, EURL ECVAM reviewed relevant case studies from the peer-reviewed literature (Bopp et al., 2016), which provided further evidence that chemicals need to be further addressed not only in single substance risk assessments but also in mixture risk assessments that cover multiple chemical classes and legislative sectors (see Box 5.1).

Currently, EURL ECVAM is performing several own case studies that will become available in 2018.
As international harmonisation is essential in this context, EURL ECVAM plays an active role in the OECD project on combined exposure (led by the OECD WPHA 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 document was completed in June 2017 and is expected to be finalised by the end of 2017.

5.1.3 Guidance Document on Physiologically Based Mathematical Models

The EURL ECVAM Strategy Document on Toxicokinetics (TK) (Bessems et al., 2015) outlines objectives to enable prediction of systemic toxicity by applying new approach methods (NAM) that consider TK.

The aim of the EURL ECVAM strategy is to replace, reduce and refine animal testing in the assessment of toxicokinetics and systemic toxicity of substances, showing a significant short to mid-term Three Rs impact, and at the same time laying the foundation for a risk assessment approach that is increasingly based on human ADME/TK data. In order to facilitate the generation, acceptance and use of TK data and in vitro and in silico methods in the regulatory domain, four main objectives were identified.

The first concerns the development of standards to characterise in vitro methods that measure individual Absorption, Distribution, Metabolism and Excretion (ADME) processes. The second objective aims to establish good kinetic modelling practices (GMP). The third objective expresses the need for publicly available databases to facilitate access to information to create and deploy Physiologically Based Kinetic (PBK®) models. The fourth objective expresses the need to develop guidance on how to generate, use and integrate these data in a regulatory setting.

Concerning GMP (objective two), PBK models are used widely throughout a number of working sectors to provide insight into the dosimetry related to observed adverse health effects in humans and other species. Despite significant advances in GMP for model development and evaluation, there remains some reluctance among regulatory agencies to use such models during the risk assessment process.

The results of the EURL ECVAM international survey disseminated to the modelling community (93 respondents from 19 countries) in January 2017 are presented in Paini et al., (2017a) and inform on the frequency of use and applications of PBK models in science and regulatory submission, on the increased use of new approaches, on the need to peer review the models and on the need for guidance (see Box 5.2).

Box 5.1 Assessing potential risks from exposure to chemical mixtures - case study review

Humans and wildlife can be exposed to an almost infinite number of different combinations of chemicals in mixtures via food, consumer products and the environment, which raises concerns for possible impacts on public and environmental health.

A review of recent literature by the JRC’s EURL ECVAM (Bopp et al., 2016) summarises the outcome of case studies covering several chemical classes. Parameters that could lead to an over- or underestimation of potential risks were identified. Case study results need to be interpreted with caution, considering the underlying assumptions, model parameters and related uncertainties. However, there is clear evidence that chemicals need to be further addressed not only in single substance risk assessments but also in mixture assessments that cover multiple chemical classes and legislative sectors.

Furthermore, several issues hampering mixture risk assessments are identified. In order to perform a mixture risk assessment, the composition of the mixture in terms of chemical components and their concentrations need to be known, and information on their uptake and toxicity are required. Screening level assessments based on conservative assumptions are generally possible. However, refining such assessments to more realistic exposure scenarios is often not feasible due to data gaps. In particular, relevant exposure and toxicity data as well as information on modes of action are often lacking.

8 PBK is synonymous of PBPK, PBPK, PBTK.
During a JRC workshop in November 2016 on the use of PBK models in risk assessment, twenty-two experts unanimously agreed that there is a need for a guidance document for the new generation of PBK models that integrate in vitro, in silico and NAM data (Paini et al., 2017b). Thus, the ultimate aim of the endorsed OECD guidance document is to address such a need so that the credibility of this new generation of models can be established in order to promote their acceptance and use in a regulatory context.

The intention is to provide harmonised guidance to model developers, reviewers, and risk assessors on i) characterisation of PBK models using non-animal ADME data, ii) assessment of the model performance and iii) reporting and documentation of model characterisation, evaluation/validation, and intended applications. Additionally EURL ECVAM was active in knowledge transfer of the COSMOS/SEURAT-1 outputs to the EU-ToxRisk partners. On 3 March 2017, a webinar was provided on the COSMOS database and KNIME workflows.

In terms of dissemination, a special issue on the Virtual Cell Based Assay (VCBA), which simulates the in vitro fate and toxicity of chemicals, was published (Comenges et al., 2015; Graepel et al., 2017; Paini et al., 2017c, d; Sala Benito et al., 2017; Whelan et al., 2017a). A JRC technical report covering application of the VCBA for simulation of chemical fate following acute exposure was also published (Proenca et al., 2017). A traineeship on the evaluation of the applicability of PBK modelling for mixtures also resulted in a publication (Desalegn et al., submitted).

The work carried out under objective 2, on GMP for PBK models, of the EURL ECVAM TK Strategy was disseminated during several meetings and events: at the NC3Rs workshop on IVIVE, the ECETOC meeting on Advances in Exposure Modelling, the ICCA-LRI/JRC workshop on exposure assessment and at a session at the

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**Box 5.2**

**Biologically based mathematical models in toxicology: current use and regulatory acceptance**

Scientists from the JRC and US Environmental Protection Agency examined how and to what extent mathematical models of biological organisms are used in toxicology, and identified a number of challenges that need to be overcome for wider use and regulatory acceptance of these non-animal approaches.

The survey focused on the use of physiologically based kinetic (PBK) models which provide a mathematical representation of how chemicals are distributed in the body following exposure (Paini et al., 2017a). This information - what the body does to a chemical – is used along with additional information, e.g. from in vitro toxicity experiments, to determine the potential health risks resulting from realistic exposure conditions.

The results of the survey indicate that PBK models are used widely in academia, industry, and government. Moreover, significant scientific and technical advances have been made in their development and application. There remains some reluctance, however, among regulatory agencies to apply these models to their full potential. One reason for this is the lack of appropriate guidance. The findings were based on the responses of 93 individuals (modelling experts and end-users) from 19 countries. The individual results are publicly accessible at: [http://bolegweb.geof.unizg.hr/questionaire/pbk](http://bolegweb.geof.unizg.hr/questionaire/pbk).

**Figure 5.2:** Geographic distribution of the PBK questionnaire results (From Paini et al., 2017a, Regulatory Toxicology and Pharmacology, 90, p. 107, under CC BY-SA 4.0).
Since March 2017, EURL ECVAM has participated in the HESI PBPK working group, to discuss and advance the utilization of PBK models, by improving PBK model platforms and model evaluation, where results from the survey were presented. Since May 2017, EURL ECVAM has also participated in the EU-ToxRisk biokinetic group, providing an overview of the tools developed in COSMOS (PBK models and VCBA).

5.2 Activities in the OECD Test Guidelines Programme

The OECD Test Guidelines (TG) for the testing of chemicals are a collection of internationally agreed testing methods used by governments, industry, research or contract laboratories and academia to assess the safety of chemical products. They are primarily used in regulatory safety testing and subsequent chemical notification and registration.

The set of TG is updated on a regular basis to keep pace with scientific developments and Member Countries’ regulatory needs. OECD-wide networks of national coordinators and national experts provide input from scientists in government, academia and industry. OECD TG should not be confused with data requirements, which are the prerogative of supra-national (EU) or national authorities.

The OECD Test Guidelines Programme (TGP), with the Mutual Acceptance of Data Agreement (MAD) is the main instrument to promote a globally harmonised regulatory safety testing of chemicals. This supports an open global market for the chemicals industry and protection of the safety of workers, consumers and the environment. OECD also considers animal welfare and is committed to the implementation of the 3Rs principles in the development of TGs.

Historically, acting on behalf of the EU with a mandate from the European Commission (DG ENVI), the JRC’s EURL ECVAM has taken a leading role in the development of OECD TG and GD based on alternative approaches. Annex 1 of this report summarises the status of adoption of OECD TGs on alternative methods from 2011 to 2017.
out that the issue was not limited to similar methods, but was a question of priorities within the TGP. The WNT supported the option to have a modified SPFS template for similar methods, require an up-front submission package, develop a modified process for peer-review, and a simplified process for adding similar methods to existing TGs.

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

5.2.2 Guidance Document on Integrated Approaches to Testing and Assessment for Serious Eye Damage/Eye Irritation

The assessment of serious eye damage/eye irritation is a basic information requirement in international regulations for the classification and/or safety assessment of chemicals, pesticides, and medicines. Under some regulations (e.g., EU Cosmetics Regulation, REACH) this information is required to be generated without the use of animal tests.

In 2002, a supplement describing a sequential testing and evaluation strategy was added to OECD TG 405 on in vivo 'acute eye irritation/corrosion'. This strategy recommended that, prior to undertaking the described in vivo test, a weight-of-evidence analysis be performed on all existing relevant data and that, where insufficient data were available, new data be developed through application of a sequential testing strategy, starting first with alternative methods, in order to avoid unnecessary testing in laboratory animals.

Since publication of the supplement in 2002, several in vitro methods for serious eye damage/eye irritation have been developed, validated and accepted by the OECD. Depending on country requirements and the results obtained with the OECD accepted methods (e.g., BCOP, ICE, FL, STE, EpiOcular™ EIT and SkinEthic™ HCE EIT), they may in many cases satisfy all information requirements for serious eye damage/eye irritation.

In addition, non-standard methods (e.g., not yet validated and/or accepted by OECD) may provide further information (e.g., persistence vs. reversibility of effects, direct identification of Category 2 chemicals) that could contribute to the full replacement of the in vivo rabbit eye test.

Although the suitability of such data for regulatory purposes needs to be judged case by case, they should be considered before conducting animal studies. For these reasons, guidance in relation to the use and generation of data for serious eye damage/eye irritation required updating and, therefore, the EC JRC's EURL ECVAM and the United States Environmental Protection Agency (U.S. EPA) proposed to jointly lead the development of a Guidance Document (GD) on an Integrated Approach on Testing and Assessment (IATA) for serious eye damage and eye irritation at the OECD.

The objective was to provide guidance on the possible use and usefulness of individual test methods and on how to best combine them to reach a scientifically sound conclusion in an effective way, at the same time minimising animal testing to the extent possible. The project was approved by WNT in April 2015.

The final GD was adopted by the WNT in April 2017 following two commenting rounds, and was published by the OECD in July 2017 (OECD, 2017). TGs 405, 437 (BCOP), 438 (ICE), 460 (FL), 491 (STE) and 492 (EpiOcular™ EIT and SkinEthic™ HCE EIT) were also revised and adopted at the same time to include a new reference to the GD and delete the Supplement from TG 405 (see also Annex 1).

5.2.3 Adoption of New Test Guidelines for Skin Sensitisation

Two new TGs for skin sensitisation testing were approved by the WNT at its April meeting in 2017, OECD TG on the U937 cell line activation Test (U-SENS™) and OECD TG on the IL-8 Luc assay.

The U-SENS™ method is proposed to address the third key event (dendritic cell activation) of the skin sensitisation AOP by quantifying the change in the expression of a cell surface marker associated with the process of activation of monocytes and DC (e.g., CD86), in the human histiocytic lymphoma cell line U937,
following exposure to sensitisers (Piroird et al., 2015). The measured expression levels of CD86 cell surface marker in the cell line U937 is then used for supporting the discrimination between skin sensitisers and non-sensitisers.

The U-SENS™ method has been evaluated in a validation study and subsequently independently peer reviewed by the EURL ECVAM Scientific Advisory Committee (ESAC) (ESAC, 2016c, see section 4.8). Considering all available evidence and input from regulators and stakeholders, the U-SENS™ was recommended by EURL ECVAM (EURL ECVAM, 2017) to be used as part of an IATA to support the discrimination between sensitisers and non-sensitisers for the purpose of hazard classification and labelling.

The IL-8 Luc assay addresses the third key event (dendritic cell activation) of the skin sensitisation AOP. In contrast to the h-CLAT, which follows the expression of cell surface markers, it quantifies changes in IL-8 expression, a cytokine associated with the activation of DC. In the THP-1-derived IL-8 reporter cell line (THP-G8, established from the human acute monocytic leukemia cell line THP-1), IL-8 expression is measured following exposure to sensitisers (Takahashi et al., 2011). The expression of luciferase is then used to aid discrimination between skin sensitisers and non-sensitisers.

The IL-8 Luc method has been evaluated in a validation study (OECD, 2017h) conducted by the Japanese Centre for the Validation of Alternatives Methods (JaCVAM), the Ministry of Economy, Trade and Industry (METI), and the Japanese Society for Alternatives to Animal Experiments (JSAAE) and subsequently subjected to independent peer review (OECD, 2017i) under the auspices of JaCVAM and the Ministry of Health, Labour and Welfare (MHLW) with the support of the International Cooperation on Alternative Test Methods (ICATM). The IL-8 Luc assay is considered useful as part of IATA to discriminate sensitisers from non-sensitisers for the purpose of hazard classification and labelling.

Based on a proposal made by the EC at the meeting of the OECD expert group on skin sensitisation held on 2 and 3 November 2016 in Paris, the OECD decided to develop key event-based TGs for non-animal test methods addressing a specific key event within an existing AOP. This approach would facilitate organising the various test methods into single documents and simplify locating methods that address the same key event.

The first example of key event based TG (TG 442E) describes in vitro assays that address mechanisms under the 3rd Key Event of the AOP for skin sensitisation, i.e., activation of dendritic cells (OECD, 2012a). The TG comprises test methods to be used for supporting the discrimination between skin sensitisers and non-sensitisers in accordance with the UN GHS. The test methods described in this TG are: the Human Cell Line Activation test (h-CLAT), the U937 cell line activation Test (U-SENS™) and the Interleukin-8 Reporter Gene Assay (IL-8 Luc assay).

The test methods included in this TG differ in relation to the procedure used to generate the data and the readouts measured and can be used indiscriminately to address countries’ requirements for test results on the 3rd Key Event on the AOP for skin sensitisation while benefiting from the Mutual Acceptance of Data.

5.2.4 Development of OECD Test Guidelines on in vitro Fish Hepatic Metabolism

The OECD project on the development of new OECD TGs on in vitro Fish Hepatic Metabolism (under the lead of USA and the EC represented by JRC’s EURL ECVAM) aims 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 rates.

The project builds on work carried out within the framework of the ILSI HESI project ‘Bioaccumulation’. ILSI HESI coordinated a multi-laboratory ring trial to assess the reliability, transferability, and predictive value of the two in vitro methods (2014-2016). Building on the protocols used, two OECD TGs and a guidance document have been developed and published for consultation with WNT experts in April and August 2017 (OECD, 2017j-m).

The fish intrinsic hepatic clearance rate derived with in vitro methods can be extrapolated to a whole-body metabolism rate constant. Inclusion of measured biotransformation rates enhances the reliability of models to estimate the Bioconcentration Factor (BCF) (Laue et al., 2014;
Nichols et al., 2013). The BCF is either predicted or measured (typically in fish, but if necessary, also in invertebrates).

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. More reliable BCF prediction models have the potential to reduce uncertainty and thus avoiding unnecessary testing on fish.

In order to support this project, EURL ECVAM has launched the development of a curated database containing in vivo and in vitro biotransformation data (see section 2.6.6).

5.2.5 Update of OECD Guidance Document 23 on Aquatic Toxicity Testing of Difficult Substances

This project initiated by the International Council on Animal Protection in OECD Programmes (ICAP) and co-led by the EC (JRC-EURL ECVAM) addresses the use of solvents in fish toxicity tests. When solvents are used, e.g., for the testing of poorly soluble chemicals, OECD test guidelines require two control groups - a water control and a solvent control.

Part 1 of the project initially aimed at updating specific sections of OECD Guidance Document 23 on Aquatic Toxicity Testing of Difficult Substances and Mixtures (OECD, 2000) with advanced methodology for media preparation and exposure systems, and by that minimising the use of solvents.

At the WNT meeting in 2016, OECD member countries agreed upon the complete revision of GD 23 (under the lead of USA) to adequately address the many comments received on all sections of GD 23 during the 1st WNT commenting round. During 2016-2017, the three parties worked closely together and the revised draft GD 23 has been published for WNT consultation in August 2017 (OECD, 2017n).

Part 2 of the project 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. It is anticipated that a Detailed Review Paper (DRP) will be prepared.

5.2.6 Revision of OECD Guidance Document 126

The project aims at 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 evaluation of data is ongoing.
5.2.7 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 (LC50) 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 (moribund state) to reduce the suffering of the fish. The evaluation of non-lethal endpoints and their predictivity is ongoing.

5.2.8 OECD Test Guideline on CYP Induction
The human cytochrome P450 (CYP) activity n-fold induction in vitro test method assesses the potential of test items to induce three cytochrome P450 (CYP) enzyme activities (CYP1A2, CYP2B6, and CYP3A subfamily) in two human-derived metabolically competent hepatic in vitro test systems: the cryopreserved human primary hepatocytes and the cryopreserved human HepaRG (HepaRG).

The method has been validated and peer reviewed (EURL ECVAM, 2014; Zuang et al., 2015) and an EURL ECVAM Recommendation is currently being prepared (see section 4.9.2).

A PBTG, prepared by EURL ECVAM, including both the CryoHepaRG™ and the human primary hepatocytes test methods, underwent a first commenting round in 2015. Subsequently, a first meeting of the OECD Expert Group on Biotransformation Assays took place in Paris, France, on 11 to 12 May 2015.

Considering the need for TGs on in vitro metabolism, the expert group suggested that a TG based on the CryoHepaRG™ model should be developed in the short-term and that other methods, based e.g. on human primary hepatocytes, could be included in the medium-term, while keeping in mind the development of AOP/IATA in the long-term.

It was considered that for the regulatory early screening phase of substances, a reproducible and easy to use model for the evaluation of the induction properties of a chemical was needed. The cryoHepaRG™ is likely to deliver more consistent results and the test system itself is more readily available than the cryopreserved human primary hepatocytes model.

In addition, it was suggested to prepare an explanatory background review document (BRD) that describes the context of use of this type of methods in a regulatory hazard and risk assessment framework for a better understanding of the value of information on the CYP inducing properties of a chemical. The need for an accompanying GD was also raised at the OECD WNT meeting in April 2017.

5.2.9 OECD Guidance Document on Good In Vitro Method Practices for the Development and Implementation of In vitro Methods for Regulatory Use in Human Safety Assessment
A Guidance Document (GD) on Good In Vitro Method Practices (GIVIMP) for the development and implementation of in vitro methods for regulatory use in human safety assessment was identified as a high priority requirement by the OECD.

The GD aims at reducing the uncertainties in cell and tissue-based method predictions by the application of good scientific, technical and quality practices from method development to method implementation for regulatory use.

The draft GIVIMP GD is coordinated by EURL ECVAM and was accepted on the work plan of the OECD test guidelines programme in April 2015 as a joint activity between the Working Group on Good Laboratory Practice (GLP) and the Working Group of the National Coordinators of the Test Guideline Programme (WNT).

During the first stage, expert input was received from European regulatory agencies (the European Food Safety Authority (EFSA), the European Medicine Agency (EMA), the European Chemicals Agency (ECHA)), the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL), the EU and OECD Working Groups on GLP, 3Rs Centres, a regulatory agency (RIVM), from scientists of large industries and SMEs and from international scientists with expertise in stem cells, cell biology, GLP and in vitro methods.

The GIVIMP GD is divided into 10 sections covering: (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.
This guidance is not intended to duplicate or replace existing OECD Guidance Documents but rather to complement them by addressing specific gaps and collecting available references and information on best scientific, technical and quality practices in one document.

On 23 and 24 March 2017, EURL ECVAM hosted a meeting with the OECD GIVIMP expert group to discuss some outstanding issues (see Box 5.2). Following on from this meeting, a new draft version of GIVIMP (v05) incorporating the feedback received during the meeting was prepared. On 4 August 2017, this GIVIMP GD draft was sent to the OECD for the second commenting round. The GD is expected to be adopted at the 30th WNT meeting to be held on 24 to 27 April 2018.

The purpose of the GIVIMP guidance document is to take into account all necessary good scientific, technical and quality practices, to ensure that the overall process from in vitro method development to in vitro method implementation for regulatory use becomes more efficient and effective.

This proposal for the development of a PBTG in the area of skin sensitisation builds on discussions held during an International Cooperation on Alternative Test Methods (ICATM) workshop on the regulatory applicability and acceptance of alternative approaches to skin sensitisation, hosted by EURL ECVAM in October 2016 (see section 7.1 and Box 7.1). More than 20 regulatory authorities from the US, EU, Korea, Japan, China, Canada, and Brazil were represented.

One major outcome of the ICATM workshop was the proposal to develop assessment criteria for non-animal DAs for skin sensitisation that will serve as alternatives to the current animal tests, namely the LLNA (OECD TGs 429, 442A and 442B) and the guinea pig tests (OECD TG 406), in the context of IATA (e.g., always considering other existing reliable and relevant information).

A set of criteria were already proposed by the regulatory experts at the workshop, and will be used by ICATM as a basis to propose assessment criteria within the OECD, in consultation with the relevant expert group and the WNT to evaluate a number of existing DAs (Annex 1 to OECD GD 256) and individual test methods (if available/applicable). These
assessment criteria will be based on key criteria such as biological plausibility, inclusion of existing validated in vitro methods covering AOP key events, consideration of the known applicability domain of the DAs, potential of the DAs to generate data that may inform potency sub-categorisation, accessible data interpretation procedures, and performance against reference chemicals.

Performance criteria will be derived from, for example, a comprehensive analysis of current animal test (LLNA) data to determine thresholds for acceptance based on i) reproducibility of the animal test and ii) concordance with human data where available.

The assessment framework will also provide criteria for selection of appropriate reference chemicals to be used to compare the performance of DAs (and individual test methods) to the LLNA. The ultimate goal will be to use the assessment framework to draft a PBTG including those DAs (and individual test methods) able to meet the acceptance criteria.

A special meeting of the OECD WNT will take place from 13 to 15 December 2017 at the JRC. On occasion of this meeting preliminary assessment criteria as well as potential ways for analysing the in vivo (LLNA and human) data will be proposed for discussion.

5.2.11 New Project Proposal on a Guidance Document for Human Hepatic Metabolic Clearance Methods
The published EURL ECVAM Toxicokinetics (TK) Strategy (Bessems et al., 2015) aims to promote the use of TK/ADME data in chemical risk assessment considering due to the increasing demand (from researchers, test method developers, regulators and end-users) to integrate TK information in chemical risk assessment.

This is based on the fact that exposure to a chemical does not automatically mean that all of the dose will be bioavailable and therefore able to cause a specific toxicity. Hence the knowledge of the chemical’s human kinetics can contribute to the better design of toxicity tests (both in vivo and in vitro) and the interpretation of toxicological findings.

Consequently, the EC-EURL ECVAM has proposed the development of an OECD GD to characterise and describe a specific class of in vitro methods for measuring hepatic metabolic clearance as a first practical step to promote the use of kinetics information. The underlying rationale is that hepatic metabolic clearance represents in many cases the main driving process of kinetics and that there are already several non-guideline in vitro methods to measure hepatic metabolic clearance which can significantly vary in their experimental settings, stage of development, intended use, reliability and relevance, etc.

With a view to enhancing the use of in vitro methods for hepatic metabolic clearance in chemical hazard and risk assessment, the objective of this GD is to establish a consistent and transparent framework. This framework will be focused on identifying the relevant elements to be considered when characterising and describing in vitro hepatic metabolic clearance methods in order to facilitate the assessment of their performance, method comparison and increase confidence in their use.

The framework and its elements are intended to be descriptive rather than prescriptive. They do not define how a method should be designed, how it should be used, or what performance it should have. Instead they provide a standardised means of characterising, describing and reporting the salient attributes of a method.

In addition, the GD is expected to enhance the communication between in vitro methods developers, end-users and regulators, by increasing the understanding of how in vitro hepatic metabolic clearance data have been generated and therefore what uncertainties need to be taken into account when applying the method for specific applications.

5.2.12 Workshop and New Project Proposal on Developmental Neurotoxicity
A clear overall consensus has been reached among the participants of the recent workshops on developmental neurotoxicity (DNT; October 2016, January 2017) that DNT is a highly relevant toxicological measure, and that the amount of data generated to date is not sufficient to provide confidence on the safety of the thousands of untested chemicals to which pregnant women, infants and children may be exposed, nor to be informative or supportive of epidemiological observations on neurodevelopmental disturbances (Fritsche et al., 2017).
The workshop participants emphasised that there is an urgent need for a problem formulation-driven, fit-for-purpose testing paradigm to supply data for risk assessment to support risk management decisions. Such a testing strategy should be developed and implemented to achieve two aims, conducted simultaneously.

The first aim is to begin using the battery of currently available alternative test methods to generate data that could be used to prioritise chemicals for further testing. The second aim is to generate data that informs risk assessment and management decisions.

Priority for the first aim should be given, where possible, to a human cell-based testing battery covering basic neurodevelopmental processes and key events identified in the existing DNT AOPs to be put into place immediately for screening and prioritisation purposes. It was suggested that the testing strategy should be flexible, able to incorporate new technologies, and adaptable to the different problem formulations. The testing battery should be part of an IATA strategy, and the process should result in an OECD GD on DNT.

Currently, the main task is to establish performance standards and readiness criteria for individual in vitro DNT assays evaluation to understand which in vitro assays are ready to be used and which test methods need further optimisation and standardisation. The evaluation of such assays will lead to development of a guidance on principles for the building of an in vitro DNT testing strategy followed by challenging not a single assay, but a battery of in vitro assays by chemical testing.

These efforts should result in development of an OECD GD on available in vitro DNT test methods and principles of building DNT testing strategies for various regulatory purposes. This task has been included in the OECD Work programme 2017 and the GD will be developed in collaboration with EFSA, EU (including JRC) and US DNT experts.

5.2.13 Integrated Approach to Testing and Assessment of Non-genotoxic Carcinogens
Non-genotoxic carcinogens contribute to an increased cancer risk through a variety of mechanisms that are not yet included in international regulatory approaches. To address this need, an OECD expert working group has been set up to develop an integrated approach to testing and assessment (IATA) of non-genotoxic carcinogens internationally (Jacobs et al., 2016).

Among other activities the working group has so far: agreed on the definition of non-genotoxic carcinogenicity, examined the current international regulatory requirements for non-genotoxic carcinogenicity risk assessment and has conducted an analysis of uncertainty in the rodent cancer bioassay (Paparella et al., 2017).

One 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 define a benchmark that new approaches should overcome.

The working group is also tasked to review, describe and assess relevant in vitro assays with the aim of tentatively organising them into levels of testing, following the AOP format, such as that possible structure(s) of the future IATA(s) can be created. A workplan for taking forward the identification of assays for non-genotoxic carcinogenicity is currently under development.

5.2.14 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 perform a retrospective analysis of data obtained from the different miniaturised versions of the Ames test and comparative evaluation with Ames results from the classic Ames test.

A Detailed Review Paper will be drafted in view of an eventual incorporation of these methods into an updated version of OECD TG 471.

Several miniaturised versions of the Ames test are used routinely as early screening method in product development. The added value for the miniaturised versions of the test is mainly based on significant reduction of test material and of costs and on the possibility for simultaneous analyses of large number of samples. The inclusion of these versions of the Ames test in an updated version of the TG would eventually allow more regulatory use and integration in genotoxicity testing.
During its kick-off meeting in February 2017, the Expert Working Group decided to first launch an exploratory survey in order to obtain better insights in (i) the use, (ii) the amount of data available for the different miniaturised Ames tests and (iii) the extent to which these data can ultimately be shared. In a second phase, this initial survey will be followed with a request for actual data sharing and then with the analysis of the data.

In this first phase, EURL ECVAM contacted its EU-NETVAL (see section 4.10) for the extension of the exploratory survey, launched in May 2017, to the 37 members.

5.2.15 Survey on Acute Systemic Toxicity
At the last OECD WNT meeting the proposal made by ICAPO, the US (through NICEATM), and the EC (through JRC-EURL ECVAM) to launch a new acute toxicity survey to complete our understanding of the international requirements for acute systemic toxicity has been approved.

The survey is also meant to gather information about the level of acceptance of alternative approaches such as read-across, (Q)SAR, potential for waiving tests, in vitro methods, or a combination of such approaches. The survey builds on previous efforts from OECD (OECD, 2001), EPAA (Seidle et al., 2010) and EURL ECVAM (Graepel et al., 2016, see Box 5.3) and intends to cover all industry sectors for oral, dermal, and inhalation acute toxicity endpoints.

The outcome of the survey will be published in a peer-reviewed journal and will hopefully be useful in guiding activities worldwide of stakeholders working to replace acute systemic in vivo tests.

5.2.16 Update of OECD Guidance Document 150
The OECD GD 150 on the assessment of chemicals for endocrine disruption was published in 2012 (OECD, 2012b).

The objectives of the Guidance Document are to support regulatory authorities’ decisions on the hazard of specific chemicals and toxicologically-relevant metabolites when they receive test results from a TG or draft TG for the screening/testing of chemicals for endocrine disrupting properties by providing guidance on how to interpret the outcome of individual tests and how to increase evidence on whether or not a substance may be an endocrine disruptor (ED). In the context of this document, an ED has

**Box 5.3**

**Opportunities to prevent new animal testing in the area of acute toxicity**

Acute systemic toxicity testing is one in vivo test that always has generated and still generates a lot of debate due to scientific and ethical considerations. In view of the upcoming final registration deadline for chemicals under the EU REACH Regulation, in May 2018, and in line with the EURL ECVAM strategy to replace, reduce and refine the use of animals in the assessment of acute mammalian systemic toxicity, JRC scientists conducted a survey directed to experts in the field of toxicity testing with the ultimate aim of understanding how data from acute systemic toxicity tests are used in practice, and whether non-animal methods and/or existing in vivo data could provide the information needed. The results of the survey were complemented with a retrospective data analysis of acute and repeated-dose toxicity studies from ECHA registration dossiers.

The overall outcome of these investigations (Graepel et al., 2016) supported the use of data from 28-day toxicity tests in order to identify non-classified chemicals under the EU CLP Regulation. The strength of this approach lies in the fact that non-classified chemicals make-up the majority of chemicals currently registered under REACH. In particular, JRC scientists have shown in their analysis that with the use of a previously established threshold of 200 mg/kg b.w./day (Bulgeroni et al., 2009), both NOAEL and LOAEL values can be used in order to identify non-classified chemicals. A higher threshold of 1000 mg/kg b.w./day, as proposed by ECHA in the revision of their guidance document on acute toxicity, would give greater confidence in the predictions. Importantly, the answers to the survey questionnaire showed also willingness to adopt waiving opportunities to chemical pesticides, they could be extended to the assessment of other chemicals, formulations and biological materials on a case-by-case basis.
been defined according to WHO (2002), i.e.,
"An ED is an exogenous substance or mixture
that alters function(s) of the endocrine system
and consequently causes adverse health
effects in an intact organism, or its progeny, or
(sub) populations."

The original version of this GD was focusing on
four endocrine modalities; estrogen receptor
mediated, androgen receptor mediated, thyroid
hormone mediated, and steroidogenesis
interference, both for human health and
wildlife populations.

In order to take into account the evolution of
science and the development of new TGs, the
GD 150 is being updated. The revised version
includes some coverage of juvenile hormone,
ecdysone or retinoid modalities as well as new
sections on e.g. cross-species extrapolations,
integrated approaches and systems, sources
of uncertainties in in vitro assays, the aspect
of multiple simultaneous modes of endocrine
actions, and regulatory experience of endocrine
assessment.

5.2.17 New Endpoints and Assays for
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 has been supporting the
European Commission’s Directorate General
Environment over a wide range of activities
aimed at improving ED detection.

The first step was to conduct a survey (Bopp
et al., 2017) to solicit views of academia,
regulators, industry and civil society
organisations on perceived gaps in test
methods and priorities for addressing the gaps
followed up by three projects focused on the
following specific aspects:
• Thyroid disruption;
• Retinoid signalling pathway;
• Temporal aspects.

Thyroid disruption is linked to neurodevelopment
and cognitive impairment with maternal
hypothyroidism associated with delayed
language development, lower intelligence
scores, hippocampal abnormalities and higher
autism scores. Thyroid cancers are the fastest
rising in both men and women (6% increase
per year) and thyroid disruption also impacts on
cardiovascular health.

Identification of thyroid disruption was
considered to be insufficiently covered by
current test methods. A specific workshop (DG
ENV, 2017) was held to discuss a number of
different aspects of improving test method
design and interpretation including the need
for screening a number of relevant targets with
in vitro methods (see section 4.2) combining
methods to cover multiple mechanisms of
thyroid disruption in a testing battery.

The scoping of a detailed review paper on the
retinoid signalling pathway, to feed into the
EC/Sweden co-led OECD project, was a first
step in investigating the need for development
of methods for assessing retinoid signalling
disruption. There are currently no in vitro
methods in the OECD Conceptual Framework
for the testing and assessment of endocrine
disruptors relevant to the retinoid pathway
although there are a number of effects such as
hypospadias, cryptorchidism, effects on testes
development or spermatogenesis, oogenesis,
oestrus cycle, craniofacial and skeletal system
defects measured in current in vivo test
guidelines that may have a retinoid component.

The temporal aspects project focused on what
are considered to be sensitive windows of
exposure such as embryo-foetal development,
post-natal period and early childhood, puberty
and senescence and the extent to which
current in vivo test guidelines might not cover
such aspects and how such gaps might be
addressed in the future.

All of these concerns were brought together
in a workshop on setting priorities for further
development and validation of test methods
and testing approaches for evaluating endocrine
disruptors at the end of May 2017. The
workshop report is in preparation. Participants
from academia, regulatory authorities, civil
society organisations and industry were asked
to identify endocrine-related diseases/disorders
and pathways of concern, gaps in current
approaches to identify substances which may
be contributing to the disease burden in human
and wildlife populations, ideas to address the
gaps, criteria for prioritising the proposals and
application of criteria to identify the priorities in
the short and long term.

The conclusions confirmed the outcome of the
JRC survey in that the diseases/disorders of
highest concern were related to thyroid disruption,
specifically neurodevelopmental impacts, female reproduction and metabolic disturbances. It was indicated that there was a need for further in vitro assays to investigate pathways outside of the Estrogenic, Androgenic and Steroidogenic (EAS) modalities measuring more downstream effects and accounting for metabolism.

5.2.18 OECD Harmonised Template 201

The OECD Harmonised Templates for reporting chemical test summaries (OHTs) 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 2016 OECD revision of the OHTs included a set 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 picklists 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).

In 2016 OHT 201 was published on the OECD website.

The JRC is currently collaborating with the OECD and ECHA to endorse the use of OHT 201 for all in vitro test results, especially those that are currently stored in less suitable “apical” (in vivo test–oriented) templates.

5.2.19 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 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 April 2017, the JRC hosted the annual face-to-face meeting of the Steering Group, and experts from the OECD secretariat, Germany, USA, Canada, Japan, the Russian Federation, and ECHA discussed the upcoming development of new eChemPortal features.

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

The OECD AOP Development Programme was launched in 2012 and is managed by the Extended Advisory Group for Molecular Screening and Toxicogenomics (EAGMST), a large group of experts from various areas of toxicology.

Experts are designated by governmental or non-governmental affiliations (academia, agencies, industry, animal welfare groups, scientific societies, etc.). They meet once a year face-to-face and hold a teleconference after six months to keep pace with new developments.

Together with US EPA, JRC/EURL ECVAM is co-chairing this group, which is leading the AOP development plan, issuing regularly updated GD for standardising the development and assessment of AOPs and supporting the build-up of an AOP knowledgebase (AOP-KB) as described in the following chapters.

5.3.1 Update on the Adverse Outcome Pathway-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-KB project. Within this project EURL ECVAM contributes ICT design and analysis know how and co-manages the project together with the US EPA.

AOP-KB consists of several modules, each tailored to specific needs, a module titled e.AOP.Portal is the uniform search interface to retrieve AOPs from all other modules; 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.

In early 2016, the introduction of ontologies to further harmonise the naming of AOP objects was discussed, and ontologies were then implemented in the AOP-KB Wiki in the second half of 2016 and first half of 2017.

The AOP-Wiki 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) was released in December 2016 with a completely revisited website style and upgraded functionalities such as a facilitated search of AOPs elements through keywords and unique identification numbers.

Due to the increasing number of AOPs and the lack of a harmonised controlled terminology, a joint effort to incorporate ontology-based information in the AOP-Wiki has been carried out by JRC/EURL ECVAM and the US EPA throughout the second half of 2016 and 2017.

The new version of the AOP-Wiki now introduced the concept of 'event component' which provides a structured representation of the KEs using terms from a selected series of existing biological ontologies (Ives et al., accepted).

One or more event components can be assigned to each KE to represent main features as Process, Object and Action through a controlled vocabulary. Biological Process and Object are used to portray the biological system whose perturbation described in the AOP is captured by the Action term. In addition, a separate Context term can be defined to represent the biological environment where the KE occurs in relation to the level of biological organisation.

As a result, all the individual KEs present in the AOP-Wiki as of December 2016 have been annotated using ontologies. In the future, AOP authors will be able to independently annotate their new KEs using the controlled terminology provided in drop-down lists.

The ontology-based annotations 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.

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 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.

5.3.2 Update of OECD Guidance Document on Developing and Assessing Adverse Outcome Pathways

The revised OECD Guidance Document on developing and assessing AOPs has been approved by the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST) at their annual meeting in June 2016.

Following this revision and to keep up with the ongoing development of the AOP framework the OECD handbook writing group, in which EURL ECVAM is involved, prepared proposal for changes/amendments to the existing User’s Handbook, including a new section called “Lessons Learned in Application of the AOP Framework to Challenging Examples” for approval at the 2017 EAGMST meeting.

New findings from the Society of Environmental Toxicology and Chemistry (SETAC) Pellston workshop in April 2017 were already integrated in this new handbook version. Three EURL ECVAM staff members participated in this Pellston Workshop for advancing the AOP framework that dealt with various key questions, which have been identified during a
preceding horizon scanning exercise among the global scientific and regulatory communities.

5.3.3 Development of Adverse Outcome Pathways relevant to Neurotoxicity

Two AOPs relevant to neurotoxicity developed by EURL ECVAM have already been endorsed by WNT and WPHA and published by OECD (Sachana et al., 2017a,b) and another two are the OECD review process:

- Inhibition of the mitochondrial complex I of nigra-striatal neurons leads to parkinsonian motor deficits (in collaboration with EFSA; AOP-Wiki: https://aopwiki.org/aops/3)
- Sodium Iodide Symporter (NIS) inhibition and subsequent adverse neurodevelopmental outcomes in mammals (AOP-Wiki: https://aopwiki.org/aops/54)

The first AOP relevant to Parkinson disease passed an internal and an external OECD reviewing process and after introducing the relevant comments, it will be forwarded to WNT for a final endorsement in 2018. The second AOP (NIS inhibition) is currently being amended taking into consideration the comments of the OECD internal reviewers and shortly will undergo the reviewing process by the external OECD reviewers.

EURL ECVAM has undertaken the first steps in applying the AOP framework for understanding and predicting developmental and adult neurotoxicity (Bal-Price et al., 2015b; Bal-Price et al., 2017) including more holistic and mechanistic understanding of the pathophysiological pathways involved in complex neurodegenerative disorders such as Parkinson disease (Bal-Price and Meek, 2017).

The current AOPs are mainly qualitative pathway descriptions, and further experimental work is required to develop the relationships between key events in quantitative terms.

These AOPs follow publically available conventions adopted in the OECD AOP development programme to permit tailored application of AOPs for a range of different regulatory purposes.

5.3.4 Activities on "Omics"

EURL ECVAM participated in an European Chemical Industry Ecology and Toxicology Center (ECETOC) workshop on “Applying ‘Omics Technologies in Chemicals Risk Assessment” organised in October 2016 to directly discuss how to overcome the obstacles that limit regulatory uptake of ‘omics data.

The workshop focused on best practices in the collection, storage, analysis and interpretation of ‘omics data. As follow up of the meeting, a series of peer reviewed papers have been published (Buesen et al., 2017; Sauer et al., 2017; Kauffmann et al., 2017; Gant et al., 2017; Bridges et al., 2017).

A proposal was also brought to the EAGMST to develop a series of guidance documents dealing with the processing of data from collection to interpretation of results.

Finally, a project proposal on the "Construction of a series of guidance documents for consistent reporting of ‘omics data from various sources” has been submitted to the OECD WNT. This project aims to develop reporting frameworks for the standardisation of reporting of ‘omics data generation and analysis, to ensure that all of the information required to understand, interpret and reproduce an ‘omics experiment and its results are reported. The project will initially focus on transcriptomics.

5.4 VICH Guidelines on Vaccines: Harmonisation of Criteria for Waiving of Target Animal/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.

Since 2008, EURL ECVAM is working on behalf of EMA with VICH experts on the development of VICH guidelines on harmonisation of criteria to waive the target animal batch safety testing for inactivated and live vaccines for veterinary use. VICH GL50 for inactivated veterinary
CHAPTER 5

Box 5.4

Two new guidelines for the reduction of animal tests for quality control of veterinary vaccines

JRC scientists supported the development of two guidelines released by the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) on the harmonisation of criteria to waive target animal batch safety tests for inactivated and live veterinary vaccines. Their implementation (from May 2018 onwards) is a major step towards international harmonisation and will reduce the number of animals used for batch release testing. The JRC’s EURL ECVAM has been working on behalf of EMA with VICH experts on the development of these guidelines.

Each batch of a vaccine has to undergo strict quality control before it is released on the market. Some of these batch release tests are carried out in animals, for example the target animal batch safety test (TABST). Since 1 April 2013, the TABST is no longer required in Europe and has been deleted from the European Pharmacopoeia monographs for veterinary vaccines. However, outside of Europe and European Pharmacopoeia countries the TABST is still requested for batch release. With the new VICH GL55 and the revised GL50(R), European manufacturers have the possibility to apply for a waiver of the TABST when exporting to the other VICH regions (Japan, North America) or countries following the VICH guidelines.

EURL ECVAM had been a driving force for this change. In 1997, EURL ECVAM launched a retrospective study analysing TABST data provided by manufacturers and control authorities. The ECVAM Scientific Advisory Committee (ESAC) peer-reviewed the results of the study and stated in 2002 that the TABST was no longer relevant and should be omitted for routine batch control.

This had been incorporated into the European Pharmacopoeia and from 2004 onwards (until its deletion in 2013) manufacturers had the possibility to waive the TABST provided that their vaccines fulfilled the waiving criteria, i.e., the manufacturer had to demonstrate to the control authorities that at least 10 batches of the given product had not failed the TABST.

In 2016, the European Medicines Agency (EMA) published the final guidance on the acceptance of Three Rs testing approaches in pharmaceuticals testing (EMA, 2016a) and several other documents (guidelines, reflection papers) drafted by the joint CVMP/CHMP expert group on the application of the 3Rs (within the former JEG 3Rs) underwent public consultation (EMA, 2016b-d).

After the mandate of the JEG 3Rs terminated at the end of 2016, EMA established the joint CVMP/CHMP working group on the application of the Three Rs in regulatory testing of medicinal products (J3RsWG), which will finalise the guidelines and reflection papers drafted by the JEG3Rs, work on 3Rs issues related to batch release testing of vaccines and other biologicals, and support the implementation of Directive 2010/63/EU (EU, 2010).

The J3RsWG 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).

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

In 2016, the European Medicines Agency (EMA) published the final guidance on the acceptance of Three Rs testing approaches in pharmaceuticals testing (EMA, 2016a) and several other documents (guidelines, reflection papers) drafted by the joint CVMP/CHMP expert group on the application of the 3Rs (within the former JEG 3Rs) underwent public consultation (EMA, 2016b-d).

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5.6 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 eight business sectors.

The partners are committed to pooling knowledge and resources to accelerate alternative approaches to animal use in regulatory testing.
The overall aim is the replacement, reduction and refinement (Three Rs) of experiments on animals. The JRC, represented by EURL ECVAM, is one of the Commission services that are members of EPAA.

The European Partnership for Alternative Approaches to Animal Testing

In the reporting period, EPAA has focused mainly on the promotion of regulatory and user acceptance. In this context, EPAA runs a number of projects that are described in the sections below. The partnership also engages in dissemination activities (see section 6.7).

5.6.1 Optimised Evaluation of Skin Sensitisation

As described in the previous EURL ECVAM report (Zuang et al., 2016), in December 2015, the EPAA skin sensitisation team started a new project aimed at comparing the performance of in vitro skin sensitisation methods based on 3D-epidermis to predict a set of “difficult” reference chemicals.

Data for twelve chemicals have been generated with the reconstructed human Epidermis (epiCS®), IL-18 test method and the SenCeeTox. Such data have been complemented with SENSIS results from the Cosmetics Europe evaluation program. The analysis of the data is ongoing.

5.6.2 Waiving of the Two-year Carcinogenicity Studies

The University of Wageningen in collaboration with the Dutch Medicines Evaluation Board has finalised the compilation and analysis of a database on active pharmaceutical ingredients.

The aim was to confirm and expand previous investigations by Sistare et al., (2011) to identify opportunities for waiving the two-year carcinogenicity studies based on in vitro genotoxicity testing and the results of (sub-)chronic toxicity studies. This approach is now under consideration by the ICH.

Moreover, the combination of the histopathological approach as proposed by Sistare and colleagues with the pharmacological approach (van der Laan et al., 2016a) has been investigated. The data for this evaluation were obtained from reports from the Medicines Evaluation Board of the Netherlands. This approach resulted in an ability to predict non-carcinogens and carcinogens at a success rate of 92% and 98%, respectively.

These findings show that by taking the results of the three to six month rat studies and the pharmacological properties of a pharmaceutical product into account, a reliable prediction of a carcinogen can be made without the need for two-year carcinogenicity studies (van der Laan et al., 2016b).

A new project has been awarded aiming at evaluating whether a similar approach as described above for pharmaceuticals can be translated and adapted to the assessment of agrochemicals. The objective of the project is to provide evidence that data from three-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.

5.6.3 Acute Toxicity

The data mining exercise aimed to enable the identification of clinical signs predictive of mortality that was initiated in 2015 is still ongoing (Zuang et al., 2016, section 5.9.2).

After completion of the statistical analysis and based on the results obtained, the team plans to finalise a decision framework document that aims to replace animals in acute toxicity testing and in those cases where animal usage cannot be avoided, to substitute clinical signs predictive of mortality at higher dose levels and thereby replace mortality as the principal endpoint.

The document will ultimately facilitate discussions with regulators and industry regarding acceptance and implementation of the new results. A peer review publication of the overall data
evaluation, which is carried out by NC3Rs in collaboration with the UK Chemicals Regulation Directorate (CRD), is foreseen.

5.6.4 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 results of BSP130 were discussed with the study participants at a workshop in September 2015. The results show that the in vitro assays are repeatable and reproducible and that there is excellent overall concordance with the mouse tests (Sinitskay et al., 2016). However, in order to fully exploit the advantage of the Vero cell assays, further work is needed.

A follow-up study (BSP130 III) with 14 participants started in 2016 aiming at the further optimisation of the Vero cell assays to increase their sensitivity and accuracy. The experimental phase will be finalised in 2017.

Adaptations of the Vero cell assays to other clostridial vaccines will be evaluated in the VAC2VAC project (see section 2.4).

5.6.5 Human Rabies Vaccine Project
Based on the outcome of discussions held at an EPAA workshop in 2012, the EPAA human rabies vaccines group organised a collaborative study to identify the most suitable ELISA for quantitation of glycoprotein-G in rabies vaccines and possible replacement of the current in vivo test for potency testing of human rabies vaccines.

Vaccine samples of different origin and composition were tested with three different ELISAs currently in use by manufacturers and control authorities. The results of the study and possible follow-up were discussed at a workshop in May 2015 (Morgeaux et al., 2017).

One out of the three ELISAs correctly quantified the antigen content of all vaccine samples including degraded samples (Morgeaux et al., 2017). After additional work carried out during 2016, the EDQM BSP accepted this ELISA for full validation and the BSP148 project “Validation of a rabies in vitro potency assay” was launched in 2017.

5.6.6 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. Due to evident differences in the current regional requirements, manufacturers may need to carry out animal tests which are no longer required in Europe, if they want to market their products outside of Europe. EURL ECVAM is a member of the project team.

At an international workshop, held in September 2015 (see Box 5.5), representatives from regulatory bodies and manufacturers discussed steps towards deletion of general safety tests and identified means towards implementation of in vitro methods for potency testing of human and veterinary vaccines.

The major recommendation, agreed by all participants, is the deletion of general safety tests, e.g., abnormal toxicity test, target animal batch safety, from regulatory requirements at a global level. Nowadays, these tests lack scientific relevance and their omission does not compromise the safety of vaccines, or any other pharmaceutical, since more adequate quality control measures are in place.

The project team is following up the recommendations in collaboration with workshop participants and relevant stakeholders (Schutte et al., 2017). For example, requests for revision have been presented to the European Pharmacopoeia Commission via the German Pharmacopoeia and drafted by the Paul-Ehrlich Institut in Germany. The revised 49 monographs have been published in Pharmeuropa for public consultation.

5.7 UN subcommittee on Globally Harmonised System of Classification and Labelling of chemicals (GHS)

On the initiative of the Netherlands and the United Kingdom, an informal working group was set up under the UN Sub-Committee of experts on GHS to further explore the use of non-animal methods for classification.

This review of the GHS would include *in vitro*, *in silico* and *in chemico* methods, as well as grouping and read-across, as a basis for hazard assessment. It was suggested to start with a ‘pilot’ hazard class, which was selected to be skin corrosion/irritation.

The current GHS chapter was re-drafted by the Netherlands and is currently under discussion in the working group.

The activity in the UN Sub-Committee related to the use of non-animal testing methods for classification of health hazards is in the scope of the EURLECVAM work programme, therefore EURLECVAM has suggested to initiate the drafting of the chapters on skin sensitisation and eye damage/irritation in collaboration with the United Kingdom and the Netherlands with support from the working group.

As the work progresses and experience is gained on the inclusion of alternatives into the GHS criteria for health hazards, and in parallel considering further adoption of alternative methods and development of defined approaches and integrated approaches to testing and assessment within the OECD test guideline programme, further

**Box 5.5**

**Recommendations to delete or replace animal tests for quality control of biologicals**

Due to their complexity of composition and heterogeneity, biologicals undergo legally required quality control (QC) before they are released on the market. Some of these QC tests involve testing on animals. However, legal requirements for the QC are not harmonised at global level, which may lead to unnecessary animal testing. For example, specific animal tests may have been deleted from requirements or replaced by non-animal approaches in Europe, whilst the same animal tests are still required outside of Europe.

In September 2015, the European Partnership for Alternative Approaches to Animal Testing (EPAA) convened an international workshop ‘Modern science for better quality control of medicinal products: Towards global harmonization of 3Rs in biologicals’, which took place in Egmond aan Zee, The Netherlands, to discuss with 45 invited international experts the relevance of current animal tests and the promotion and acceptance of non-animal methods. In particular, the usefulness of general safety tests (e.g., abnormal toxicity test, target animal batch safety test) has been discussed and participants agreed that these tests are no longer useful to ensure the safety of biologicals for human or veterinary use. Therefore, they recommended their deletion from all legal requirements and guidance documents at a global level.

Workshop participants further addressed batch potency testing of e.g., diphtheria, tetanus and erysipelas vaccines. Potency tests are performed to demonstrate that the given vaccine batch induces protective immunity. It was acknowledged that non-animal methods are now available and should be used provided that they are appropriately validated. However, some jurisdictions may not accept non-animal tests. Consequently, seeking a common understanding on scientific principles of their use was identified as an overarching goal. Moreover, the development of new non-animal approaches may be an effective means to increase and facilitate international harmonisation (Schutte et al., 2017).
evaluation of how (and when) to tackle other more complex systemic health hazards will be discussed with a view to developing a plan for consideration by the UN Sub-Committee in its programme 2019-2020.

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

The International Cooperation on Cosmetics Regulation (ICCR) is a voluntary international group of cosmetics regulatory authorities from Brazil, Canada, the European Union, Japan and the United States founded in 2007. It discusses common issues on cosmetics safety and regulation and is in dialogue with relevant cosmetics industry trade associations.

The ad hoc ICCR Joint Regulators-Industry Working Group (JWG) on “Integrated Strategies for Safety Assessments of Cosmetic Ingredients” aims to outline principles that underpin the integration of novel methods and data for the safety assessment of cosmetic ingredients, or ‘Next Generation’ risk assessment.

The scope of new approach methodologies (NAMs) considered included in silico methods (such as (quantitative) structure activity relationships ((Q)SARs) and other computational modelling approaches), in chemico methods and in vitro tests.

EURL ECVAM has contributed and is continuing to contribute to the working group and writing of an ICCR report that is intended to help build integrated safety assessments for cosmetics-related substances without generating animal data.

Part 1 of the report summarises major overarching principles for an integrated strategy for risk assessment of cosmetics ingredients incorporating NAMs. It was adopted at the 11th ICCR Annual Meeting in July 2017 and is publicly available from the ICCR website (Amaral et al., 2017).

The four main overriding principles are:

1) human safety risk assessment as overall goal,
2) the assessment being exposure led,
3) hypothesis driven and 4) designed to prevent harm (e.g., distinguishing between adversity and adaptation). The other five principles describe how ‘Next Generation’ risk assessments should be conducted and documented.

Work continues on Part 2 which will illustrate how NAMs may be used in the cosmetic safety evaluation process, related to the principles, with examples of methods and their current strengths and limitations.

5.9 Nanocomput - Computational Models for the Regulatory Assessment of Nanomaterials

In March 2017, EURL ECVAM completed the Nanocomput project, the main aims of which were to review the current status of computational methods that are potentially useful for predicting the properties of engineered nanomaterials, and to assess the applicability of these approaches for regulatory purposes, with emphasis on REACH (Worth et al., 2017b).

The 39-month project was carried out for European Commission Directorate General Internal Market, Industry, Entrepreneurship and SMEs (DG GROW) and was supported by a steering group with representatives from DG GROW, DG ENV and ECHA (see Box 5.6).

Emphasis was placed on Quantitative Structure-Property Relationship (QSPR) and Quantitative Structure-Activity Relationship (QSAR) models, as well as grouping and read-across. In addition, a diverse array of compartment-based mathematical models were reviewed.

These models comprised toxicokinetic (TK), toxicodynamic (TD), in vitro and in vivo dosimetry, and environmental fate models. The computational models were reviewed in a systematic manner, with the results (model characteristics) being made available in the form of an Excel database. For the QSPR and QSAR models (predicting physicochemical properties and biological activities, respectively), the QSAR Model Reporting Format (QMRF) was used. For the compartment-based models, new reporting templates were developed.

Overall, it was found that the model landscape is sparsely populated, especially in the case of models that are directly predictive of regulatory endpoints.

In relation to grouping and read-across, a comprehensive review of grouping approaches was carried out. This was supplemented with two case studies on the genotoxicity of nano titania (TiO$_2$) and multi-walled carbon nanotubes (MWCNT).
Review of computational models for the safety assessment of nanomaterials

Manufactured nanomaterials are being increasingly included in a variety of products because of novel characteristics related to their small size and surface properties. However, concerns have been raised about their potential adverse effects on environment and human health. This has led to an increasing interest in assessing the potential risks of nanomaterials, particularly using non-animal approaches, including computational methods.

Considerable scientific progress has been made towards the challenges of predicting the hazardous properties of nanomaterials using non-animal methods, although issues remain in translating these developments into regulatory practice. Therefore, JRC scientists carried out an extensive review of the current status of computational methods used for modelling nanomaterials.

This review, which was published in a report based on a 39-month project, Nanocomput, was carried out by the JRC on behalf of DG GROW (Worth et al., 2017b). It also includes an assessment of the applicability of these computational approaches, with emphasis on the safety assessment needs of the REACH regulation. This was supplemented with two case studies on the genotoxicity of nano titania (TiO₂) and multi-walled carbon nanotubes (MWCNT). They illustrate the applicability and usefulness of ECHA’s guidance on read-across as well as the Read Across Assessment Framework (RAAF), which provides a systematic means of evaluating the uncertainties in a read-across prediction.

Overall, the report shows that considerable progress has been made towards addressing the challenges of modelling nanomaterials. However, there is still a fragmentation in the scientific results, and limited public access to high quality data. A lack of detailed guidance on how to apply existing approaches is preventing the uptake and use of computational models in regulatory decision making. Recommendations for further research and development are therefore made. Supplementary materials are available at http://europa.eu/!nu77WH

The case studies served to explore and illustrate the practical application of ECHA’s guidance on grouping and read-across, including the possible use of chemoinformatic techniques to support a grouping hypothesis. The case studies also explored and illustrated the applicability of ECHA’s Read Across Assessment Framework (RAAF) in analysing and documenting the uncertainties in read-across prediction. Finally, based on systematic reviews of the scientific literature, as well as the outputs of the EU-funded research projects, recommendations for further research and development were also made.

The results and experience gained in the project was well received when presented to Member State authorities and other stakeholders at the Competent Authorities Sub Group on nanomaterials (CASG Nano) in March 2017, and at the ECHA Nanomaterials Working Group (NMWG) meeting in May 2017. The results of the project should be of interest to scientists who are interested in the state-of-the-art in the field of computational nanotoxicology, as well as assessors and regulators who are interested in the practical application of different modelling approaches and tools.

5.10 International Life Sciences Institute/Health and Environmental Sciences Institute (ILSI-HESI) Update

5.10.1 Update Genetic Toxicology Technical Committee Activities

The aims of the ILSI/HESI Genetic Toxicology Technical Committee (GTTC) are to discuss how to: i) develop follow-up strategies for determining the relevance of test results to human health; ii) promote the development, integration and use of new/emerging technologies and scientific knowledge in genetic toxicology testing strategies for hazard and risk assessment; iii) and boost regulatory and OECD acceptance for new assays.

During the annual meeting that took place in Alexandria, USA in May 2017, the different working groups presented their progress. Among other activities, an analysis of most appropriate in vivo follow-up testing, a literature review of genotoxicity of nanomaterials, quantitative analyses of genotoxicity and the clean sheet testing strategy were presented.
This latter activity foresees the development of case studies for the different sectors to test the recently published clean sheet strategy for genotoxicity (Dearfield et al., 2017). A new working group was set up on MoA and the development of AOPs related to genotoxic MoA pathways. In this context, methods to determine the mode of action of genotoxic agents, and how to use new technologies to establish the mode of action of genotoxicity for new chemical entities.

5.10.2 Workshop on Advances and Roadblocks for Use of Genomics Data in Cancer Risk Assessment for Drugs and Chemicals

A workshop to feature multi-sector and international perspectives on current and potential applications of genomics in cancer risk assessment was organised by the Health and Environmental Sciences Institute (HESI), Health Canada and Mc Gill University in Montreal in May 2017.

The workshop specifically addressed: areas in the current cancer risk assessment paradigm where genomics approaches will have a chance to add value in the future; insights gained through genomics data or molecular approaches and roadblocks for implementation of genomics in cancer risk assessment.

Transcriptomics-based tests are used by companies for guiding internal decisions and huge improvements have been made in terms of technology and with the advent of the new generation of RNA sequencing. Nevertheless, there is still limited implementation of transcriptomics in regulatory decision-making.

Several potential applications were presented, among which, clarification of MoA, classification, calculation of points of departure (PoD) and prioritisation. The targeted use of transcriptomics tests, e.g., for MoA determination was the preferred one. It was also underlined that the application may consistently differ whether the transcriptomics tests are applied to data-rich or data-poor chemicals.

Among the roadblocks identified there was the fear by industry to submit data due to uncertainty on how these data would be used by regulators. This is linked to the difficulty in data interpretation. It was thus felt necessary to find a mechanism to promote submissions of transcriptomics data. Also validation and lack of regulatory guidance were considered as roadblocks.

A guidance document on reporting formats for transcriptomics data was highlighted as a priority. A new OECD project proposal on the construction of a series of guidance documents for consistent reporting of ‘omics data from various sources, with an initial focus on transcriptomics, has been proposed by the UK/Canada in November 2017 (see also section 5.3.4).
6 Dissemination of Information on Alternatives

The availability of information on alternatives that is readily accessible is of fundamental importance both to those who use alternatives as well as those tasked with the evaluation of projects and their compliance with the Three Rs (e.g., within the framework of Directive 2010/63/EU).

In order to disseminate information on alternative approaches and enhance overall progress in their use, several database services are available at EURL ECVAM. They are described in the following sections and available on the EURL ECVAM website.
6 Dissemination of Information on Alternatives

6.1 EURL ECVAM Databases

6.1.1 In vitro methods: DB-ALM—EURL ECVAM’s Database service on Alternative Methods to animal experimentation

The DB-ALM provides ready-to-use and evaluated information on the application and development status of advanced and alternative methods in a standardised manner. Information at various levels of detail is provided and defined according to predetermined criteria for data content by experts in the field.

Current focus is given to in vitro methods and non-experimental approaches used for safety assessments of chemicals and/or formulations, but also includes methods for testing drugs or biologicals or for research purposes.

Since 2015, the DB-ALM Method Summary data sector provides a harmonised framework for adequately describing alternative methods in an OECD recommended accepted format (OECD Guidance Document No. 211 (OECD, 2014d)) for describing non-guideline in vitro test methods.

Growing interest in the DB-ALM was observed in 2016 with a total of over 5000 registrations from 82 countries with more than 360 new registrations. The usage increased by 20% compared to the year before with over 37,500 visits to the website contents and more than 3100 documents downloaded in 2016, being the fourth highest during 10 years of public access.

The DB-ALM is referenced in formal OECD documents, scientific books and cited in scientific articles. ECHA suggests the DB-ALM as useful information source and the OECD recommended it for the storage and dissemination of non-guideline in vitro test methods and protocols of in vitro guideline methods.

Table 6.1: The online information content originates from research projects, validation studies or individual submissions and covers as of June 2017.

<table>
<thead>
<tr>
<th>Information Sector</th>
<th>Number of Documents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topic Summaries</td>
<td>5</td>
</tr>
<tr>
<td>Method Summaries</td>
<td>180</td>
</tr>
<tr>
<td>Protocols</td>
<td>159</td>
</tr>
<tr>
<td>Method Evaluations, EU projects, Validation studies</td>
<td>90</td>
</tr>
<tr>
<td>Test Results (individual investigations)</td>
<td>9128</td>
</tr>
<tr>
<td>Contacts to People active in the field of alternative methods</td>
<td>94</td>
</tr>
<tr>
<td>Bibliographic References</td>
<td>74/5</td>
</tr>
</tbody>
</table>
Further information can be obtained from the DB-ALM Progress Report 2014-2016 (Janusch Roi et al., 2016).

6.1.2 In silico methods: QSAR Model Database

The JRC QSAR Model Database is a freely accessible web application that enables users to submit, publish, and search for peer-reviewed summary descriptions of QSAR Models. An internationally accepted format is used, known as the QMRF (QSAR Model Reporting Formats) to ensure the provision of comprehensive and consistent information.

Developers and users of QSAR models can submit to the dedicated mailbox (JRC-COMPUTOX@ec.europa.eu) information on QSARs by using a downloadable QMRF editor which is reviewed for adequacy and completeness by JRC-EURL ECVAM before publishing it through the JRC database.

At the time of writing (October 2017), the online information content of JRC QSAR Model Database covers 147 QSAR Model Descriptions grouped according to OECD defined (regulatory) endpoints as indicated in Figure 6.1 out of which 54 QSAR Reports were published during the past two years (2016 & 2017). A progress report summarising the activities of the QSAR model database during 2016-2017 can be made available on request.

As an indication of usage, in the last 12 months, the JRC QSAR Model database had a total of 8297 visitors, with an average of 23 per day (if a visitor comes the first time or visits a page more than 30 minutes this will be recorded as a new visit).

The public users worldwide in 2017 originate from North America (56%), Europe (24%), Asia (16%), South America, Africa, Oceania and Central America.

The year 2017 shows a steady and continued interest in the JRC QSAR Model database with a peak in May and a decrease towards the end of the year.
Towards the end of 2017 the launch of the upgraded JRC QSAR Model database is expected.

6.1.3 Tracking System for Alternative Test Methods towards Regulatory acceptance (TSAR)

The end of 2016 saw the public launch of the revised Tracking System for Alternative methods towards Regulatory acceptance (TSAR) on occasion of the Commission’s scientific conference on “Non-animal Approaches - The Way Forward” held on 6-7 December in Brussels (see Box 6.2).

The revised TSAR provides enhanced visibility of the progress a test method makes from first being proposed for validation through to its eventual acceptance for regulatory safety and efficacy testing of chemicals or biological agents.

TSAR disseminates information on test methods not only under consideration by EURL ECVAM but by all member organisations of the International Collaboration on Alternative Testing Methods (ICATM) representing the EU, Canada, USA, Republic of Korea, Japan, Brazil and China.

This first release of the revised TSAR includes the majority of methods submitted to the EURL ECVAM back until 2008. The remaining methods will progressively be made available for public access together with those from all ICATM partners.


The EURL ECVAM Search Guide continues to encounter success in and outside Europe and is also applied in America, with particular emphasis on South America, used as a resource for higher education in academic institutions in life sciences and by national authorities for scientific project evaluations that might involve animal use.

It entered the Asian region in 2014 where it was translated and re-published as a handbook and e-book in Korean. Based on the success reached of the Search Guide in Korea, a second edition was produced and published in 2016 and included in a national survey on the 3Rs. It was sent to all Institutional Animal Care and Use Committees (IACUCs) (about 350) of the Animal Research Institutions together with government agency, Animal Protection Division in Korea. A total of 532 responses were received. The survey results were presented at the 10th World Congress on Animal use in Life sciences in Seattle, US.

A second translated version, this time in Portuguese was finalised in autumn 2017 together with Brazilian Authorities within the framework of ICATM.

The EURL ECVAM Search Guide has specifically been developed to inform and support untrained database users in finding high quality information on relevant alternative methods.
and strategies from the large amount of available information resources in an easy, yet systematic, and efficient way during project preparations in biomedical sciences.

6.1.5 EURL ECVAM Genotoxicity and Carcinogenicity Database of Ames Positive Chemicals

The EURL ECVAM Genotoxicity and Carcinogenicity Consolidated Database of Ames positive chemicals, launched at the end of 2014, is a structured and highly curated database compiling available genotoxicity and carcinogenicity data for 726 Ames positive chemicals originating from different sources (Kirkland et al., 2014).

In 2016 and 2017, the database was again used in a wide field of applications by third party actors (see below). The database was constructed following a recommendation of an EURL ECVAM Workshop on “Can in vitro mammalian cell genotoxicity test results be used to complement positive results in the Ames test and help predict carcinogenic or in vivo genotoxic activity?” (Kirkland et al., 2014). Its development implemented part of the published EURL ECVAM strategy aimed at avoiding and reducing animal use in genotoxicity testing (Corvi et al., 2013).

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 in late 2014, the EURL ECVAM Genotoxicity and Carcinogenicity Database serves as a reference database for both the regulatory and scientific communities as demonstrated by a number of on-going activities. The database has 1) contributed to various activities of the Genetic Toxicology Technical Committee of ILSI/HESI on in vivo follow up studies and more recently to the genotoxicity project on Clean Sheet-Industrial Chemicals Case Study Subgroup; 2) been considered the starting point for a major project launched by the Cefic Long-Range Research Initiative (LRI-B18); 3) been considered for a recent re-evaluation of the TTC values for compounds that are genotoxic and/or carcinogenic (Boobis et al., 2017); 4) been the basis to perform analysis of the sensitivity of unscheduled DNA synthesis (UDS), transgenic and comet genotoxicity assays to detect carcinogens to support the recent Scientific Opinion drafted by EFSA on “Reflection on interpretation of some aspects related to genotoxicity assessment” (EFSA, 2017).

Genotoxicity and carcinogenicity results from the DB have been also considered for a recent research project aimed at testing the sensitivity of a transcriptomic biomarker for classifying agents as genotoxic (DNA damaging) and non-genotoxic in human lymphoblastoid TK6 cells (Yauk et al., 2016).

The related publication is still among the most downloaded articles from Mutation Research - Genetic Toxicology and Environmental Mutagenesis Journal since its first appearance (Kirkland et al., 2014).

The database has been linked to two other JRC databases, CheLIST (see 6.1.7) and ChemAgora (see 6.1.8) and, in the past year, to information published in the updated recommended list of genotoxic and non-genotoxic chemicals (Kirkland et al., 2016). This allows retrieving additional information on the chemicals of interest using a single platform.

The DB is a living project with possibilities of continuous update as new genotoxicity and carcinogenicity data are made available. A major extension of the database will be soon available with an enrichment of curated data from more than 200 new chemicals with Ames negative results.

An Ad hoc EURL ECVAM Expert Meeting was held at JRC Ispra on 14-15 September for the finalisation and expert review of the data.
6.1.6 EURL ECVAM Skin Sensitisation Database

The EURL ECVAM Skin Sensitisation Database is a collection of 269 organic chemicals with corresponding skin sensitisation-related results. These include data from \textit{in chemico} (Direct Peptide Reactivity Assay, DPRA, OECD TG 442C) and \textit{in vitro} methods (KeratinoSens™, OECD TG 442D and human Cell Line Activation Test, h-CLAT, OECD TG 442E), Local Lymph Node Assay (LLNA) and human classifications (according to Basketter \textit{et al.}, 2014) assorted molecular descriptors and other QSAR predictions (e.g., from Dragon, TIMES, etc.).

The source for the Skin Sensitisation Database includes submissions to EURL ECVAM and information available in the public domain. The data currently held in the EURL ECVAM Skin Sensitisation Database are stored locally in an Excel sheet, where they will be supplemented with assorted links to other Databases (e.g., CheLIST and ChemAgora).

Ultimately, the DB will be published on the JRC Science Hub.

6.1.7 Update on 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 has published 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.

6.1.8 Update on ChemAgora

People in need of a comprehensive overview of what information is available about a certain chemical often struggle with the heterogeneity of that information, scattered across numerous locations, in different formats and stored under often conflicting identifiers.

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. Hyperlinks to the exact third party pages are provided, where more information about the chemical can be found. The tool also provides a list of synonyms the chemical is known under.

Using ChemAgora, third party databases can be searched by an identifier originally not available in these repositories. Thus, ChemAgora is not only useful for getting an overview of what is currently known about a substance, but also adds value to third party systems.

Making access to information about 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 the reporting period, the following activities led to improvements of ChemAgora:

- A similarity search option was added, which can be started from the structure editor page. The feature is based on the ChEMBL Data Web Services with the possibility to choose between 90%, 80% and 70% Tanimoto similarity cut offs.
- The user interface was adapted to user feedback.
- The third-party repositories were extended including:
  - CREST (Chemical attributes, Regulatory approaches and Experimental STudies from a mixture toxicology perspective)
  - CompTox Chemistry Dashboard from US-EPA

6.1.9 Endocrine Active Substances Information System (EASIS)

The term ‘endocrine active substance’ (EAS) is used to describe 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, the Endocrine Active Substances Information System, is a web-based application 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, e.g. not only with Endocrine Disruptors.

On 3 October 2016, EASIS was officially launched and is now available at https://easis.jrc.ec.europa.eu.

JRC staff presented and promoted EASIS in numerous workshops and conferences, and has now started to identify more relevant data that can be managed and made available through EASIS.

6.2 Knowledge Sharing Activities

During 2016, the JRC’s EURL ECVAM carried out a study (Holley et al., 2016) of available knowledge on the 3Rs to understand how supply of such knowledge can better meet demand.

The work undertaken in this study underpins the first of four actions which were identified by the Commission in response to the European Citizens’ Initiative (ECI) “Stop Vivisection” and set out to assess the current situation regarding the sharing of knowledge relevant to the 3Rs between various sectors and communities with a view to accelerate the development and uptake of non-animal approaches in research and testing.

The results of this study were presented at the Commission’s scientific conference on “Non-animal Approaches – The Way Forward” held on 6-7 December in Brussels (see Box 6.2).

In order to map available knowledge, the JRC profiled over 800 knowledge sources relevant for the 3Rs and compiled them into an inventory covering different types of knowledge sources both explicit (e.g., websites, publications, databases, etc.) and more tacit (e.g., organisations, events, expert groups, etc.).

A public survey was also conducted which aimed to elicit input from people working in areas related to the 3Rs to understand what knowledge sources they use, how they access them, and how they are currently being used to further the 3Rs. The wealth of information provided by the 351 survey respondents, a third of which replied on behalf of their organisations, has been invaluable to identify

READ MORE

EASIS - Endocrine Active Substances Information System

EASIS.jrc.ec.europa.eu

Stop Vivisection

ec.europa.eu/
citizens-initiative/
public/initiatives/
successful/
details/2012/000007
CHAPTER 6

Reducing animal testing through better knowledge sharing

The JRC carried out a study of available knowledge on the replacement, reduction and refinement (the 3Rs) of animal procedures used in research and testing to understand how supply of such knowledge can better meet demand. Findings show that although much 3Rs knowledge exists, its sharing can be improved through better coordination, communication and outreach, and by more emphasis on targeted education and training initiatives.

In 2015, the Commission identified four actions to accelerate the development and uptake of non-animal approaches, in response to the European Citizens’ Initiative (ECI) “Stop Vivisection”. The work undertaken in this study underpins the first action that set out to assess the current situation regarding the sharing of knowledge relevant to the 3Rs between various sectors and communities with a mind to accelerate the development and uptake of non-animal approaches in research and testing.

Inventory of knowledge sources

In order to map available knowledge, the JRC profiled over 800 knowledge sources relevant for the 3Rs and compiled them into an electronic inventory. The inventory covers different types of knowledge sources both explicit (e.g., websites, publications, databases, etc.) and more tacit (e.g., organisations, events, expert groups, etc.) and identifies who they typical target and how they share their content. Such a structured inventory has also allowed a comprehensive meta-analysis of potential knowledge gaps and redundancies.

Public survey

The public survey aimed to elicit input from people working in areas related to the 3Rs to understand what knowledge sources they use, how they access them, and how they aid them in their activities to further the 3Rs.

The wealth of information provided by the 351 survey respondents, a third of which replied on behalf of their organisations, has been invaluable to identify current practice, preferences and opportunities for enhancing knowledge sharing. Notably, many of the survey respondents stated that the available means of knowledge exchange are adequate, whilst two thirds stated that knowledge sources are lacking in their area.

Room for improvement

As described in the JRC report, although there are many 3Rs relevant knowledge sources available, their impact could be greater. There needs to be better awareness and coordination between existing knowledge sources. In addition, much of the vast amount of relevant information needs to be better structured and curated.

The knowledge sources also need to have a greater outreach, to increase the beneficiaries of the knowledge and to bring about more dialogue across sectors and between different groups working with animals and alternative methods.

Education and training opportunities relating to the 3Rs need to be increased and improved, extending across three levels of learning: professional, undergraduate and school-goers. Educators need more dedicated teaching resources and these should be freely available to them and their students.

What knowledge exists and how it can be best exploited can be better communicated to wider sections of potential users. And although there are many examples of good practice of knowledge exchange, in general people require more guidance and trust in the sources which are available.

Findings will feed strategies to accelerate uptake of 3Rs

The findings provide a strong evidence-base on which to formulate collaborative strategies to accelerate the uptake of the 3Rs and reduce the reliance on animal testing via enhanced knowledge sharing. This is important since most focus and investment until now has been in knowledge generation (e.g., via research programmes) rather than in better exploitation of already existing knowledge.

The results of this study inform knowledge providers on how their knowledge can be shared more effectively to accelerate progress in the 3Rs, as well as knowledge-users on the many existing knowledge sources that they might not be familiar with but which could help them in their daily work.

This report also provides a strong basis for the formulation of further activities underpinning the actions in the Commission Communication in response to the ECI, some of which may be supported as part of a one million euro EU pilot project adopted by the European Parliament which seeks to promote the use of alternatives to animal testing in the EU through information sharing and education activities.

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The main conclusions of the study show that although much 3Rs knowledge exists, its sharing can be improved through better coordination, communication and outreach, and by more emphasis on targeted education and training initiatives. The findings provide a strong evidence-base on which to formulate collaborative strategies to accelerate the uptake of the 3Rs and reduce the reliance on animal testing via enhanced knowledge sharing.

**6.3 JRC Summer School on Alternative Approaches for Risk Assessment**

On 16 to 19 May 2017, EURL ECVAM hosted a Summer School on alternative approaches for risk assessment with the aim of providing comprehensive training on state-of-the-art alternative (non-animal) approaches to predictive toxicology and to promote their use within modern chemical risk assessment practice.

The programme was tailored for post-graduate students (PhD/Master) and early-career scientists working in relevant fields. Over 100 students from 29 countries (universities located in 19 countries) aged between 22 and 42 years participated. All together there were 146 registered participants.

EURL ECVAM also established a JRC summer school alumni group to keep the momentum going and to maintain the established professional network.

**6.4 Training Activities on Read-Across**

As part of the dissemination of information on alternative approaches, EURL ECVAM contributes to training activities related to the principles and application of these methods.

EURL ECVAM was involved in the Society of Toxicology (SOT) Continuing Education Course "Read-Across: Case Studies, New Techniques, and Guidelines for Practical Application", with a presentation on applying the Read-Across Assessment Framework to identify and address uncertainty. The outreach includes over 200 registered participants at the course on site at the 56th SOT Annual Meeting on 12 March 2017 and continued availability for training as a recording in the SOT CEd-Tox online courses programme.

Similarly, EURL ECVAM contributed with a presentation on the principles and application of collaborative strategies to accelerate non-animal alternatives in different areas of research and testing.
of read-across to the training event on health-based guidance value development which was held in connection with the WHO Chemical Risk Assessment Network meeting on 19 June 2017 at EFSA in Parma, Italy (see section 7.3).

6.5 Training Activities on Adverse Outcome Pathways

EURL ECVAM is strongly committed to the development and dissemination of AOPs. As a part of the OECD AOP training subgroup involved in the preparation of training material and various training activities, it continued with the planned AOP training courses for EFSA (via a Service Level Agreement), for both EFSA staff and external experts at EFSA premises in Parma. During the reporting period, three such courses were held in November 2016 and in April and June 2017 for a total of 49 participants. The last course is foreseen in November 2017.

The courses aim at creating awareness of the AOP framework, the principles and practices underlying AOP development, description, and evaluation, and to provide the participants with the theory and hands-on skills to facilitate use and application of AOPs. Equally important is to get valuable feedback from risk assessors that in turn can shape further development of the concept to increase the utility for regulators. The courses are consistently highly rated by attendees and EFSA organisers.

Further, EURL ECVAM has established strong ties with the EU-ToxRisk project (see section 2.2) and has provided several training modules on SOP design, validation, and AOPs at their summer school for PhD students, postdoc collaborators, and principal investigators (see section 6.3 and Box 6.3).

In September 2016, a two-day AOP training course has been given for EU-ToxRisk project collaborators (principal investigators, Postdocs, and PhD students) in collaboration with the OECD AOP training group.

6.6 European School Traineeship

In 2017, EURL ECVAM offered a project to the students of the European School in Varese, Italy. The project, “Bringing scientific alternatives to animal testing to your classroom”, helped students to explore the science behind alternatives to animal testing and, using what they had learnt, to develop an exciting learning activity to engage and inform fellow students.
Covering chemistry, biology and toxicology in the context of alternatives, the students discovered what chemicals are used in everyday products, why they need to be tested and how in vitro cell systems and computational methods can be used instead of animals.

The students also had the opportunity to see how EURL ECVAM assesses the reliability of these methods and were introduced to the scientific research that is important to develop them. At the end, they produced a poster which could be used to inform other students and gave feedback on the traineeship.

The project was a good opportunity as well for EURL ECVAM to increase its outreach activities and to engage with a different target audience. Many colleagues participated in this traineeship and gained valuable insight into how we can work to improve our communications with young scientists and to present our work in a way which is more understandable to non-scientists.

6.7 Dissemination Activities of EPAA

6.7.1 EPAA 3Rs Prizes

The EPAA Awards are granted to young scientists (3Rs Science Prize) or laboratory technicians and animal caretakers (3Rs Technician 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.

In 2016, the EPAA granted the 3Rs Science Prize to a scientist from Wageningen University (former grant holder at EURL ECVAM), for his work on a reverse dosimetry approach using Physiological based kinetic (PBK) modelling.

Through this technique, a known tissue concentration can be converted to an external dose as applied to the organism in question, e.g., a human, enabling the translation of in vitro concentrations to an exposure in vivo. While so far this approach has been successfully applied to the prediction of toxic effects of several chemicals in rodents based on in vitro data produced in rodent embryonic stem cells, future studies will investigate the prediction of effects in humans.

6.7.2 Other Dissemination Activities

In 2016 EPAA launched a process to identify the most important scientific challenges to the wider development and adoption of non-animal methods and engaged in a discussion on possibilities to overcome these challenges in progressing towards the ultimate goal of full replacement of animal testing.

The main results of this process were presented and further discussed at EPAA’s Annual Conference in December 2016, of which the report is available online. The process will lead to an amendment of the current EPAA Action Programme (2016 – 2020), which presumably will be published by the end of 2017, and possibly to new activities within EPAA.

In the reporting period, EPAA presented its work and projects in a number of events, such as the 2016 annual conferences of the Federation of Laboratory Animal Science Associations (FELASA) and the European Society for Alternatives to Animal Testing (EUSAAT) as well as at the Danish 3Rs Symposium (September 2016) and the 10th Congress on Alternatives and Animal Use in the Life Sciences (Seattle, USA, August 2017).
It is essential to maintain and strengthen international cooperation on alternative test methods in the areas of validation studies, independent peer review, and development of harmonised recommendations and test guidelines in order to ensure that alternative methods/strategies are more readily accepted for regulatory purposes worldwide.

International cooperation is also necessary to ensure that new alternative test methods/strategies adopted for regulatory use will provide equivalent or improved protection for people, animals, and the environment, while replacing, reducing or refining animal use whenever scientifically feasible. This chapter updates on EURL ECVAM involvement in various international cooperation initiatives.
7 International Cooperation on Alternative Test Methods

7.1 ICATM Workshop and Meeting 2016
EURL ECVAM, in collaboration with the International Cooperation on Alternative Test Methods (ICATM), hosted a two-day workshop on the international regulatory applicability and acceptance of alternative non-animal approaches to skin sensitisation assessment of chemicals used in a variety of sectors on 4 to 6 October 2016 at the JRC in Ispra, Italy (see Box 7.1).

The workshop convened representatives from more than 20 regulatory authorities from the European Union (EU), United States (US), Canada, Japan, South Korea, Brazil, and China, to facilitate a common understanding of the available non-animal methods (e.g., *in vitro*, *in chemico*, *in silico* and read-across) and their role within Defined Approaches (DAs). Working together to identify potential obstacles, the international and cross-sector group defined a series of steps that should be taken to support the regulatory application of DAs.

The participants initially focused on the current regulatory requirements for skin sensitisation in different regions by chemical sector (e.g., pesticides, cosmetics, pharmaceuticals, industrial chemicals, etc.) that could be potentially satisfied with the use of non-animal approaches.

Following the workshop, members of the International Cooperation on Alternative Test Methods attended a one-day meeting with EURL ECVAM. Representatives from organisations from China, Japan, Brazil, South Korea, the US and Canada followed up on how to implement the actions decided at the workshop, and also on how to build on current strategies to facilitate and accelerate global progress in the development, validation, acceptance and use of alternative methods to animal testing.

| Box 7.1 |

**International workshop on alternative non-animal approaches to skin sensitisation assessment of chemicals**

On 4-6 October 2016 EURL ECVAM, in collaboration with the International Cooperation on Alternative Test Methods (ICATM), hosted a two day workshop on the international regulatory applicability and acceptance of alternative non-animal approaches to skin sensitisation assessment of chemicals used in a variety of sectors.

The workshop brought together regulators and alternative method experts from Europe, China, the United States, Brazil, Canada, South Korea and Japan, working in a variety of sectors, to facilitate a common understanding of the non-animal approaches (i.e., *in vitro*, *in chemico*, *in silico* and read-across) that are available in the area and their current proposed use.

The participants focused on the current regulatory requirements for skin sensitisation in different regions by chemical sector (i.e., pesticides, cosmetics, pharmaceuticals, industrial chemicals, etc.) that could be potentially satisfied with the use of non-animal approaches. Working together to identify potential obstacles, the international and cross-sectorial group was able to define initial steps which should be taken to support the regulatory application of these approaches.
There was general consensus among the workshop participants that in order to maximise regulatory consideration and acceptance of data generated with DAs, international harmonisation and standardisation will be necessary. This should ideally be achieved through the development of an evaluation framework that allows an independent assessment of the DAs currently reported in Annex I to OECD GD 256 (OECD, 2016f) and any other upcoming promising DAs.

The area of skin sensitisation has in recent years been at the centre of concerted efforts to replace animal testing. Progress has been made in the development, validation and regulatory adoption of in chemico and in vitro methods, and various in silico approaches (e.g., (Q)SAR models and expert systems) are available (see section 4.9.1 and section 5.2.3).

In parallel, efforts have been made to develop defined approaches to testing and assessment using data generated with these methods, and to derive predictions that would replace information traditionally generated with animal models that can be used in the context of Integrated Approaches to Testing and Assessment (IATA). The outcomes of the workshop will be presented in two peer-reviewed publications (Casati et al., 2017; Daniel et al., 2017).

Following the workshop, members of the International Cooperation on Alternative Test Methods attended a one-day meeting with EURL ECVAM. Representatives from organisations from China, Japan, Brazil, South Korea, the US and Canada followed up on how to implement the actions decided at the workshop, and also on how to build on current strategies to facilitate and accelerate global progress in the development, validation, acceptance and use of alternative methods to animal testing.

The main action discussed and agreed upon was the submission to the OECD of a project proposal for the development of a PBTG for DAs for skin sensitisation testing and assessment. Such a proposal (co-led by the EU, US and Canada) was submitted to the OECD in November 2016 and following revision based on feedback from Member Countries, was approved on 27 April 2017 for inclusion in the OECD workplan (see section 5.2.10).

### 7.2 Overview of EURL ECVAM – NICNAS Training Webinar Series on “Alternative Approaches to Assessing Systemic Toxicity”

EURL ECVAM has delivered a series of webinars on alternative approaches to assessing systemic toxicity to the National Industrial Chemicals Notification and Assessment Scheme (NICNAS) Office of Chemical Safety, Australian Government Department of Health. The following topics were presented:

#### 7.2.1 Skin Sensitisation

The presentation provided an overview of the state-of-the-art in the area. This included a description of the skin sensitisation AOP and of the adopted methods and other methods in the validation/adoption pipeline. The definitions and concepts of IATA and DAs including an overview of current discussions and activities...
CHAPTER 7

at the OECD on their regulatory use and acceptability were provided.

The DAs reported in Annex 1 to the OECD GD 256 (OECD, 2016f) were briefly illustrated as well as the new legal requirements for the endpoint as established within the REACH legislation and how these have been addressed in the revised European Chemicals Agency (ECHA) guidance on information requirements and chemical safety assessment.

7.2.2 Genotoxicity Testing

Several in vitro tests are available at different stages of development and acceptance, yet they are not considered at present sufficient to fully replace animal tests needed to evaluate the safety of substances. The webinar focused on the presentation of recent activities that have taken place which aim at the overall improvement of the traditional genotoxicity testing paradigm (Corvi and Madia, 2017) (see Box 7.2).

These include the improvement of existing tests, the development of novel tests, as well as the establishment and exploration of approaches to optimise in vitro testing accuracy. Furthermore, useful tools, such as databases or reference chemical lists (see section 6.1.5) have been developed to support advances in this field.

7.2.3 Non-Animal Methods: How to Judge their Reliability and Relevance

The principles and process of validation were first established in the 1990s and gained international recognition with the adoption of OECD Guidance Document No. 34 in 2005 (OECD, 2005). If these principles and processes were successful in pioneering the regulatory acceptance of alternative methods for less complex endpoints, an evolution of current practices is needed to embrace emerging technologies and the increased complexity of endpoints.

Indeed, validation needs to keep pace with scientific progress, the availability of new tools and techniques, the need for data integration to address complex endpoints, and the growing demands for better protection of human health and the environment, to ensure that it continues to be fit for purpose and to add value rather than hindering progress.

In 2004, a “Modular Approach to the ECVAM Principles on Test Validity” was proposed with the objective of making the validation process more flexible by breaking down its various steps into seven independent modules, and defining for each module the information needed for assessing the scientific validity of a test method (Hartung et al., 2004).

Box 7.2

Improved performance of in vitro genotoxicity testing?
The evaluation of genotoxicity is an essential component of the safety assessment of all types of substances (industrial chemicals, pharmaceuticals, pesticides, food additives, cosmetics ingredients, etc.) for the protection of human and animal health. In general, the assessment of genotoxic hazards to humans begins with non-animal (in vitro) tests followed in some cases by animal testing.

A variety of well-established in vitro assays are available at different stages of development and regulatory acceptance. However, they are not considered at present sufficient to fully replace animal tests currently used to evaluate the safety of substances for regulatory purposes.

A strategy to reduce and avoid animal use in genotoxicity testing had previously been described by the JRC-hosted EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), based on the regulatory needs across different EU legislations, state of the science, and recent efforts undertaken by various organisations. Moreover, considerable activities have been carried out in the last decade worldwide with the aim of optimising strategies for genotoxicity testing.

These recent achievements together with the implementation of the EURL ECVAM strategy plan are described in this review.

They include the improvement of existing tests, the development of novel tests, as well as the establishment and exploration of approaches to optimise in vitro testing accuracy (Kirkland et al., 2014a, Kirkland et al., 2014b). Furthermore, useful tools, such as databases or reference chemical lists, have been developed by the JRC in collaboration with other scientists to support advances in this field. Some of these activities have also led to the revision of OECD Test Guidelines and regulatory guidance (Corvi and Madia, 2017).
This modular approach to validation should be further exploited to encourage more innovation and flexibility in study design and to increase efficiency in validation. A more systematic consideration of the uncertainty associated with both in vivo and non-animal data is also an important step in building further confidence in the validation of alternative approaches and their regulatory acceptance.

This webinar provided a historical overview of the establishment of the principles of the scientific validation of alternative methods for toxicity testing as well as the challenges and opportunities for adapting the validation practices to keep pace with scientific progress whilst ensuring the protection of human health and the environment and best serve the needs of society.

7.2.4 SEURAT-1 Read-Across Case Studies and ab initio Case Study
The presentation started with a brief introduction to the EU FP7 research initiative SEURAT-1 (see section 2.1), which focused on the complex area of alternative approaches to repeated dose toxicity testing, and the conceptual framework for safety assessment developed. It was followed by two more detailed presentations that covered the cross-cluster SEURAT-1 read-across case studies (Berggren et al., 2015; Schultz et al., 2015; Mellor et al., 2017; Przybylak et al., 2017; Schultz and Cronin, 2017; Schultz et al., 2017a,b) and the SEURAT-1 ab initio case study (Berggren et al., 2017).

7.2.5 Exposure Assessment - Route to Route extrapolation
The webinar introduced the concept of exposure assessment. To assess risk, exposure and hazard factors are needed. For estimation of exposure factors the following steps were highlighted: (i) data collection (of diaries, monitoring of environment, biomonitoring); (ii) organisation of knowledge by using defined frameworks like Aggregate Exposure Pathways (AEP) and hazard Adverse Outcome Pathways (AOPs); (iii) simulation of chemicals fate and transport and human exposure by predictive models; Finally, (iv) route to route extrapolation was explained by means of an approach applicable to cosmetics compounds, called the margin of internal exposure (MOIE).

In the MOIE approach PBK models developed for human and rodents for simulating caffeine exposure via oral and dermal route were applied (Bessens et al., 2017). The models developed as part of the COSMOS Project within the SEURAT-1 research initiative, were implemented as KNIME workflows and are freely available. Furthermore, the evaluation of the applicability of the Threshold of Toxicological Concern (TTC) concept to cosmetics-related substances was discussed, including building of a cosmetics-ingredient enriched TTC dataset and extrapolation to the dermal route of exposure relevant to cosmetics (Williams et al., 2016; Yang et al., 2013; Yang et al., 2017).

7.2.6 EU Activities relevant to the Identification and Assessment of Endocrine Disruptors
The webinar started with the need for assessing EDs in general, followed by past and present activities in the EU regarding endocrine disruptors and the scientific criteria for EDs. This was followed by the JRC work (Munn et al., 2016) on the ED impact assessment, explaining how the data has been captured and codified, and the automatically generated matrix can be used to get an easy overview of the available evidence. This is really useful in assessing whether a compound is likely to be an ED or not and to identify clear data gaps. Next, the AOP framework (and its practical applications) was presented, including IATA and the use of AOPs for hazard identification (as was done for the EDs).

An overview of the test methods that are available to predict the ED potential of chemicals was presented, focusing on the methods listed in the OECD Guidance Document 150 (as part of the OECD Conceptual Framework for Testing and Assessment of Endocrine Disruptors (OECD, 2012b)); these methods range from simple (Q)SAR and in vitro tests to multigenerational in vivo tests. The webinar ended with an overview of the activities by the US EPA, including the recent analysis of the Endocrine Disruptor Screening Programme (EDSP) and the ED work covered by ToxCast with the development of the ER and AR models (to predict level 3 mechanistic in vivo assays) (Browne et al., 2015).

7.3 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) introduced the concept of exposure factors and the following steps were highlighted: (i) data collection (of diaries, monitoring of environment, biomonitoring); (ii) organisation of knowledge by using defined frameworks like Aggregate Exposure Pathways (AEP) and hazard Adverse Outcome Pathways (AOPs); (iii) simulation of chemicals fate and transport and human exposure by predictive models; Finally, (iv) route to route extrapolation was explained by means of an approach applicable to cosmetics compounds, called the margin of internal exposure (MOIE).

International Programme on Chemical Safety (IPCS) introduced the concept of exposure factors and the following steps were highlighted: (i) data collection (of diaries, monitoring of environment, biomonitoring); (ii) organisation of knowledge by using defined frameworks like Aggregate Exposure Pathways (AEP) and hazard Adverse Outcome Pathways (AOPs); (iii) simulation of chemicals fate and transport and human exposure by predictive models; Finally, (iv) route to route extrapolation was explained by means of an approach applicable to cosmetics compounds, called the margin of internal exposure (MOIE).
(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 85 institutions from 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 involved as an observer, and contributes to the Network Coordination Groups on Mode of Action (MoA) and Combined Exposures.

The MoA Human Relevancy Framework was developed by WHO/IPCS as a tool for Weight of Evidence analysis and was updated in 2013 (Meek et al., 2014), including a MoA roadmap showing the use of MoA knowledge in human health risk assessment as well as MoA analysis templates. WHO/IPCS further follows up work on MoA and is planning activities for training purposes.

The Network Coordination Group on Combined Exposures was set up at the first WHO CRAN meeting in Paris in 2014. It has compiled an inventory with information on combined exposure activities of the Network participants to find synergies and opportunities for cooperation between Group members and is further monitoring ongoing developments.

The Second Meeting of the WHO CRAN was hosted by the European Food Safety Authority (EFSA) on 20-22 June 2017, with attendants from 63 chemical risk assessment institutions from 39 countries. It addressed themes including combined exposures to multiple chemicals, human biomonitoring, identifying new and emerging risks, interplay of existing methodologies, new scientific approaches for regulatory safety assessment as well as prioritising chemicals and settings of concern for risk assessment. The draft strategic plan for enhancing chemical risk assessment capacity in the Network was discussed.

EURL ECVAM contributed to the meeting with a keynote presentation on establishing the credibility of predictive toxicology approaches for regulatory safety assessment, as well as providing a thought starter document on new science in chemical risk assessment for one of the break-out discussions.

The meeting concluded with plans for future activities, including scoping activities on emerging risks, raising awareness of new approaches for chemical risk assessment and sharing experience to promote harmonised approaches, as well as developing training and case studies in order to foster integration of the use of new methods and existing methods.
Current EU-funded projects such as EU-ToxRisk aim for animal-free toxicological assessments for complex areas such as repeated dose and reproductive toxicology. These assessments are based on human cell responses and a comprehensive mechanistic understanding of cause-consequence relationships of chemical adverse effects. These projects try to integrate cell biology, ‘omics technologies, systems biology and computational modelling for the risk assessment of chemicals without relying on animal testing.

Similarly, other EU projects investigate the applicability of these novel, non-animal tools in the assessment of combined effects of chemicals on humans and the environment. New approaches and methods, such as the AOP concept, in vitro methods, ‘omics techniques, quantitative structure activity relationships (QSARs), read-across, toxicokinetic and dynamic energy budget (DEB) modelling, and IATA contribute to a more mechanistically based prediction of mixture effects and at the same time reduce the reliance on animal testing.

Besides R&D projects that are focused on human health effects, other projects related to fish toxicity and bioaccumulation are currently ongoing such as e.g., the development of AOPs for chronic fish toxicity testing, the potential use of the eco-TTC concept for environmental risk assessment and the development of a tiered testing strategy for fish bioaccumulation testing based on in vitro approaches.

In the area of acute systemic toxicity, activities are dedicated to the collection and evaluation of mechanistic information on eight potential organs identified as relevant for acute systemic toxicity. The overall aim is to identify properties that are required for in vitro test methods for target organ toxicity testing which are not covered by cytotoxicity assays. An improved understanding of toxicological mechanisms in this area will allow the development of IATA for acute systemic toxicity.

In the area of carcinogenicity, the current efforts focus on providing insight into carcinogenicity mechanisms and the integration of all available information on relevant endpoints, including from epidemiology, traditional and alternative toxicology test systems, and from novel data streams (e.g., ‘omics, high through technologies).

IATA have been developed and internationally approved in the areas of skin irritation/corrosion, serious eye damage/eye irritation and skin sensitisation. For the latter, a number of DAs integrating data from internationally adopted in vitro methods with other relevant information have been proposed and documented by the OECD under the leadership of JRC’s EURL ECVAM.

With the aim to further enhance regulatory consideration and adoption of DAs, EURL ECVAM in collaboration with ICATM hosted a workshop on the international regulatory applicability and acceptance of alternative non-animal approaches, e.g., DAs, to skin sensitisation assessment of chemicals used in a variety of sectors. The workshop led to the conclusion that international harmonisation and standardisation of DAs are needed. EURL ECVAM therefore submitted together with US and Canada, a new project proposal to the OECD on the development of a PBTG on DAs and individual methods for skin sensitisation testing.

In the area of toxicokinetics, EURL ECVAM focuses its efforts on the development of an OECD GD to characterise and describe in vitro methods for measuring hepatic metabolic clearance, since the latter represents in many cases the main driving process of kinetics and several in vitro methods exist in that area. In parallel, another OECD GD on Physiologically Based Kinetic (PBK) models is currently being prepared with a view to provide harmonised guidance to model developers, reviewers, and risk assessors.
In the area of vaccines, projects like “Vaccine batch to vaccine batch comparison by consistency testing” (VAC2VAC) focus on the development, optimisation and evaluation of non-animal methods, e.g., physicochemical and immunochemical methods, cell-based and other assays for routine batch quality, safety and efficacy testing of vaccines in order to avoid severe animal testing.

At international level, efforts are focused on the development and adoption of VICH guidelines on harmonisation of criteria to waive the target animal batch safety testing for inactivated and live vaccines for veterinary use.

Information on *in vitro* and *in silico* data as well as on test methods are disseminated through a variety of databases hosted by JRC-EURL ECVAM.

EURL ECVAM also invested in communication, education and training activities on alternatives for young scientists during the JRC Summer School and the European School traineeship with the aim to raise awareness of new modern risk assessment approaches to post-graduate students and early-career scientists.


Ives, C., Campia, I., Wang, R.-L., Wittwehr, C., & Edwards, S. Creating a Structured AOP Knowledgebase via Ontology-Based Annotations, Applied In Vitro Toxicology. (accepted)


List of abbreviations and definitions

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ADME</td>
<td>Absorption, distribution, metabolism and excretion</td>
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<tr>
<td>AEP</td>
<td>Aggregate Exposure Pathway</td>
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<tr>
<td>ANSES</td>
<td>Agence Nationale Sécurité Sanitaire Alimentaire Nationale</td>
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<tr>
<td>AOP</td>
<td>Adverse Outcome Pathway. It is a mechanistic knowledge framework that describes a logical sequence of causally linked events at different levels of biological organisation, which follows exposure to a chemical and leads to an adverse health effect in humans or wildlife.</td>
</tr>
<tr>
<td>AOP-KB</td>
<td>Adverse Outcome Pathway-Knowledge Base</td>
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<tr>
<td>AR</td>
<td>Androgen Receptor</td>
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<td>ARTA</td>
<td>Androgen Receptor Transactivation Assays</td>
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<tr>
<td>BCF</td>
<td>Bioconcentration Factor</td>
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<tr>
<td>BSP</td>
<td>Biological Standardisation Programme</td>
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<td>CAAT</td>
<td>The Center for Alternatives to Animal Testing</td>
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<tr>
<td>CARACAL</td>
<td>Competent Authorities for REACH and CLP</td>
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<tr>
<td>CASG Nano</td>
<td>Competent Authorities Sub Group on nanomaterials</td>
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<tr>
<td>Cefic</td>
<td>The European Chemical Industry Council</td>
</tr>
<tr>
<td>CHMP</td>
<td>Committee for Medicinal Products for Human Use (EMA)</td>
</tr>
<tr>
<td>CLP</td>
<td>Classification, Labelling and Packaging</td>
</tr>
<tr>
<td>CosEU</td>
<td>Cosmetic Europe</td>
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<tr>
<td>CPSC</td>
<td>The Consumer Product Safety Commission (USA)</td>
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<tr>
<td>CRAN</td>
<td>Chemical Risk Assessment Network (WHO)</td>
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<tr>
<td>CRD</td>
<td>Chemicals Regulation Directorate (UK)</td>
</tr>
<tr>
<td>CREST</td>
<td>Chemical attributes, Regulatory approaches and Experimental STudies from a mixture toxicology perspective</td>
</tr>
<tr>
<td>CVMP</td>
<td>Committee for Medicinal Products for Veterinary Use (EMA)</td>
</tr>
<tr>
<td>CYP</td>
<td>Human cytochrome P450</td>
</tr>
<tr>
<td>DA</td>
<td>Defined Approach. It consists of a fixed Data Interpretation Procedure (DIP) applied to data generated with a defined set of information sources to derive a result that, depending on the regulatory requirements, can be used to support an assessment in replacement of standard animal test(s).</td>
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<tr>
<td>DB-ALM</td>
<td>DataBase on ALternative Methods</td>
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<tr>
<td>DEB</td>
<td>Dynamic Energy Budget</td>
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<tr>
<td>DG ENV</td>
<td>Directorate General for Environment (EU)</td>
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<tr>
<td>DG GROW</td>
<td>Directorate General for Internal Market, Industry, Entrepreneurship and SMEs (EU)</td>
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<tr>
<td>DIP</td>
<td>Data Interpretation Procedure</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DNT</td>
<td>developmental neurotoxicity</td>
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<tr>
<td>DRP</td>
<td>Detailed Review Paper</td>
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<tr>
<td>EAGMST</td>
<td>Extended Advisory Group for Molecular Screening and Toxicogenomics</td>
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<tr>
<td>EAS</td>
<td>Endocrine Active Substance</td>
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<tr>
<td>EASIS</td>
<td>Endocrine Active Substances Information System</td>
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<tr>
<td>EC</td>
<td>European Commission</td>
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<tr>
<td>ECCC</td>
<td>Environment and Climate Change Canada</td>
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<tr>
<td>ECETOC</td>
<td>The European Chemical Industry Ecology and Toxicology Centre</td>
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<td>ECHA</td>
<td>European Chemicals Agency</td>
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<tr>
<td>ED(s)</td>
<td>Endocrine Disruptor(s)</td>
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<tr>
<td>EDC</td>
<td>Endocrine Disruptive Compound</td>
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<tr>
<td>EDQM</td>
<td>European Directorate for the Quality of Medicines &amp; HealthCare (Council of Europe)</td>
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<tr>
<td>EDSNP</td>
<td>Endocrine Disruptor Screening Programme</td>
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<tr>
<td>EFPIA</td>
<td>European Federation of Pharmaceutical Industries and Associations</td>
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<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
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<tr>
<td>EIT</td>
<td>Eye Irritation Test</td>
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</table>
EIVS  Eye Irritation Validation Study
ELISA  Enzyme-Linked Immunosorbent Assay
EMA  European Medicines Agency
EPAA  European Partnership for Alternatives to Animal Testing
ER  Estrogen Receptor
ESAC  ECVAM Scientific Advisory Committee
ESTAF  The ECVAM Stakeholder Forum (see also PARERE)
EU  European Union
Eurometaux  European non-ferrous metals association
EUSAAT  European Society for Alternatives to Animal Testing
ESTIV  The European Society of Toxicology In Vitro
EU-NETVAL  European Union Network of Laboratories for the Validation of Alternative Methods
EURL ECVAM  European Union Reference Laboratory for Alternatives to Animal Testing
FELASA  Federation of Laboratory Animal Science Associations
FELS  Fish Early Life-Stage
FCP  Fixed Concentration Procedure
GARD  Genomic Allergen Detection Test
GFP  Green Fluorescent Protein
GHS  Globally Harmonised System of Classification and Labelling of chemicals
GIVIMP  Good In Vitro Method Practices
GLP  Good Laboratory Practice
GMP  Good Kinetic Modelling Practices
GPS  GARD Prediction Signature
GTTC  Genetic Toxicology Technical Committee (ILSI/HELSI)
HBM4EU  The European Human Biomonitoring Initiative
HCE  Human Corneal Epithelium
HESI  Health and Environmental Sciences Institute
HET-MN  Hen's Egg Test for Micronucleus Induction
HPLC  High-Performance (or Pressure) Liquid Chromatography
IACUC  Institutional Animal Care and Use Committee
ICAPD  International Council on Animal Protection in OECD Programmes
ICATM  International Cooperation on Alternative Test Methods
IARC  International Agency for Research on Cancer
IATA  Integrated Approaches to Testing and Assessment. They are frameworks used for hazard identification, hazard characterisation and/or safety assessment of a chemical or group of chemicals, which strategically integrates and weights all relevant existing data and guide the targeted generation of new data where required to inform regulatory decision-making regarding potential hazard and/or risk.
ICCA  The International Council of Chemical Associations
ICCR  International Cooperation on cosmetics Regulation
ICH  International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ILSI/HESI  International Life Sciences Institute / Health and Environmental Sciences Institute
IP  Intellectual Property
iPSC  induced Pluripotent Stem Cell
IPCS  International Programme on Chemical Safety
ISO  The International Organization for Standardization
IVIVE  In vitro to in vivo extrapolation
IWGT  International Workshops on Genotoxicity Testing
JaCVAM  Japanese Centre for the Validation of Alternatives Methods
JEG 3Rs  Joint Expert Group for Reduction, Replacement and Refinement
JSAAE  Japanese Society for Alternatives to Animal Experiments
JWG  Joint Working Group
Three Rs replace, reduce, and refine the use of animals. This means that animal studies should be either replaced by methods not involving animals, or adapted to reduce the number of animals needed, or refined so as to minimise pain, suffering or distress experienced by the animal, or to increase their welfare. 3Rs and Three Rs is used interchangeably in this report.

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>J3RsWG</td>
<td>The 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</td>
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<tr>
<td>KE</td>
<td>Key Event</td>
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<td>KER</td>
<td>Key Event Relationship</td>
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<tr>
<td>LC-MS</td>
<td>Liquid Chromatography-Mass Spectrometry</td>
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<td>LLNA</td>
<td>Local Lymph Node Assay</td>
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<td>LRI</td>
<td>Cefic’s Long-Range Research Initiative</td>
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<tr>
<td>MAD</td>
<td>Mutual Acceptance of Data (OECD)</td>
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<td>mESC</td>
<td>mouse Embryonic Stem</td>
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<tr>
<td>METI</td>
<td>Ministry of Economy, Trade, and Industry (Japan)</td>
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<tr>
<td>MHLW</td>
<td>Ministry of Health, Labour, and Welfare (Japan)</td>
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<td>MIE</td>
<td>Molecular Initiating Event</td>
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<td>MoA</td>
<td>Mode of Action</td>
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<td>MOIE</td>
<td>margin of internal exposure</td>
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<td>MWCNT</td>
<td>Multi-walled Carbon Nanotube</td>
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<td>NAM</td>
<td>new approach methods</td>
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<td>NCSRs</td>
<td>National Centre for the Replacement, Refinement &amp; Reduction of Animals in Research (UK)</td>
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<td>NICEATM</td>
<td>The NTP Interagency Center for the Evaluation of Alternative Toxicological Methods</td>
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<td>NICNAS</td>
<td>National Industrial Chemicals Notification and Assessment Scheme</td>
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<td>NIS</td>
<td>Sodium/Iodide Symporter</td>
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<td>NMWG</td>
<td>Nanomaterials Working Group (ECHA)</td>
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<td>OECD</td>
<td>The Organisation for Economic Co-operation and Development</td>
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<td>OHT</td>
<td>OECD Harmonised Template</td>
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<tr>
<td>PBK</td>
<td>Physiologically Based Kinetic (also PBPK, PBBK, PBTK)</td>
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<td>PBT</td>
<td>Persistent, Bioaccumulative and Toxic</td>
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<td>PBTG</td>
<td>Performance-Based Test Guideline (OECD)</td>
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<td>PARERE</td>
<td>Preliminary Assessment of Regulatory Relevance network (see also ESTAF)</td>
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<td>PoD</td>
<td>Point of Departure</td>
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<td>QMRF</td>
<td>QSAR Model Reporting Formats</td>
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<td>(Q)SAR</td>
<td>(Quantitative) Structure Activity Relationship</td>
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<td>QSAR</td>
<td>Quantitative Structure Property Relationship</td>
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<td>RAAF</td>
<td>Read Across Assessment Framework (ECHA)</td>
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<td>R&amp;D</td>
<td>Research &amp; Development</td>
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<tr>
<td>REACH</td>
<td>European Regulation (EC) no 1907/2006 Registration, Evaluation, Authorisation and Restriction of Chemicals</td>
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<tr>
<td>RhCE</td>
<td>Reconstructed human Cornea-like Epithelium</td>
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<td>RIVM</td>
<td>National Institute for Public Health and the Environment (The Netherlands)</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>SAICM</td>
<td>Strategic Approach to International Chemicals Management</td>
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<tr>
<td>SETAC</td>
<td>Society of Environmental Toxicology and Chemistry</td>
</tr>
<tr>
<td>SIT</td>
<td>Skin Irritation Test</td>
</tr>
<tr>
<td>SME</td>
<td>Small and Medium-sized Enterprises</td>
</tr>
<tr>
<td>SOT</td>
<td>Society of Toxicology</td>
</tr>
<tr>
<td>SPSF</td>
<td>Standard Project Submission Form (OECD)</td>
</tr>
<tr>
<td>SVM</td>
<td>Support Vector Machine</td>
</tr>
<tr>
<td>TD</td>
<td>Toxicodynamic</td>
</tr>
<tr>
<td>TG</td>
<td>Test Guideline (OECD)</td>
</tr>
<tr>
<td>TGP</td>
<td>Test Guidelines Programme (OECD)</td>
</tr>
<tr>
<td>Three Rs</td>
<td>Replace, Reduce and Refine the use of animals. This means that animal studies should be either replaced by methods not involving animals, or adapted to reduce the number of animals needed, or refined so as to minimise pain, suffering or distress experienced by the animal, or to increase their welfare. 3Rs and Three Rs is used interchangeably in this report.</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TK</td>
<td>Toxicokinetics</td>
</tr>
<tr>
<td>TSAR</td>
<td>Tracking System on Alternative Methods</td>
</tr>
<tr>
<td>TTC</td>
<td>Threshold of Toxicological Concern</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra Performance Liquid Chromatography</td>
</tr>
<tr>
<td>UDS</td>
<td>Unscheduled DNA Synthesis</td>
</tr>
<tr>
<td>US EPA</td>
<td>United States Environmental Protection Agency</td>
</tr>
<tr>
<td>VAC2VAC</td>
<td>&quot;Vaccine batch to vaccine batch comparison by consistency testing’</td>
</tr>
<tr>
<td>VICH</td>
<td>Veterinary International Cooperation on Harmonization</td>
</tr>
<tr>
<td>WHO</td>
<td>The World Health Organisation</td>
</tr>
<tr>
<td>WNT</td>
<td>Working Group of the National Coordinators of the Test Guideline Programme (OECD)</td>
</tr>
<tr>
<td>WPHA</td>
<td>Working Party on Hazard Assessment (OECD)</td>
</tr>
</tbody>
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Table 1 summarises the status of adoption of OECD test guidelines on alternative methods from 2011 to 2017. 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](http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicalsandrelateddocuments.htm)

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Toxicity area</th>
<th>Test method description</th>
<th>Acceptance status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Skin corrosion</td>
<td>Reconstructed human Epidermis (Rhe) test methods, as included in OECD TG 431/EU TM B.40 bis</td>
<td>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 on 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 (EpiDermTM, SkinEthicTM and EpiCS®) for the correct prediction of Sub-Cat.1A.</td>
</tr>
<tr>
<td>2</td>
<td>Skin corrosion</td>
<td>Transcutaneous Electrical Resistance (TER) test method, as included in OECD TG 430/EU TM B.40</td>
<td>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)</td>
</tr>
<tr>
<td>3</td>
<td>Skin corrosion</td>
<td><em>In vitro</em> Membrane Barrier Test Method for Skin Corrosion, as included in OECD TG 435/EU TM B.40</td>
<td>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 on 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)</td>
</tr>
<tr>
<td>Nr.</td>
<td>Toxicity area</td>
<td>Test method description</td>
<td>Acceptance status</td>
</tr>
<tr>
<td>-----</td>
<td>---------------------</td>
<td>------------------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>4</td>
<td>Skin irritation</td>
<td>Reconstructed human Epidermis (RhE) test methods, as included in OECD TG 439/EU B.46</td>
<td>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 on 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)</td>
</tr>
<tr>
<td>5</td>
<td>Serious eye damage/eye irritation</td>
<td>Fluorescein Leakage (FL) test method, as included in OECD TG 460</td>
<td>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</td>
</tr>
<tr>
<td>6</td>
<td>Serious eye damage/eye irritation</td>
<td>Bovine Corneal Opacity and Permeability (BCOP) test method, as included in OECD TG 437/EU TM B.47</td>
<td>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</td>
</tr>
<tr>
<td>7</td>
<td>Serious eye damage/eye irritation</td>
<td>Isolated Chicken Eye (ICE) test method, as included in OECD TG 438/EU TM B.48</td>
<td>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</td>
</tr>
<tr>
<td>8</td>
<td>Serious eye damage/eye irritation</td>
<td>Cytosensor Microphysiometer (CM) test method</td>
<td>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</td>
</tr>
<tr>
<td>9</td>
<td>Serious eye damage/eye irritation</td>
<td>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</td>
<td>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</td>
</tr>
<tr>
<td>10</td>
<td>Serious eye damage/eye irritation</td>
<td>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</td>
<td>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</td>
</tr>
<tr>
<td>Nr.</td>
<td>Toxicity area</td>
<td>Test method description</td>
<td>Acceptance status</td>
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<tr>
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</tr>
<tr>
<td>11</td>
<td>Skin sensitisation</td>
<td><em>In chemico</em> skin sensitisation: Direct Peptide Reactivity Assay (DPRA), as included in OECD TG 442C</td>
<td>Adopted as a new TG in 2015</td>
</tr>
<tr>
<td>12</td>
<td>Skin sensitisation</td>
<td><em>In vitro</em> skin sensitisation: ARE-Nrf2 Luciferase test method (KeratinoSens™), as included in OECD TG 442D</td>
<td>Adopted as a new TG in 2015</td>
</tr>
<tr>
<td>13</td>
<td>Skin sensitisation</td>
<td><em>In vitro</em> 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</td>
<td>Adopted as a new TG in 2016 as &quot;<em>In vitro</em> Skin Sensitisation: human Cell Line Activation Test (h-CLAT)&quot;; 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</td>
</tr>
<tr>
<td>14</td>
<td>Carcinogenicity</td>
<td><em>In vitro</em> Syrian Hamster Embryo (SHE) Cell Transformation Assay (CTA) as included in OECD GD No. 214</td>
<td>Adopted as a new GD in 2015</td>
</tr>
<tr>
<td>15</td>
<td>Carcinogenicity</td>
<td><em>In vitro</em> Bhas 42 Cell Transformation Assay (CTA) as included in OECD GD no 231</td>
<td>Adoption as a new GD in 2016</td>
</tr>
<tr>
<td>18</td>
<td>Genotoxicity</td>
<td><em>In vitro</em> Mammalian Cell Gene Mutation Test using Hprt and xprt genes as included in OECD TG 476</td>
<td>OECD TG 476 (originally adopted in 1984) &quot;<em>In vitro</em> Mammalian Cell Gene Mutation Test&quot; 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 thymidine 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)</td>
</tr>
<tr>
<td>19</td>
<td>Genotoxicity</td>
<td><em>In vitro</em> Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene as included in OECD TG 490</td>
<td>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.</td>
</tr>
<tr>
<td>Nr.</td>
<td>Toxicity area</td>
<td>Test method description</td>
<td>Acceptance status</td>
</tr>
<tr>
<td>-----</td>
<td>---------------------</td>
<td>----------------------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>20</td>
<td>Endocrine disruption</td>
<td>H295R Steroidogenesis Assay</td>
<td>Adopted as TG 456 in 2011</td>
</tr>
</tbody>
</table>
| 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) |
| 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 (hER) 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 as included in OECD TG 236                        | Adopted in 2013                                                                     |

1 These test methods were initially proposed to be included in Test Guidelines. It was later decided to include them in Guidance Documents.
Annex 2 — ICATM Alternative Test Methods Validation and Status of Regulatory Acceptance

<table>
<thead>
<tr>
<th>Method</th>
<th>Current Status</th>
<th>Lead Organisation</th>
<th>International Acceptance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dermal Corrosion Test Methods</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CORROSITEX Skin Corrosion Test</td>
<td>Completed</td>
<td></td>
<td>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.</td>
</tr>
<tr>
<td>EpiSkin™, EpiDerm™ SCT, SkinEthic™ RHE, epiCS® Skin Corrosion Tests</td>
<td>Completed</td>
<td></td>
<td>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.</td>
</tr>
<tr>
<td>In vitro Reconstructed human Epidermis (RhE) test methods: LabCyte EPI-MODEL24 SCT</td>
<td>JSAAE sponsored validation study is ongoing. The experimental part was finalised in autumn 2017</td>
<td>JaCVAM</td>
<td></td>
</tr>
<tr>
<td>Method</td>
<td>Current Status</td>
<td>Lead Organisation</td>
<td>International Acceptance</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>----------------------------------------</td>
<td>-------------------</td>
<td>--------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Dermal Irritation Test Methods</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>In vitro</em> Reconstructed human Epidermis (Rhe) test methods: EpiSkin™, EpiDerm™ SIT, SkinEthic™ RHE and LabCyte EPI-MODEL24 SIT</td>
<td>Completed</td>
<td></td>
<td>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.</td>
</tr>
<tr>
<td><em>In vitro</em> reconstructed human epidermis (Rhe) test methods: Korean epidermis model</td>
<td>KoCVAM sponsored validation study is ongoing</td>
<td>KoCVAM</td>
<td></td>
</tr>
<tr>
<td><em>In vitro</em> reconstructed human full-thickness model test methods: LbL model</td>
<td>JSAAE sponsored validation study is ongoing</td>
<td>JaCVAM</td>
<td></td>
</tr>
<tr>
<td><strong>Phototoxicity Test Methods</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test method battery to predict phototoxicity (yeast growth inhibition phototoxicity assay and red blood cell photohemolysis assay)</td>
<td>Japanese Regulatory Acceptance Board recommended additional work be performed</td>
<td>JaCVAM</td>
<td></td>
</tr>
<tr>
<td><em>In vitro</em> test method based on reactive oxygen species (ROS) and photostability</td>
<td>Completed</td>
<td>ICH S10 (2014)</td>
<td>Included in the OECD TGP work plan in 2016.</td>
</tr>
<tr>
<td><strong>Ocular Toxicity Test Methods</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine Corneal Opacity and Permeability (BCOP) Test Method</td>
<td>Completed</td>
<td>OECD TG 437 (2009)</td>
<td>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.</td>
</tr>
<tr>
<td>Method</td>
<td>Current Status</td>
<td>Lead Organisation</td>
<td>International Acceptance</td>
</tr>
<tr>
<td>-----------------------------------------------------------------------</td>
<td>-------------------</td>
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<td>-----------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Isolated Chicken Eye (ICE) Test Method</td>
<td>Completed</td>
<td></td>
<td><strong>OECD TG 438 (2009)</strong>&lt;br&gt;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 draft under consideration to include histopathology and modified decision criteria.</td>
</tr>
<tr>
<td>Use of Histopathology as an additional endpoint in Ocular Safety Testing</td>
<td>Completed</td>
<td></td>
<td><strong>OECD GD 160 (2011)</strong>&lt;br&gt;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.</td>
</tr>
<tr>
<td>Cytotoxicity test: SIRC CVS</td>
<td>Peer review coordinated by JaCVAM is ongoing</td>
<td>JaCVAM</td>
<td></td>
</tr>
<tr>
<td>Cytotoxicity test: three-dimensional dermal model (MATREX)</td>
<td>JaCVAM-sponsored validation study in the planning stage</td>
<td>JaCVAM</td>
<td></td>
</tr>
<tr>
<td>Cytotoxicity test: Short Time Exposure (STE) test</td>
<td>Completed</td>
<td></td>
<td><strong>OECD TG 491 (2015)</strong>&lt;br&gt;Updated version to include a new reference to OECD GD 236 on an IATA for Serious Eye Damage and Eye Irritation adopted in 2017.</td>
</tr>
<tr>
<td>Use of anaesthetics, analgesics, and humane endpoints for routine use in TG 405</td>
<td>Completed</td>
<td></td>
<td><strong>OECD updated TG 405 (2012)</strong>&lt;br&gt;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.</td>
</tr>
<tr>
<td>In vitro approach for categorisation of antimicrobial cleaning products: recommendations for further studies</td>
<td>Completed. EPA/OPP 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 <a href="https://www.epa.gov/pesticides">https://www.epa.gov/pesticides</a> for the details of the scope of the policy).</td>
<td>ICCVAM</td>
<td></td>
</tr>
</tbody>
</table>

2 Environmental Protection Agency/Office of Pesticide Programme
<table>
<thead>
<tr>
<th>Method</th>
<th>Current Status</th>
<th>Lead Organisation</th>
<th>International Acceptance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosensor Microphysiometer® (CM) Test method</td>
<td>The draft TG was submitted to OECD for comments including a set of Performance Standards</td>
<td>EURLECVAM</td>
<td>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.</td>
</tr>
<tr>
<td>Fluorescein Leakage (FL) test method</td>
<td>Completed</td>
<td></td>
<td>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</td>
</tr>
<tr>
<td>Vitrigel-EIT</td>
<td>Validation and peer review coordinated by JaCVAM completed</td>
<td>JaCVAM</td>
<td>Included in the OECD TGP work plan in 2017</td>
</tr>
<tr>
<td>Reconstructed human Cornea-like Epithelium (RhCE) for eye irritation LabCyte Cornea-model</td>
<td>Validation and peer review coordinated by JaCVAM completed. Undergoing commenting at OECD level for inclusion in TG 492.</td>
<td>JaCVAM</td>
<td>Included in the OECD TGP work plan in 2017</td>
</tr>
<tr>
<td>OptiSafe</td>
<td>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.</td>
<td>NICEATM</td>
<td></td>
</tr>
<tr>
<td>In vitro reconstructed human Cornea-epithelium model (RhCE) test method: Korean Cornea-model</td>
<td>KoCVAM-sponsored validation study is ongoing</td>
<td>KoCVAM</td>
<td></td>
</tr>
<tr>
<td>Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) Test Method</td>
<td>Validation study sponsored by Brazilian Ministry of Science, Technology Innovation and Communication (MCTIC). Preliminary phase of validation study ongoing</td>
<td>BraCVAM</td>
<td></td>
</tr>
<tr>
<td>Method</td>
<td>Current Status</td>
<td>Lead Organisation</td>
<td>International Acceptance</td>
</tr>
<tr>
<td>--------------------------------------------------------------</td>
<td>-----------------------------------------</td>
<td>-------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Updated Murine local lymph node assay (LLNA) for skin sensitization (20% reduction)</td>
<td>Completed</td>
<td>Update to TG 429 OECD (2010)</td>
<td>ISO (2010)</td>
</tr>
<tr>
<td>Reduced LLNA (rLLNA)</td>
<td>Completed</td>
<td>Update to TG 429 OECD (2010)</td>
<td></td>
</tr>
<tr>
<td>Harmonized performance standards for the LLNA</td>
<td>Completed</td>
<td>Update to TG 429 OECD (2010)</td>
<td></td>
</tr>
<tr>
<td>Nonradioactive LLNA protocol (LLNA: BrdU-Flow Cytometry)</td>
<td>Validation study and peer review coordinated by KoCVAM completed. Undergoing commenting at OECD level for inclusion in TG 442B.</td>
<td>KoCVAM</td>
<td>Consideration to include the method into draft TG 442B</td>
</tr>
<tr>
<td>In vitro skin sensitisation assay (DPRA)</td>
<td>Completed</td>
<td>OECD TG 442C (2015)</td>
<td></td>
</tr>
<tr>
<td>In vitro skin sensitisation assay KeratinoSens™</td>
<td>Completed</td>
<td>OECD TG 442D (2015). Draft updated version under consideration to include an adaptation to animal-free conditions.</td>
<td></td>
</tr>
</tbody>
</table>

**Immunotoxicity (Allergic Contact Dermatitis) Test Methods**
<table>
<thead>
<tr>
<th>Method</th>
<th>Current Status</th>
<th>Lead Organisation</th>
<th>International Acceptance</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro skin sensitisation assay SENS-IS</td>
<td>External validation study finalised</td>
<td>EURL ECVAM</td>
<td>Included in the OECD TG work plan in 2016</td>
</tr>
<tr>
<td>In vitro skin sensitisation assay Genomic Allergen Rapid Detection (GARDskin)</td>
<td>External validation study ongoing</td>
<td>JaCVAM</td>
<td>Included in the OECD TG work plan in 2016</td>
</tr>
<tr>
<td>In vitro skin sensitisation assay Vitrigel-SST</td>
<td>MAFF³-sponsored validation study is pending</td>
<td>JaCVAM</td>
<td></td>
</tr>
<tr>
<td>In vitro skin sensitisation assay, Amino acid derivative reactivity assay (ARDA)</td>
<td>JCIA⁴ and JSAAE⁵ validation study is ongoing; peer review of study results foreseen for spring 2018</td>
<td>JaCVAM</td>
<td></td>
</tr>
<tr>
<td>IL-2 Luc assay for the evaluation of the immunotoxic potential of chemicals</td>
<td>JaCVAM validation study is ongoing</td>
<td>JaCVAM</td>
<td></td>
</tr>
<tr>
<td>Electrophilic allergen screening assay (EASA)</td>
<td>Validation study coordinated by NICEATM is currently being planned. Testing began in spring 2017 and will continue through 2018.</td>
<td>NICEATM</td>
<td></td>
</tr>
</tbody>
</table>

### Acute Toxicity Test Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Current Status</th>
<th>Lead Organisation</th>
</tr>
</thead>
</table>

³ Ministry of Agriculture, Forestry and Fisheries
⁴ Japan Cosmetic Industry Association
⁵ Japanese Society for Alternatives to Animal Experiments
<table>
<thead>
<tr>
<th>Method</th>
<th>Current Status</th>
<th>Lead Organisation</th>
<th>International Acceptance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vitro</em> cytotoxicity test (3T3 Neutral Red Uptake) for identifying substances with acute oral LD50 &gt; 2000 mg/kg b.w.</td>
<td>EURL ECVAM ESAC peer review completed, and EURL ECVAM Recommendation published in 2013</td>
<td>EURL ECVAM</td>
<td></td>
</tr>
<tr>
<td>EpiAirway human reconstructed lung epithelium for identifying acute inhalation toxicity</td>
<td>Validation study currently being planned. ICCVAM agency representatives serving on the VMT</td>
<td>NICEATM/ICCVAM</td>
<td></td>
</tr>
<tr>
<td><em>Toxicokinetic Test Methods</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>In vitro</em> hepatic biotransformation – CYP induction: Hepa RG and cryopreserved human hepatocytes</td>
<td></td>
<td>EURL ECVAM</td>
<td>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.</td>
</tr>
<tr>
<td><em>Endocrine Disruptor Test Methods</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stably transfected human estrogen receptor-α transcriptional activation assay for detection of estrogenic agonist-activity of chemicals (STTA and BG1-Luc assays)</td>
<td>Completed</td>
<td>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</td>
<td></td>
</tr>
<tr>
<td>H295R Steroidogenesis assay</td>
<td>Completed</td>
<td>OECD TG 456 (2011)</td>
<td></td>
</tr>
<tr>
<td>BG1Luc® human estrogen receptor transcriptional activation assay: agonist and antagonist protocols</td>
<td>Completed</td>
<td>OECD TG 457 (2012)</td>
<td>TG 457 has been deleted in parallel to TG 455 updates (see previous table entry)</td>
</tr>
<tr>
<td>CertiChem MCF-7 cell proliferation assay for the detection of human estrogen receptor agonists and antagonists</td>
<td>International validation study completed. Protocol must be revised for adequate transferability.</td>
<td>NICEATM</td>
<td>Not in the OECD TGP work plan anymore</td>
</tr>
<tr>
<td>CertiChem MDA-Kb2 assay for the detection of human androgen receptor agonists and antagonists</td>
<td>NICEATM coordinated single lab validation study ongoing</td>
<td>NICEATM</td>
<td></td>
</tr>
<tr>
<td>Method</td>
<td>Current Status</td>
<td>Lead Organisation</td>
<td>International Acceptance</td>
</tr>
<tr>
<td>-----------------------------------------------------------------------</td>
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<td>-------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Stably transfected CHO Androgen receptor-α transcriptional activation assay for detection of androgenic agonist and antagonist activity of chemicals (AR-STTA)</td>
<td>Completed</td>
<td></td>
<td>OECD TG 458 (2016)</td>
</tr>
<tr>
<td>MELN® human estrogen receptor transcriptional activation assay: agonist and antagonist protocols</td>
<td>Validation stopped</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stably Transfected Transactivation in vitro Assay to detect Androgen Receptor Agonists and Antagonists</td>
<td>Validation study ongoing</td>
<td>EURL ECVAM</td>
<td>Included in the OECD TGP work plan in April 2013</td>
</tr>
<tr>
<td>Transactivation assay for the detection of compounds with (anti)androgenic potential using 22Rv1/MMTV cells</td>
<td>Validation study ongoing</td>
<td>Ministry of Food and Drug Safety (MFDS) South Korea</td>
<td></td>
</tr>
<tr>
<td>Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) In Vitro Assays to Detect Chemicals with ER Binding Affinity</td>
<td>Completed</td>
<td></td>
<td>OECD TG 493 (2015)</td>
</tr>
</tbody>
</table>

**Genetic Toxicity Test Methods**

<table>
<thead>
<tr>
<th>Test</th>
<th>Status</th>
<th>OECD TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro mammalian cell micronucleus test</td>
<td>Completed</td>
<td>487 (2010), updated TG adopted in 2014</td>
</tr>
<tr>
<td>In vitro mammalian cell chromosome aberration assay</td>
<td>Completed</td>
<td>473 (1997), updated TG adopted in 2014</td>
</tr>
<tr>
<td>In vivo comet assay</td>
<td>Completed</td>
<td>489 (2014)</td>
</tr>
<tr>
<td>Method</td>
<td>Current Status</td>
<td>Lead Organisation</td>
</tr>
<tr>
<td>-----------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td><em>In vitro</em> comet assay</td>
<td>Validation study for the <em>in vitro</em> comet assay stopped</td>
<td>JaCVAM</td>
</tr>
<tr>
<td>Genotoxicity assays (micronucleus and comet) in 3D skin models</td>
<td>Validation study ongoing</td>
<td>Cosmetics Europe (lead); EURL ECVAM support</td>
</tr>
</tbody>
</table>

**Carcinogenicity Test Methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Status</th>
<th>Lead Organisation</th>
<th>OECD GD</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vitro</em> Bhas 42 cell transformation assay (CTA)</td>
<td>Completed</td>
<td>JaCVAM</td>
<td>231 (2016)</td>
</tr>
<tr>
<td><em>In vitro</em> Syrian hamster embryonic cells (SHE) cell transformation assays (CTAs)</td>
<td>Completed</td>
<td>JaCVAM</td>
<td>214 (2015)</td>
</tr>
</tbody>
</table>

**Reproductive Test Methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Status</th>
<th>Lead Organisation</th>
<th>OECD GD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hand-1 Luc assay</td>
<td>METI?-sponsored validation is completed. The peer review is on-going</td>
<td>JaCVAM</td>
<td>Included in the OECD TGP work plan in 2017</td>
</tr>
</tbody>
</table>

**Acute Aquatic Toxicity Test Methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Status</th>
<th>OECD GD</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Zebrafish Embryo Acute Toxicity test (ZFET)</em></td>
<td>Completed</td>
<td>236 (2013)</td>
</tr>
</tbody>
</table>

**Fish Bioaccumulation Test Methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Status</th>
<th>Lead Organisation</th>
<th>OECD GD</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vitro</em> Fish Hepatic Metabolism - Two <em>in vitro</em> systems for deriving information on biotransformation and improving reliability of predicted bioconcentration factors (BCF)</td>
<td>Ring trial conducted under the auspices of the OECD completed; ring trial report, two draft test guidelines and draft guidance document underwent two WNT commenting rounds in 2017 (April, August)</td>
<td>US and European Commission (through JRC-EURL ECVAM)</td>
<td>Included in the OECD TGP work plan in April 2014</td>
</tr>
</tbody>
</table>

6 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.

7 Ministry of Economy, Trade and Industry
25 years of EU Reference Laboratory for Alternatives to Animal Testing
Dedicated to 3Rs – Replacement, Reduction and Refinement of animal experiments

1991
Foundation of ECVAM, the European Centre for the Validation of Alternative Methods, at the European Commission’s Joint Research Centre in Ispra, Italy.

1994
ECVAM opening Symposium “The Validation of Replacement Alternative Methods”.

1997
First Statement of the ECVAM Scientific Advisory Committee on an in vitro method for phototoxicity testing of chemicals.

1999
Third World Congress on Alternatives and Animal Use in Life Sciences organised by ECVAM with signing of the Three Rs Declaration of Bologna.

2000
Inclusion of first in vitro replacement tests for skin corrosion and phototoxicity testing into EU law.

2002
International recognition of ECVAM’s validation process at OECD conference.

2004
ECVAM publishes its Modular Approach to Validation of alternative methods to achieve more flexibility while retaining scientific rigour.

2006
Launch of DB-ALM, the ECVAM Database Service on Alternative Methods.

2010
Alternative methods for full replacement of skin irritation, skin corrosion, phototoxicity and skin penetration testing accepted as OECD Test Guidelines and included in the EU Test Methods Regulation.

2011
ECVAM becomes EURL ECVAM, the EU Reference Laboratory for Alternatives to Animal Testing.
Publication of 1st edition of the EURL ECVAM Search Guide, a means for effective retrieval of information on alternative methods.

2016
OECD endorses three Adverse Outcome Pathways developed by EURL ECVAM signalling a clear shift towards knowledge-driven design of Integrated Approaches to Testing and Assessment using alternative methods.

Updates to REACH Regulation making in vitro methods the default route to satisfy information requirements for serious eye damage and eye irritation, skin corrosion and irritation, and skin sensitisation.

EURL ECVAM has organised 86 workshops on the development, validation and acceptance of alternative methods & has validated around 50 alternative methods | There are 37 members in the network of validation laboratories | 56 Statements/Opinions have been issued by the EURL ECVAM Scientific Advisory Committee | EURL ECVAM has led the development of 14 OECD Test Guidelines and Guidance Documents on alternative methods | DB-ALM has over 5000 registered users, from 82 countries & contains over 300 method descriptions.
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