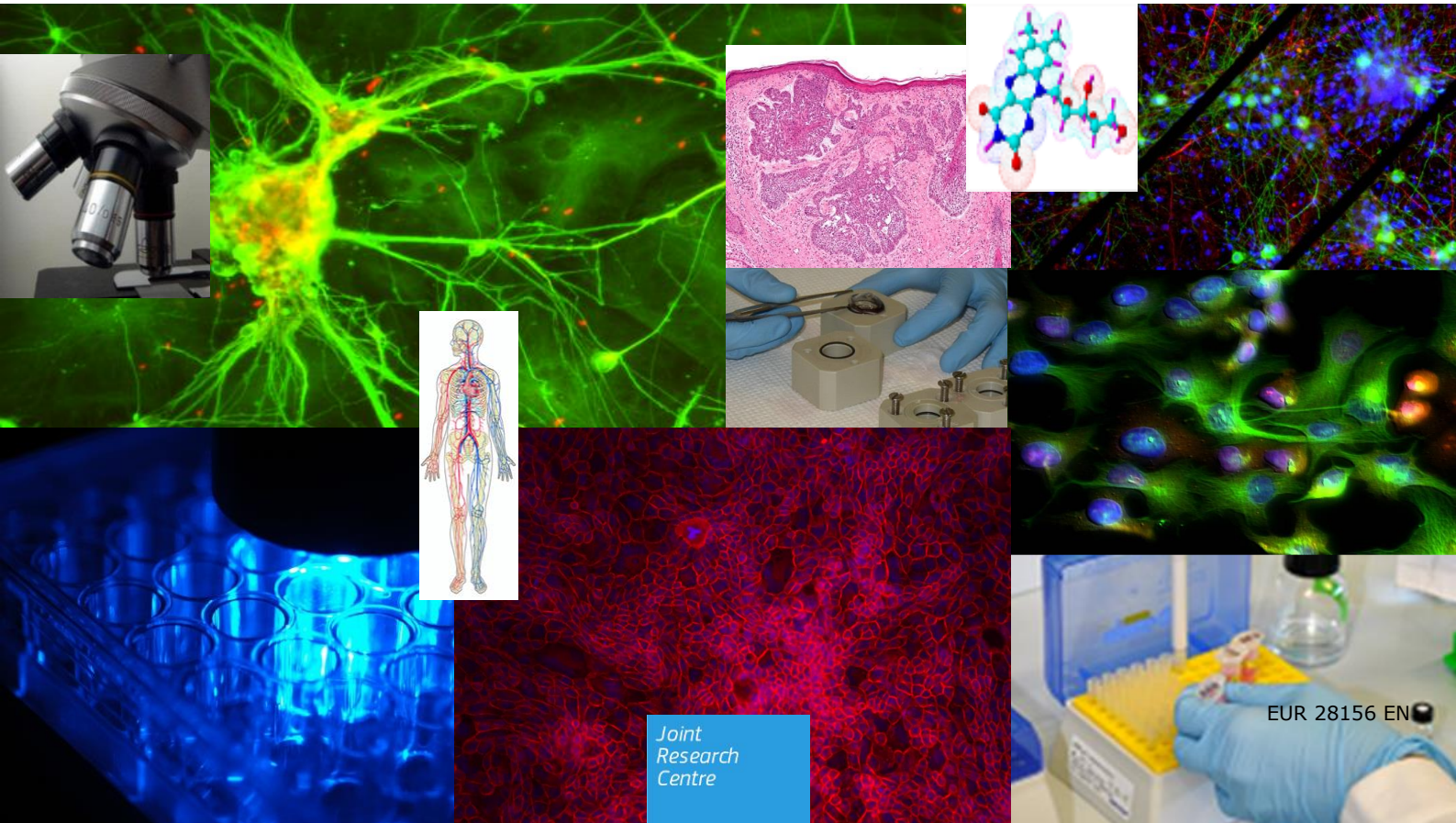
European Commission

JRC SCIENCE FOR POLICY REPORT

EURL ECVAM Status Report on the Development, Validation and Regulatory Acceptance of Alternative Methods and Approaches (2016)

Valérie Zuang, David Asturiol Bofill, João Barroso, Susanne Belz, Elisabet Berggren, Camilla Bernasconi, Stephanie Bopp, Ann-Charlotte Bostroem, Mounir Bouhifd, Silvia Casati, Sandra Coecke, Tom Cole, Raffaella Corvi, Marlies Halder, Tracey Holley, Tomislav Horvat, Annett Janusch-Roi, Aude Kienzler, Brigitte Landesmann, Federica Madia, Anne Milcamps, Sharon Munn, Alicia Paini, Taina Palosaari, Anna Price, Pilar Prieto, Andrea Richarz, Jutta Triebe, Clemens Wittwehr, Andrew Worth and Maurice Whelan

2016



EUR 28156 EN

Contact information
Name: Valérie Zuang
Address: via Enrico Fermi 2749. TP 126
I-21027 Ispra (VA), Italy
E-mail: valerie.zuang@ec.europa.eu

JRC Science Hub
https://ec.europa.eu/jrc

JRC103522

EUR 28156 EN

PDF	ISBN 978-92-79-63031-6	ISSN 1831-9424	doi: 10.2787/400140
Print	ISBN 978-92-79-63030-9	ISSN 1018-5593	doi: 10.2787/644905

Luxembourg: Publications Office of the European Union, 2016

Status of alternative approaches to animal testing

Abstract
Replacement, Reduction and Refinement of animal testing is anchored in EU legislation. Alternative non-animal approaches facilitate a shift away from animal testing. Cell-based methods and computational technologies are integrated to translate molecular mechanistic understanding of toxicity into safety testing strategies.

Table of contents

Executive Summary

Policy context

The European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) is legally anchored in Directive 2010/63/EU on the protection of animals used for scientific purposes[1] which defines its duties (Article 48 and Annex VII). The Directive mandates the application of scientifically valid alternative approaches and establishes mechanisms to speed up their development, validation and uptake. Other pieces of EU legislation such as the Cosmetics Regulation[2] and the REACH Regulation[3] foresee the increased use of alternative methods that either Replace, Reduce or Refine (the "Three Rs") animal testing. Thus the Three Rs are firmly anchored in EU legislation.

This EURL ECVAM status report provides an update on the development, validation and regulatory acceptance and use of alternative approaches and their dissemination since the last report published in September 2015. It informs EURL ECVAM stakeholders and the public on ongoing activities in the field and provides input to the annual Commission report on alternatives prepared in the framework of the Cosmetics Regulation and to the review of Directive 2010/63/EC. The report is also relevant to the European Citizen's initiative (ECI) "Stop Vivisection"[4] that calls for a regulatory framework that shifts away from animal experimentation and makes compulsory the use, in biomedical and toxicological research, of data derived from alternative methods that are directly relevant for the human species.

Key conclusions

The current research and development activities in the field of alternatives predominantly focus on the integration of a variety of testing and non-testing methods such as *in vitro* technologies, bioinformatics and computational toxicology into so-called Integrated Approaches to Testing and Assessment (IATA). Ideally such IATA are based on Adverse Outcome Pathways (AOP), 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.

The ultimate goal of these activities is to deliver reliable, animal-free hazard and risk assessments of chemicals.

Discussions in various stakeholder and regulatory fora revealed that independent scientific and purpose-driven validation remains crucial for the regulatory use and wider adoption of alternative approaches.

In the future however, validation efforts may have to focus on defining and evaluating standards for classes of methods to be used within defined approaches to testing and assessment, rather than only on the validation of individual methods.

Main findings

For complex endpoints, the lack of suitable and mechanistically based methods and their optimal integration in regulatory testing frameworks remains a challenge. Therefore EU-funded research projects continue in this area.

For the quality control of vaccines, an area which consumes a considerable amount of animals, a number of recently launched research projects aim to provide data to support

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[1] Directive 2010/63/EU OJ L276, 20.10.2010, p.33-79 http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32010L0063

[2] https://ec.europa.eu/growth/sectors/cosmetics/legislation_en

[3] https://echa.europa.eu/regulations/reach/legislation

[4] http://ec.europa.eu/citizens-initiative/public/initiatives/successful/details/2012/000007

the consistency approach for routine batch quality control, and safety and efficacy testing of established vaccines for human and veterinary use.

With regard to validation and regulatory acceptance, advanced areas such as local toxicities and genotoxicity are covered with alternative methods and approaches. In the area of skin sensitisation progress has been made in the development, validation and regulatory adoption of various alternative approaches. Recent efforts focused on the development of defined approaches to testing and assessment using data generated with these methods.

IATAs are also being developed for non-genotoxic carcinogens and for serious eye damage/eye irritation. Complementary IATA case studies support the use of alternative methods within IATA by developing guidance and tools. EURL ECVAM regularly engages with regulators (through PARERE[5]), stakeholders (through the ECVAM Stakeholder Forum ESTAF[6]) and validation laboratories (through EU-NETVAL[7]) as well as with its international partners (e.g. ICATM[8], OECD[9] and USEPA[10]) to better understand the different requirements and to find global solutions.

Related and future JRC work

Three Rs knowledge sharing opportunities are being investigated with the aim to explore how sharing of knowhow and access to resources could be enhanced to accelerate overall progress in the Three Rs. This is explored in every domain where animals are used for a scientific purpose, be it for basic biological research, toxicological testing, or for training and education purposes.

Quick guide

Alternative non-animal methods do not use live animals for safety, efficacy and quality control testing of chemicals and products. They include *in vitro* (test tube) test methods and models based on human cell and tissue cultures, computer models and simulations (also called *in silico* methods) as well as stem cell and genetic testing methods.

The Three Rs concept is the requirement to **R**eplace, **R**educe and **R**efine the use of animals wherever possible. 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. The Three Rs are firmly anchored in all EU legislations.

IATA 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.

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[5] PARERE Network (*P*reliminary *A*ssessment of *RE*gulatory *RE*levance) established under Article 47(5) of Directive 2010/63/EU

[6] https://eurl-ecvam.jrc.ec.europa.eu/about-ecvam/scientific-advice-stakeholders-networks/estaf-ecvam-stakeholder-forum

[7] European Union Network for the Validation of Alternative Methods (EU NETVAL) https://eurl-ecvam.jrc.ec.europa.eu/eu-netval established under Article 47(2) of Directive 2010/63/EU

[8] The International Cooperation on Alternative Test Methods (ICATM) includes governmental organisations from the EU, US, Japan, Canada, South Korea, Brazil and China. ICATM partners are working together to promote enhanced international cooperation and coordination on the scientific development, validation and regulatory use of alternative approaches

[9] Organisation for Economic Cooperation and Development (OECD)

[10] United States Environmental Protection Agency (USEPA)

1 Introduction

The EURL ECVAM status report provides an update on the progress made in the development, validation and regulatory acceptance and use of alternative methods and approaches and their dissemination since the last report published in September 2015. It is informing on ongoing research and development activities, validation studies, EURL ECVAM Scientific Advisory Committee (ESAC) scientific peer reviews, EURL ECVAM Recommendations, EURL ECVAM strategies as well as on activities which promote the regulatory acceptance 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 and covers the period October 2015 to September 2016.

It is intended to inform EURL ECVAM stakeholders and any interested parties on on-going activities in the field of alternative approaches and serves multiple purposes. This includes providing input to the annual Commission report on the progress made in the development, validation and regulatory acceptance of alternative methods/approaches prepared in the framework of Regulation 1223/2009 on cosmetic products and to the review of Directive 2010/63/EC on the protection of animals used for scientific purposes.

2 Research and Development Activities on Alternative Methods

2.1 SEURAT-1

SEURAT-1, the cluster of FP7 projects on safety assessment of chemicals replacing animal testing for repeated dose toxicity, was a private-public co-sponsored initiative equally shared between Cosmetics Europe and the European Commission (EC). The five research projects finalised their activities at the end of 2015, and the final results were presented at the SEURAT-1 symposium on the 4th December 2015 in Brussels. It was recognised that the initiative had successfully addressed the overall goal on safety assessment for regulatory use in a series of case studies. This was also demonstrated through the application of the ECHA read-across assessment framework (RAAF; see 2.1.2) to two of the SEURAT-1 case studies that were presented and discussed at the Topical Scientific Workshop at ECHA, 19th – 20th April 2016. In addition, SEURAT-1 provided an extremely rich toolbox developed by the different projects with a multitude of expertise resulting in a large variety of alternative methods, techniques and compiled information, which is available through ToxBank [11], DB-ALM [12], COSMOS Space [13], COSMOS KNIME WebPortal [14] and COSMOS Database [15] (being updated beyond the project and including an extended TTC dataset and a TTC Workflow). More information can be found on the SEURAT-1 public web-page[16].

COACH, the coordination action, is continuing its work in 2016, mainly to disseminate the SEURAT-1 achievements, and also to finalise some of the case study activities. A final workshop is planned at the JRC, Ispra, in collaboration with EURL ECVAM and the H2020 project, EU-ToxRisk (see 2.2), to further explore the current status of regulatory

[11] http://toxbank.net/
[12] EURL ECVAM's Database on Alternative Methods: https://ecvam dbalm.jrc.ec.europa.eu/
[13] http://cosmosspace.cosmostox.eu/
[14] https://knimewebportal.cosmostox.eu/
[15] https://cosmosdb.eu/cosmosdb.v2/
[16] http://www.seurat-1.eu/

use of alternative approaches in toxicology for the safety assessments of chemicals and possibilities for further progress.

2.1.1 Toxicokinetic Modelling and KNIME workflows

During the final year (2015) of the five-year COSMOS project[17], nine physiologically-based kinetic (PBK) models coded in R language were implemented into Konstanz Information Miner (KNIME) workflows (see

[17] http://www.cosmostox.eu/

Table 2.1). A PBK model is a mathematical model for predicting the absorption, distribution, metabolism and excretion (ADME) of a compound in humans and other animal species. The model can be used to simulate relevant time profiles concentration of selected chemicals and its metabolites. PBK models are used to predict *in vivo* toxicokinetics (e.g. time-course of blood or tissue concentrations of a chemical) based on *in vitro* measurements of the underlying ADME processes (*in vitro* to *in vivo* extrapolation; IVIVE), as well as to extrapolate existing animal blood time-courses of a chemical for one exposure route to another exposure route (route-to-route extrapolation; RtR), e.g. oral-to-dermal extrapolation. Route-to-route extrapolation is a common part of human risk assessment. Data from oral animal toxicity studies are used to assess the safety of specific exposure scenarios. PBK modelling approaches can be used to predict animal as well as human internal exposure dose metric, called "Margin of Internal Exposure (MOIE)" (Bessems *et al.,* submitted).

In addition, the Virtual Cell Based Assay (VCBA), developed by the JRC to model the fate and toxicity of chemicals in *in vitro* assays, was made publicly accessible by implementation as web-accessible KNIME workflows. Following a multi-scale modelling approach, PBK models were coupled with VCBA models to enable realistic estimates of *in vivo* effects from *in vitro* toxicity data. This work will be published in a special issue of Toxicology *in vitro* entitled *The Virtual Cell Based Assay* (Zaldivar *et al.,* in press, Graepel *et al.,* submitted). The description of the PBK and bioaccumulation models and VCBA were introduced in DB-ALM[18] (see 6.2.1) as method summaries/protocols nos. 161, 162 and 163. A summary of the overall COSMOS achievements in the area of toxicokinetic modelling was published in Toxicology (Bois *et al.,* 2016).

The above-mentioned computational workflows (among others) can be accessed via the KNIME WebPortal[19], following registration through COSMOS Space[20]. COSMOS Space is a platform for the sharing of predictive toxicology resources (e.g. data sets, models, workflows, documentation). The workflows were presented at the SEURAT-1 Stakeholder event held in Brussels in December 2015.

The EURO-mix consortium[21] is evaluating the potential use of the VCBA and the PBK models developed within COSMOS to refine *in vitro* testing and achieve point of departure for risk assessment.

[18] https://ecvam-dbalm.jrc.ec.europa.eu/
[19] http://knimewebportal.cosmostox.eu
[20] http://cosmosspace.cosmostox.eu
[21] http://www.euromixproject.eu/

Table 2.1 List of the biokinetics workflows developed in COSMOS and available in the COSMOS KNIME WebPortal at http://knimewebportal.cosmostox.eu.

Type	Chemical	Exposure	Species	Output	
PBK	Caffeine	Oral, Dermal	Human, rat	Time	kinetics
PBK	Coumarin	Oral, Dermal	Human, rat	Time	kinetics
PBK	Ethanol	Oral, Inhalation	Human, rat	Time	kinetics
PBK	Estragole	Oral, Dermal	Human	Time	kinetics
PBK	Hydroquinone	Oral, Dermal	Human, rat	Time	kinetics
PBK	Isopropanol	Oral, Dermal	Human, rat	Time	kinetics
PBK	Methyliodide	Oral, Dermal	Human, rat	Time	kinetics
PBK	Nicotine	Intra-venous	Human	Time	kinetics
PBK	Styrene	Inhalation	Human	Time	kinetics
QIVIVE	Caffeine	Oral, Dermal	Human	Time-dose	kinetics
QIVIVE	Coumarin	Oral, Dermal	Human	Time-dose	kinetics
QIVIVE	Estragole	Oral, Dermal	Human	Time-dose	kinetics
Type	Chemical	Exposure	Cell Type	Output	
VCBA	30 chemicals	*in vitro*	HepaRG, 3T3 Balbc, HepG2, A549	Time-dose	kinetics/dynamics

2.1.2 Evaluation of the SEURAT-1 Read-Across Case Study on β-Olefinic Alcohols According to the ECHA Read-Across Assessment Framework (RAAF)

The chemical properties and toxicity of a target substance with no data can be inferred from substances with known properties/existing data by read-across, based on the grouping of similar substances. This approach is increasingly used for filling data gaps for regulatory submissions such as REACH dossiers. The European Chemicals Agency (ECHA) has set up the ECHA Read-Across Assessment Framework (RAAF) to provide guidance for a structured analysis of these read-across submissions and assessment of the justifications. The RAAF and its Assessment Elements were used to systematically analyse the read-across argumentation and the contribution of New Approach Methodologies (NAM) - defined as including any *in silico*, *in chemico* or *in vitro* techniques supporting the substance evaluation - to reduce uncertainties in selected case studies, including two from the SEURAT-1 Initiative (Berggren *et al.,* 2015). The analysis of the β-olefinic alcohols case study led by JRC highlighted the major sources of uncertainty, in particular the need for more (quantitative) kinetic and metabolism pathway data, demonstrated the support by NAM of the read-across hypothesis, and showed further opportunities to use NAM, e.g. through targeted testing (Richarz, 2016). The study was presented and discussed at the ECHA Topical Scientific Workshop on New

Approach Methodologies in Regulatory Science, held on 19-20 April 2016 in Helsinki, Finland[22] (ECHA, 2016b).

2.2 EU-ToxRisk

EU-ToxRisk is a Horizon2020 consortium of 39 partners funded by the European Commission to work on the integration of *in vitro* non-animal methods and *in silico* computational technologies to translate molecular mechanistic understanding of toxicity into safety testing strategies[23]. The ultimate goal is to deliver reliable, animal-free hazard and risk assessment of chemicals. The project started with a meeting in Egmond aan Zee on 13th to 15th January 2016[24] and is building further on the activities started by the SEURAT-1 initiative. EU-ToxRisk continues to evaluate methodologies for repeated dose toxicity, but also for developmental and reproductive toxicity. The project is built up around different case studies, to better capture possibilities and shortcomings in safety assessment applications. A first summer school and a second consortium meeting took place in Egmond aan Zee in the last week of June 2016. In September 2016, EU-ToxRisk organised a meeting with the US Tox21[25] to proceed with a fruitful US-European collaboration on a more efficient toxicity testing applying new approaches that was started with SEURAT-1.

2.3 Development of a Defined Approach/Predictive Model for Skin Sensitisation Prediction

Within the framework of the recently developed OECD guidance documents on the reporting of defined approaches within Integrated Approaches to Testing and Assessment (IATA)(OECD 2016a; OECD 2016b), EURL ECVAM has developed a case study on the prediction of skin sensitisation.

In order to develop the defined approach, a dataset with high quality data from the direct peptide reactivity assay (DPRA) (Gerberick *et al.,* 2004; EURL ECVAM 2013), KeratinoSens™ (Natsch and Emter 2008; Emter *et al.,* 2010; EURL ECVAM 2014), and the human cell-line activation test (h-CLAT) (Ashikaga *et al.,* 2006; Sakaguchi *et al.,* 2006; EURL ECVAM 2015) was compiled and curated in collaboration with the test method developers. The dataset comprises 269 chemicals with data generated with one or more of the test methods and complemented with molecular descriptors and predictions from various *in silico* tools (i.e. OECD QSAR ToolBox, Derek Nexus, Toxtree, Dragon, Vega, TIMES, and ADMET Predictor) and LLNA data.

The modelling exercise consisted of finding the best combination of descriptors to predict skin sensitisation hazard (sensitiser/non-sensitiser) using the LLNA results as reference classifications. In order to do so, an algorithm (C4.5) was used that combines the best descriptors into decision trees, which are simple and easily interpretable predictive models based on a sequence of descriptors (properties of each chemical) and their threshold values. Therefore, the algorithm determines the optimal descriptors, order and thresholds for predicting skin sensitisation potential for a list of chemicals and corresponding descriptors/properties.

Different models were built for different subsets of the original dataset depending on the availability of *in chemico/in vitro* data. It was found that skin sensitisation hazard was

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[22] https://echa.europa.eu/view-article/-/journal_content/title/topical-scientific-workshop-new-approach-methodologies-in-regulatory-science; presentation: https://echa.europa.eu/documents/10162/22301701/presentation_bos_richarz_en.pdf; summary of the discussion: https://echa.europa.eu/documents/10162/22301701/bos_2_en.pdf

[23] http://cordis.europa.eu/project/rcn/198787_en.html

[24] https://www.eurtd.com/eu-toxrisk/2016/kick-off-meeting/

[25] http://tox21.org/

best predicted by decision trees based on molecular descriptors and *in silico* predictions, and that the most discriminating descriptor was the amount of protein-hapten adduct formation provided by TIMES-SS (Dimitrov *et al.,* 2005).

In order to provide a defined approach with the lowest reasonable number of false negative predictions (highest sensitivity), the two decision trees with the highest accuracy and highest specificity were combined into a final consensus model. Thus, the defined approach (Asturiol *et al.,* 2016) consists of a consensus of two classification trees that are both based on descriptors that account for protein reactivity and structural features. The defined approach has an accuracy of 0.93, sensitivity of 0.98, and specificity of 0.85 for 269 chemicals. In addition, the defined approach provides a measure of confidence (very high, high, low, very low) associated with each prediction. The dataset and model will be made publicly available on the EURL ECVAM website.

2.4 Generation and Use of High Throughput Screening Data in EU Projects

High throughput screening (HTS) data has been used in several EU projects such as the NanoMILE Project which is a large collaborative project under the European Commission's 7th Framework Programme. The project aims to establish a fundamental understanding of the mechanisms of nanomaterial interactions with living systems and the environment across the entire life cycle of nanomaterials and in a wide range of target species. Due to the diversity of manufactured nanomaterials (MNM) regarding chemical composition, surface modification, size, and other parameters the number of materials to be tested for safe application is steadily increasing. For effective safety screening of numerous MNMs it is necessary to speed up the testing by using *in vitro* test systems and applying High Throughput/ High Content methods.

The HTS facility at EURL ECVAM laboratory was used to establish a screening platform based on High Throughput /High Content Imaging techniques in two stages:

a) Screening for the most relevant MNMs and endpoints (using both classical and novel biomarkers) and,

b) Further validating the utility of novel mechanistic pathway specific biomarkers and developing future test methods based on high throughput profiling of MNMs.

The materials tested were selected from a library of different MNMs of different sizes, different chemistry and coatings/modifications. To date, more than 100 MNMs from the NanoMILE library have been screened at the EURL ECVAM HTS facility. Since the liver is a major target organ for MNM toxicity, a human hepatoma cell line, HepaRG, was used as *in vitro* cell model. Cells cultured *in vitro* represent ideal high-throughput and ethically acceptable systems to assess toxicity.

Similarly, data were generated at the HTS facility for the SEURAT-1 project (see 2.1). The case study "Mode of Action (MoA)-based Classification Model for Repeated Dose Liver Toxicity" was developed to compare the performances of several classification models for hepatotoxicity, which is mainly related to three major liver adverse outcomes associated with repeated dose exposure: cholestasis, fibrosis and steatosis. HepaRG cells were exposed to 90 selected reference compounds (75% known hepatotoxicants and 25% known non-hepatotoxicants), including cosmetics, pharmaceuticals, pesticides and environmental chemicals. The read-out of the *in vitro* endpoints was performed using high content screening. In this automated multiparametric analysis the following key events were assessed: mitochondrial damage, reactive oxygen species formation (indicator of oxidative stress), formation of neutral lipid droplets (indicator of chemically induced steatosis), apoptosis and cell viability. Concentration-response data for each of these parameters are generated to determine the lowest concentration at which the effect is observed.

2.5 Fish Toxicity and Bioaccumulation R&D Projects

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

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

As a follow-up to the CEllSens [26] 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 ECO8.3-NC3Rs-EAWAG[27]). A paper on the outcome of the ring trial is in preparation.

In addition, the method had been submitted to EURL ECVAM in early 2014 and the test submitter had been invited to provide a full submission (see 3.1 of EURL ECVAM Status report, Zuang V. *et al.,* 2015).

An ISO guideline "Water quality - Determination of acute toxicity of chemicals and water samples to a fish gill cell-line (RT gill-W1)" is in preparation.

2.5.2 Development of AOPs for Chronic Fish Toxicity Testing

Several research groups are working on the identification and description of potential AOPs relevant to chronic fish toxicity, which is currently assessed with fish early life-stage (FELS) test (OECD TG 210, 2013a). A CEFIC LRI-funded project (LRI-ECO20-UA[28]) aimed at mapping FELS-relevant AOPs and developing an *in vitro* toolbox and zebrafish embryo based assays to test for AOP-specific events and responses predictive for FELS chronic toxicity. A summary of the outcome of the project is available on the CEFIC LRI website[29]. The overall objective of the recently started follow-up project LRI-ECO20.2[30] 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.

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

This new CEFIC LRI-funded project (LRI-ECO34)[31] combines various *in vitro* approaches using fish cell lines to estimate chemical uptake and biotransformation with toxicokinetic and quantitative structure activity relationship models to develop a tiered approach for the assessment of the bioaccumulation potential of chemicals.

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[26] http://cefic-lri.org/projects/eco8-development-of-a-strategy-to-predict-acute-fish-lethality-using-fish-cell-lines-and-fish-embryos/

[27] http://cefic-lri.org/projects/eco8-3-nc3rs-eawag-round-robin-test-of-the-rtgill-w1-cell-line-assay-to-study-its-robustness-in-establishment-and-inter-laboratory-comparability/

[28] http://cefic-lri.org/projects/lri-eco20-ua-development-of-an-alternative-testing-strategy-for-the-fish-early-life-stage-test-for-predicting-chronic-toxicity/

[29] http://cefic-lri.org/wp-content/uploads/2014/03/LRI-ECO20-UA_Executive-summary_March2016.pdf

[30] http://cefic-lri.org/projects/eco20-2-development-of-an-alternative-testing-strategy-for-the-fish-early-life-stage-test-for-predicting-chronic-toxicity-assay-validation/

[31] http://cefic-lri.org/projects/eco34-a-tiered-testing-strategy-for-rapid-estimation-of-bioaccumulation-by-a-combined-modelling-in-vitro-testing-approach/

2.5.4 Threshold of Toxicological Concern in Aquatic Toxicity Assessment

The Threshold of Toxicological Concern (TTC) approach is based on the premise that there is a general exposure limit for chemicals below which no significant risk to human health or the environment is expected. It is well established for assessing human safety of substances present in low levels in food and feed (Kroes *et al.,* 2004; EFSA Scientific Committee, 2012).

The potential usefulness of the TTC approach for various applications in environmental toxicity and risk assessment has been explored and reported by several groups (for example by: (de Wolf *et al.,* 2005; Gross *et al.,* 2010; Williams *et al.,* 2011; Gutsell *et al.,* 2015)).

An international collaboration under the ILSI HESI has been established to address challenges relating to the development and application of useful ecotoxicological threshold of concern (eco-TTC) concepts. Belanger *et al.,* 2015, outline the major challenges of data collection, quality control, data characterisation and analysis. EURL ECVAM is contributing to this initiative.

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

The assessment of aquatic toxicity is an important component of the environmental hazard and risk assessment of all types of chemicals, and is therefore included in several pieces of EU chemicals legislation. Aquatic toxicity refers to the effects of chemicals on organisms living in the water and is usually determined by testing on organisms representing the three trophic levels, i.e. 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 a long-term exposure to the chemical is expected.

In the light of the EU Directive 2010/63 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* [32] , 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.

EURL ECVAM published the report on *Scientific options for avoiding chronic fish testing on the basis of existing data and extrapolation approaches* in May 2016 (Kienzler *et al.,* 2016a).

[32] https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-strategy-papers/strategy-fish

2.6 Quality Control of Vaccines

2.6.1 Launch of the VAC2VAC project

The new VAC2VAC project[33] - "Vaccine batch to vaccine batch comparison by consistency testing" – officially launched on 1st March 2016, brings together 20 public and private partners including the JRC represented by EURL ECVAM.

VAC2VAC 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). The project will provide data to support 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 will develop, optimise and evaluate 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 will also support project activities related to international dissemination, harmonisation and regulatory acceptance of consistency approaches.

Over the last decade EURL ECVAM, in collaboration with international experts and EPAA, organised several workshops on the use of the consistency approach for the quality control of vaccines (Metz *et al.,* 2007; Hendriksen *et al.,* 2008; De Mattia *et al.,* 2011) and supported the EPAA Vaccines Consistency Project (De Mattia *et al.,* 2015; see also 5.9.3).

2.6.2 EURL ECVAM Report on Replacement, Reduction and Refinement of Animal Testing in the Quality Control of Human Vaccines

EURL ECVAM released in 2016 a report to inform its stakeholders on ongoing activities in development and validation of Three Rs methods for the quality control of vaccines for human use (Halder, 2015). The focus of the report is on methods for lot release testing (e.g. safety, pyrogenicity, potency) and projects related to the implementation of the consistency approach to established vaccines such as diphtheria, tetanus, pertussis and rabies vaccines.

Vaccines are recognised as a highly cost effective tool for preventing infectious diseases. They are derived from biological sources and due to the complexity of composition and heterogeneity of products, vaccine lots undergo legally required quality control before they are released. Traditionally, laboratory animals have played an important role in quality control of vaccines and still, many laboratory animals are used in Europe for this purpose. Over the last decades, Three Rs methods to classical animal tests have been developed by control authorities, academia and vaccine manufacturers.

As the report shows, progress has been achieved and new approaches to quality control such as the consistency approach have the potential to further reduce animal use.

2.7 Reference Lists of Genotoxic and Non-Genotoxic Chemicals

Reference chemical selection is a key step in the development, optimisation and validation of alternative test methods. A first reference list, of genotoxic and non-genotoxic chemicals, published in 2008 (Kirkland *et al.,* 2008), has become an internationally recognised resource for scientists and has been used for a variety of purposes, including the development of new assays, the optimisation of existing test

[33] www.vac2vac.eu

protocols, the implementation of automated high throughput assays, and the design of validation studies. In addition, the reference list has proven invaluable in the attempt to reduce misleading positive results obtained from some *in vitro* methods.

In light of newly available data, it was considered appropriate to update this list of genotoxic and non-genotoxic chemicals recommended for assessing the performance of new or improved *in vitro* genotoxicity test methods. The list was updated to fit the following different sets of characteristics (Kirkland *et al.,* 2016):

> Group 1: Chemicals that should be detected as positive in *in vitro* mammalian cell genotoxicity tests. Chemicals in this group are all *in vivo* genotoxins at one or more endpoints, either due to DNA-reactive or non DNA-reactive mechanisms.
>
> Group 2: Chemicals that should give negative results in *in vitro* mammalian cell genotoxicity tests. Chemicals in this group are usually negative *in vivo* and non-DNA-reactive. They are either non-carcinogenic or rodent carcinogens with a non-mutagenic mode of action.
>
> Group 3: Chemicals that should give negative results in *in vitro* mammalian cell genotoxicity tests, but have been reported to induce gene mutations in mouse lymphoma cells, chromosomal aberrations or micronuclei, often at high concentrations or at high levels of cytotoxicity. Chemicals in this group are generally negative *in vivo* and negative in the Ames test. They are either non-carcinogenic or rodent carcinogens with an accepted non-mutagenic mode of action. This group contains comments as to any conditions that can be identified under which misleading positive results are likely to occur.

These recommended lists, which now contain a total of 69 chemicals of different structural classes and modes of action and the supporting data are expected to make an important contribution to the development and acceptance of new and refined *in vitro* genotoxicity test methods with improved predictivity and technical performance.

2.8 Activities within the European Partnership for Alternative Approaches to Animal Testing

The European Partnership for Alternative Approaches to Animal Testing (EPAA) is a public-private collaboration between the European Commission, European trade associations and companies from seven business sectors.

In 2015 the EPAA celebrated its 10th anniversary and launched a new five-year Action Programme (2016 - 2020)[34] at its Annual Conference in December 2015, whose report is available online[35].

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

EPAA recently concluded one research project on toxicokinetics (see 2.8.1), while other activities have focussed on regulatory acceptance (see 5.9), (pre)validation (see 5.9.3, 5.9.4 and 5.9.5) and training & dissemination (see 6.10).

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[34] See: https://circabc.europa.eu/sd/a/1ccf4a54-b374-4ab1-bf6c-b95200f62aab/action-programme-2016-2020.pdf

[35] See: https://circabc.europa.eu/sd/a/385c4f37-e8d3-4b82-8652-47a64513b58b/EPAA_AC2015_Report.pdf

2.8.1 Toxicokinetics - Exposure Prediction tool

Starting in 2014, an EPAA project was carried out for the development of an exposure prediction tool converting *in vivo* to *in vitro* exposure data and vice-versa. The project was completed mid-2016 and aimed at (i) the further development of the existing web-based Model Equation Generator MEGen[36] established for the generation and analysis of physiologically based kinetic (PBK) models and (ii) the generation of a prototype of a free-to-use, web-based, open-source tool ("RVis") that would enable *in vitro/in vivo* exposure predictions.

The EPAA project developed a prototype (beta-release) of the RVis application which is currently being tested by EPAA partners and other experts. EURL ECVAM participated in the technical advisory team to the project and is involved in the group of experts testing the application prior to enter a second development phase. The latter will be part of a future project funded by Cefic's programme LRI[37] and will result in the provision of the final open-access version of RVis to be made freely available online. The final tool will also be downloadable to the user's PC in order to avoid the need for uploading proprietary information to the web and thus assuring the confidentiality of the user's data.

2.8.2 EPAA Awards/Prizes

The EPAA Awards are granted to young scientists (Three Rs Science Prize) or laboratory technicians and animal caretakers (Three Rs 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 2015, the EPAA granted the Three Rs Technicians Prize to a laboratory supervisor, working for a company in Germany, for her work in developing and establishing a multi-organ chip for the co-culture of organ equivalents for long-term (up to 28 days) substance testing. The chip allows flexible arrangements of 2 to 10 different organ equivalents and a connection to two different circuits reflecting the circulatory and excretory systems. The Prize winner presented her work to the participants of the EPAA Annual Conference in December 2015.

3 Test Method Submissions

Since the last EURL ECVAM status report published in September 2015, six new test submissions were evaluated by EURL ECVAM, three pre-submissions (EDITOX, Bioelution and Ashland RHE SIT) and three full submissions (SkinEthic HCE EIT, Sterlab RHE SIT and SENS-IS). Moreover, EURL ECVAM discussed the results of the PARERE consultation on a test method for teratogenicity testing (devTOXqP, see 5.1) and reviewed the validation project plan of the Genomic Allergen Detection (GARD) method (see 3.7.1 of EURL ECVAM status report 2015, Zuang et al., 2015). A database that tracks the status of a test method from its submission through its final adoption into a regulatory framework (EU, OECD and related standards) is currently under development (TSAR, see 6.2.3).

[36] See: George Loizou and Alex Hogg, "MEGen: A Physiologically Based Pharmacokinetic Model Generator", Front. Pharmacol. 2011; 2: 56 (online: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3212724/)
[37] http://cefic-lri.org/

3.1 EDITOX: Assessent of the Risk for Chemicals to Induce Psychiatric Adverse Side Effects (Depression and/or Suicide)

The test method consists of the quantitative analysis of RNA editing modifications of the serotonin receptor 2C (5-HT2cR) induced by pharmaceutical compounds using SH-SY5Y human neuroblastoma cell line. The test is based on the observation that anomalies of serotonin biology in brain appear to be a characteristic feature underlying depression and/or suicidal behaviour. This assumption has been suggested based on analyses of postmortem brain tissue of suicide victims where distinct epigenetic alterations of the RNA editing activity on 5-HT2cR pre-mRNA has been observed. The test method can quantitatively analyse variations of the relative proportion of the 32 mRNA editing isoforms of 5-HT2cR using a next generation sequencing approach. The submitter claims that predictive performances of the submitted test outcompete current test methods using behavioural animal models. The test submission is currently under evaluation by EURL ECVAM.

3.2 Bioaccessibility Testing (Bioelution)

A pre-submission of an *in vitro* method (bioelution) was received in 2016. The method is not a toxicity test but will provide the fraction of a substance that dissolves under surrogate physiological conditions and is potentially available for absorption into systemic circulation. This is termed bioaccessibility, which can be considered as a conservative estimate of bioavailability. The method estimates relative bioaccessibility of metal ions from various metal-containing materials in simulated gastric fluid, by comparing the target and the reference material. The *in vitro* bioelution method is then proposed by the test submitter to generate bioaccessible metal ion data for the oral route of exposure, which is relevant to systemic effects of metal-containing materials. The test submission is currently under evaluation by EURL ECVAM.

3.3 Ashland Reconstructed Human Epidermis Skin Irritation Test

EURL ECVAM received a pre-submission on the Ashland skin irritation test in May 2015 proposing the conduct of a Performance Standards-based validation study. A reply was sent to the test method submitters identifying a number of shortcomings that should be addressed before embarking on the validation study and therefore, the submission was not progressed. Seeing that the submitters' intention is to include their test method in OECD TG 439, EURL ECVAM also suggested that they contact a National Coordinator (NC) for the OECD Test Guidelines Programme to seek support for a submission to the OECD and discuss the steps involved in such a submission. Following this expedited route for regulatory acceptance, the developers of the Ashland skin irritation test would be able to submit their validation report directly to the OECD for review by the appropriate expert group after successful completion of their validation study, without the need to go through further evaluation by EURL ECVAM and peer review by the EURL ECVAM Scientific Advisory Committee (ESAC). EURL ECVAM may still contribute, together with other OECD member countries/organisations, to the review of the validation study report via the usual OECD process.

3.4 SkinEthic™ Human Cornea Epithelium (HCE) Eye Irritation Test

In December 2008, two reconstructed human eye tissue models for *in vitro* assay of eye irritation potential, EpiOcular™ EIT and SkinEthic™ HCE, were sponsored for validation as alternatives to the traditional *in vivo* standard practice (Draize test) with rabbits (OECD 2012). The eye irritation validation study (EIVS) was conceived as a ring trial of comparative performance among six participant laboratories, testing selected chemicals to evaluate reliability (reproducibility of results obtained *in vitro*) and relevance (predictive capacity of effects documented *in vivo*). Neither test method was able to comply fully with the acceptance criteria set by the validation management group

(VMG). Therefore, further optimisation was recommended. With minor refinement to the EpiOcular™ EIT protocol, the method was successfully validated in 2013. The SkinEthic™ HCE protocol was subject to more comprehensive revision, followed by another validation ring trial (three laboratories) completed in 2015. In November 2015 the revised SkinEthic™ HCE test method was submitted for assessment by EURL ECVAM and formal validation by ESAC peer review.

The SkinEthic™ HCE method is suited to so-called Top-Down/Bottom-Up assessment (Scott *et al.,* 2010) particularly relevant for chemicals used in human exposure products, such as cosmetics ingredients which are banned from animal testing. In this context the SkinEthic™ HCE method is applicable as a first step in Bottom-Up discrimination of 'non-irritants (GHS no category; UN Nations GHS, 2013) or as a confirmatory last step in Top Down identification of 'irritants' (GHS categories 1 and 2). However, the method is not intended to differentiate category 1 from 2.

The follow-up validation (ring trial, three laboratories) evaluated within/between laboratory reproducibility (WLR/BLR) and predictive capacity (PC) including 120 chemicals (60 liquids, 60 solids) with an additional 80 chemicals (45 liquids, 35 solids) tested by the lead laboratory. Overall, WLR/BLR was greater than 90%. PC compares *in vitro* viability with documented *in vivo* classification (true versus false predictions) respective of irritant classification (C) and non-irritant classification (NC). Overall sensitivity (correct prediction of C) was 98% (liquids) and 92% (solids). Overall specificity (correct prediction for NC) was 69% (liquids) and 77% (solids). Overall accuracy (correct prediction, C or NC) was 85% (liquids) and 84% (solids).

The SkinEthic™ HCE method and validation study underwent scientific peer review by ESAC in June 2016 with a positive outcome (see 4.7)

3.5 Sterlab Reconstructed Human Epidermis Skin Irritation Test

EURL ECVAM received a full submission on the Sterlab RHE for skin irritation testing in June 2015. A reply was sent to the test method submitters raising several issues for their consideration and a revised submission was received in May 2016. In reply to this latest submission EURL ECVAM clarified its recent position not to proceed with a formal assessment and peer-review of the Sterlab RHE nor of any other methods similar to the already validated and accepted RhE-based skin irritation test methods described in OECD TG 439 (so-called "me-too" methods). Seeing that the submitters' aim is to include their test method in OECD TG 439, EURL ECVAM believes that a direct submission to the OECD would expedite the acceptance of the method and therefore recommended that the developers of the Sterlab RHE engage directly with a National Coordinator (NC) of the OECD Test Guideline Programme and seek the NC's immediate support to include the Sterlab RHE in the OECD Work Programme. Following this expedited route for regulatory acceptance, the developers of the Sterlab RHE would be able to submit their validation report directly to the OECD for peer-review by the appropriate expert group without the need to go through further evaluation by EURL ECVAM and peer review by the EURL ECVAM Scientific Advisory Committee (ESAC). EURL ECVAM may still contribute, together with other OECD member countries/organisations, to the review of the validation study report via the usual OECD process.

3.6 SENS-IS Skin Sensitisation Test

The SENS-IS assay provides an *in vitro* method for identifying potential skin sensitising substances. The test method is based on a reconstructed human skin model as the test system and on the analyses of the expression of three groups of genes by a toxicogenomic approach [Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)] measuring fold increase in the expression levels of genes over a calibrator control (Cottrez *et al.,* 2015). Besides providing information on the potential or not of a chemical to act as sensitiser, the method also allows to classify substances predicted to

be sensitisers in one of four potency classes (weak, moderate, strong and extreme) on the basis of the lowest concentration tested.

The Sens-is test method underwent an external validation study coordinated by ImmunoSearch (Grasse, France) to evaluate the within- and between-laboratory reproducibility and the preliminary predictive capacity of the method using results generated with 19 chemicals tested in three different laboratories (Cottrez *et al.,* 2016). Additional evidence on the predictive capacity of the method for positive/negative classification and for potency subcategorisation is available for a total of 150 substances tested at the ImmonoSearch laboratory. The information generated during the validation study has been submitted to EURL ECVAM and is currently under evaluation for adequateness to enter the peer-review process.

The development of a Test Guideline for the SENS-IS is already included in the OECD work program. The fact that the test method includes proprietary elements that may lead to a situation of market monopoly was originally seen as a reason of concern. The issue of intellectual property (IP) in Test Guidelines was discussed in occasion of the 27th meeting of the OECD Working Group of the National Coordinators for the Test Guidelines Programme (WNT) in 2015. Generally, the WNT agreed that "it is important that the Test Guidelines Programme continues to develop test methods that make use of the best available technology, provided there is transparency on important mechanisms that enable users to understand how test results are generated and interpreted, i.e. avoiding a 'black box' test system. It was recognised that the possible monopoly situation by one test method developer may be unavoidable from time to time, until similar methods are developed; this situation should not be seen as a problem provided there is no abuse and there is transparency in the functioning of the test method".

4 Validation of Alternative Methods

4.1 Androgen Receptor Transactivation Assay AR-CALUX

In view of developing an OECD Performance Based Test Guideline (PBTG) for Androgen Receptor Transactivation Assays (ARTAs), EURL ECVAM is carrying out a validation study of the *in vitro* method AR-CALUX. This method is a reporter-based assay where osteosarcoma cells, stably transfected with a human androgen receptor, will express luminescence when presented with chemicals that have (anti)androgenic potential. The method was submitted by the Dutch company BioDetection Systems (BDS). From EURL ECVAM's network of specialised laboratories, the European Union Network for the Validation of Alternative Methods (EU-NETVAL), three facilities are participating in this validation study. A Validation Management Group to provide oversight on the study has been established. So far, EURL ECVAM has carried out an experimental assessment of the method; conducted a GLP study in order to refine the assay and establish transfer criteria; provided a training for the three partner facilities at the JRC laboratories in Ispra; coordinated the first phase of the validation study, the transfer phase, where the three participating labs have applied the method in their own laboratories. This phase was recently finalised. Besides the AR-CALUX method, the PBTG on ARTAs and related Performance Standards will also be based on other ARTAs either already validated or in the process of a validation: the AR STTA using the AR-EcoScreen cell line (led by Japan, recently accepted and issued as TG 458, see Annex 1) and the ARTA using the 22Rv1/MMTV cell line (led by Korea, see Annex 2). EURL ECVAM participates in the latter study as a member of the VMG, providing support with the chemicals selection, study design and data interpretation.

4.2 Micronucleus Test and Comet assay in Reconstructed Skin Models for Genotoxicity Testing

The validation of methods for genotoxicity testing in reconstructed human 3D skin models, coordinated by Cosmetics Europe, is still ongoing (Aardema *et al.,* 2010;Reus *et al.,* 2013). The experimental work for the micronucleus test in 3D epidermis model has been finalised and data are being evaluated using the assessment criteria considered in the updated OECD Test Guidelines for genotoxicity. For the comet assay in full-thickness skin models the between-laboratory reproducibility and predictive capacity is still being assessed. Both the micronucleus and the comet assay in reconstructed skin models will not be considered as stand-alone assays, but they will be used within an animal-free testing strategy for genotoxicity to follow-up the *in vitro* testing battery results.

4.3 Hen's Egg test for Micronucleus Induction (HET-MN) for Genotoxicity Testing

The hen's egg test for micronucleus induction (HET-MN; Wolf *et al.,* 2008) 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 enables metabolic activation, elimination and excretion of xenobiotics, including those that are mutagens or pro-mutagens. The predictive capacity of the assay is currently being evaluated by a German consortium (Greywe *et al.,* 2012).

4.4 Ongoing Validation Studies for 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; a multi-dose serological assay for rabies vaccine for veterinary use; *in vitro* methods for the testing of *Clostridium septicum* vaccines, the BINACLE assay for *in vitro* detection of toxicity in tetanus vaccines) or are planned for 2016 and beyond (e.g. ELISAs for tetanus/diphtheria vaccines, ELISA for potency testing of human rabies vaccines).

More information on the BSP, its background and work programme is available at https://www.edqm.eu/en/BSP-Work-Programme-609.html.

As reported in last year's EURL ECVAM status report, the results of a validation study on alternative methods to the murine histamine sensitisation test (HIST) for safety testing of acellular pertussis vaccines indicated that the "indirect CHO-cell based assay" is a suitable alternative for replacement of HIST. The final outcome of the study has recently been published (Isbrucker *et al.,* 2016) and inclusion of the method into the relevant monographs is ongoing.

In collaboration with EPAA, EDQM discussed the results of Phase 2 of the project on *in vitro* methods for the testing of *Clostridium septicum* vaccines at a workshop in September 2015 (Sinitskaya *et al.,* 2016). Phase 3 has been launched and more details are given under 5.9.4.

4.5 Update on the European Union Network for the Validation of Alternative Methods (EU NETVAL)

EURL ECVAM established a network of 37 highly qualified laboratories (EU-NETVAL)[38] to (1) respond to the provision of Directive 2010/63/EU asking to set up a network of suitable specialised and qualified laboratories to carry out validation studies, (2) generate *in vitro* method information that is reliable, relevant and based on current best quality and scientific practices, (3) increase the European Commission's validation capacity of *in vitro* methods and (4) provide a laboratory network knowledgeable on the routine implementation of good *in vitro* method practices for regulatory use in human safety assessment (Coecke *et al.,* 2014). The network has recently expanded to include thirteen new test facilities (bringing the total to 37). Fifteen countries are now represented in the network (from EU Member States and European Free Trade Association countries) following the call for membership of EU-NETVAL which 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. These are considered important to address specific aims and objectives identified in EURL ECVAM's strategies to achieve Three Rs impact in different areas of regulatory safety testing.

EU-NETVAL members support validation studies through the execution of one or more specific tasks[39]. The first pilot validation project of selected EU-NETVAL test facilities is the generation of experimental data using the *in vitro* AR-CALUX method to support the development of an OECD Performance-based Test Guideline and associated performance standards for Androgen Receptor Transactivation Assays (ARTA) for the detection of compounds with (anti)androgenic potential (see 4.1). Also defined in the Terms of Reference[39], the network contributes to the development of guidance documents and training materials supporting good *in vitro* method development and practices. They have provided input to the technical guidance document, Good *In Vitro* Method Practice (see 5.6.11) and are currently in the process of reviewing this document in view of submission to the OECD.

[38] https://eurl-ecvam.jrc.ec.europa.eu/eu-netval-test-facilities
[39] https://eurl-ecvam.jrc.ec.europa.eu/eu-netval/EU-NETVAL-tor-november-2013.pdf

In addition to the wide range of techniques and capabilities, the network offers an opportunity to share knowledge and to collaborate for the promotion of the development and use of alternative approaches. A survey was carried out from November 2015 until 15th January 2016 to inform a report on the training needs and competences within EU-NETVAL and to identify opportunities to share knowledge and best practice. Twenty-nine test facilities responded to the survey out of a total of the thirty-six which were members at the time of the survey. A summary of the responses will be presented in a report along with the opportunities for collaborative training that EURL ECVAM has identified.

4.6 EURL ECVAM Guidance on the Validation of Alternative Methods to Animal Testing

Nowadays, an increasing number of validation studies are conducted by external parties (e.g. industry, academia, consortia). Often these studies are conducted by experts in specific technologies, but not in validation. Therefore, EURL ECVAM is preparing a Guidance Document that will support validation studies in terms of study design, management, reporting, and transparency. Hence, it will facilitate evaluation, peer-review, regulatory acceptance and international recognition of methods which have undergone a prospective validation study. After a brief introduction to validation (What is it about? Why is it necessary?), the document will guide the test submitter through the four stages of a prospective validation study: planning, conducting, analysing and reporting. Criteria for a preliminary assessment will also be covered, to evaluate at an early stage whether the method is ready for validation. While there are excellent documents on validation, regulatory acceptance and minimum reporting requirements of novel methods (i.e. OECD guidance document No. 34, 2005) this guidance attempts to provide assistance on how to practically carry out validation.

4.7 EURL ECVAM Scientific Advisory Committee Peer Reviews

From April to June 2016, the EURL ECVAM Scientific Advisory Committee (ESAC) 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 Performance Standards-based validation study of the epiCS® Skin Irritation Test (SIT) coordinated by CellSystems GmbH, (ii) the validation of the Ocular Irritection® test method for prediction of serious eye damage/eye irritation potential of chemicals coordinated by Secam Services & Consultation on Alternative Methods Sagl., (iii) the validation study of the SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT) coordinated by L'Oréal, (iv) the validation study of the U-SENS™ test method for skin sensitisation testing coordinated by L'Oréal, and (v) the Performance Standards-based validation study of the LuSens test method for skin sensitisation testing coordinated by BASF. Two ESAC Rapporteurs coordinated the peer-review of the epiCS® SIT, whereas the peer-review of the other four methods was 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. The ESAC Working Group on eye irritation met at EURL ECVAM on 11-13 May 2016 to conduct the review of the SkinEthic™ HCE EIT and of the Ocular Irritection® assay. The ESAC Working Group on skin sensitisation met at EURL ECVAM 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 will be published by EURL ECVAM during autumn 2016.

4.8 EURL ECVAM Recommendations

There are currently two EURL ECVAM Recommendations in preparation and one planned. One relates to the use of non-animal methods and approaches based on data integration for skin sensitisation assessment including the recently peer reviewed LUSENS and U-SENS test methods (see 4.7). The other EURL ECVAM Recommendation is on the human cytochrome P450 (CYP) n-fold induction *in vitro* test methods which underwent independent scientific peer review last year (see 4.6.3 of EURL ECVAM status report 2015, Zuang *et al.,* 2015). A third recommendation related to reconstructed human cornea-like epithelium test methods for serious eye damage/eye irritation including the EpiOcular™ EIT and the recently peer reviewed SkinEthic™ HCE EIT (see 4.7) methods is also planned.

The draft EURL ECVAM recommendation on skin sensitisation may be supplemented by relevant points raised at the upcoming International Cooperation on Alternative Methods (ICATM) meeting on the international regulatory applicability and acceptance of alternative approaches to skin sensitisation assessment of chemicals (see 7). Once finalised, all draft recommendations will follow the normal consultation process of EURL ECVAM's network of regulators (PARERE; see 5.1.1), its Stakeholder Forum (ESTAF; see 5.1.2) and the International Cooperation on Alternative Methods (ICATM; see 7).

5 Promoting the Regulatory Acceptance and use of Alternative Methods and Approaches

5.1 PARERE (Preliminary Assessment of Regulatory Relevance) and ESTAF (ECVAM Stakeholder Forum) Annual Meetings

EURL ECVAM organises annual meetings with the Preliminary Assessment for Regulatory Relevance network (PARERE) and the EURL ECVAM Stakeholder Forum (ESTAF). In 2015, the meetings took place on 20th and 21st October.

5.1.1 PARERE Meeting 2015

At this meeting, PARERE members gave an overview on the status of the establishment of the PARERE network in their respective countries, including the involvement of national bodies/agencies/experts, as well as a brief description of its functioning and difficulties encountered.

The PARERE consultation process on a test method for teratogenicity testing (devTOX^{qP}) was launched in 2014. EURL ECVAM presented the results of this consultation on the regulatory relevance of the method. The summary of the PARERE consultation has been finalised and transferred to the test submitter.

In relation to the priority needs identified by EURL ECVAM, discussions covered:

- Bioelution Test Method
- Reproductive and developmental toxicity

Participants provided their views on these points and were asked to continue these discussions within their networks to generate ideas for these areas.

5.1.2 PARERE ESTAF Joint Meeting 2015

The joint meeting of PARERE and ESTAF on 20th October focused on Integrated Approaches to Testing and Assessment (IATA), including a workshop. The aim of the workshop was to discuss key issues relating to the development, evaluation, acceptance and use of IATA with a view to identifying the pros and cons of different options for promoting the acceptance of IATA.

EURL ECVAM presented four case studies (serious eye damage/eye irritation, skin sensitisation, endocrine disruption and acute systemic toxicity) to demonstrate the concept of an IATA in relation to each area and to prepare the groups for the workshop. The meeting and workshop summary records have been published by EURL ECVAM[40].

5.2 Promoting the Regulatory Acceptance in the context of REACH

5.2.1 Update of REACH Annexes to Reflect Scientific Progress:

5.2.1.1 Skin Corrosion/Irritation and Serious Eye Damage/Eye Irritation

In June 2016, new amendments to the REACH Annexes VII and VIII regarding skin corrosion/irritation (point 8.1 of Annexes VII and VIII) and serious eye damage/eye irritation (point 8.2 of Annexes VII and VIII) entered into force (EC, 2016a), following the favourable vote in December 2015 of the REACH Committee on the proposal from the European Commission to change the standard information requirement under Annex VIII to *in vitro* studies. The driver of these amendments was the significant scientific progress made in recent years in the development, validation and regulatory acceptance of alternative test methods for the assessment of skin corrosion/irritation and serious eye damage/eye irritation.

The revised legal text points out that for skin corrosion/skin irritation, adequate information for the classification and risk assessment of a substance may be obtained in most cases solely on the basis of *in vitro* studies. A conclusion may be drawn on the basis of one *in vitro* test method, if the result allows an immediate decision on classification or non-classification, or from a combination of two *in vitro* test methods, one for skin irritation and one for skin corrosion. *In vivo* studies may still be required in some exceptional cases for substances manufactured or imported in quantities of 10 tonnes or more, e.g. when the substance tested falls outside the applicability domain of the *in vitro* test methods or when no conclusive results can be obtained from a comprehensive set of *in vitro* data. For serious eye damage/eye irritation, a set of *in vitro* test methods exists which would be sufficient in many cases to obtain information adequate for classification and risk assessment of substances. A conclusion about the potential of a substance to cause such eye effects may be drawn on the basis of one test method, if the result allows an immediate decision on classification or non-classification, or from a combination of two or more test methods. *In vivo* studies may still be required in some cases for substances manufactured or imported in quantities of 10 tonnes or more, e.g. when the substance tested falls outside the applicability domain of the test methods or when no conclusive results can be obtained from a comprehensive set of *in vitro* data.

In addition, the standard information requirements and adaptation rules in points 8.1, and 8.2 of Annex VII, and the adaptation rules in points 8.1 and 8.2 of Annex VIII were also revised in order to remove redundancies with rules set by Annex VI and Annex XI and in the introductory parts of Annexes VII and VIII as regards the review of available data, the waiving of studies for a toxicological endpoint if the available information indicates that the substance meets the criteria for classification for that toxicological endpoint, or to clarify the intended meaning as regards the waiving of studies for substances that are flammable under certain conditions.

5.2.1.2 Skin Sensitisation

The regulatory adoption by the OECD of the first three non-animal methods for skin sensitisation testing, the Direct Peptide Reactivity assay (DPRA; OECD, 2015a), the KeratinoSens™ (OECD, 2015b) and the human Cell Line Activation Test (h-CLAT; OECD, 2016c) addressing key mechanisms of the skin sensitisation pathway, was the driver of

[40] https://eurl-ecvam.jrc.ec.europa.eu/about-ecvam/scientific-advice-stakeholders-networks/parere

the revision of the REACH information requirements for the endpoint, laid down in Annex VII (≥1t/year) of the REACH regulation.

In April 2016, the REACH Committee voted in favour of a proposal from the European Commission to make the adopted *in chemico*/*in vitro* methods the default requirement (EC, 2016a). The revised provisions are unequivocal in asking that each of the following skin sensitisation key events should be addressed: 1) molecular interaction with skin proteins, 2) inflammatory responses in keratinocytes and 3) activation of dendritic cells. An *in vivo* study should only be conducted if the non-animal test methods are not applicable, or if the test results are not adequate for classification and risk assessment. In such cases, the murine local lymph node assay (LLNA) still needs to be used. The revised legal text also foresees that a substance predicted to have skin sensitisation hazard should be further evaluated to assess whether it can be presumed to have the potential to produce significant sensitisation in humans (Cat. 1A).

The revised legal text, accessible at: http://eur-lex.europa.eu/legal-content/FR/TXT/?qid=1475073772590&uri=CELEX:32016R1688, will entry into force in October 2016 and, therefore, will have important implications for the REACH 2018 registration deadline.

5.2.1.3 Acute Systemic Toxicity

In June 2016, the REACH amendment concerning acute dermal toxicity (point 8.5 of Annex VIII) has been published (EC, 2016b), following the favourable vote in December 2015 of the REACH Committee on the proposal from the European Commission to waive an acute dermal toxicity study for those substances which are non-toxic via the oral route. The driver of this revision was the existing evidence from several scientific analyses of available data indicating that substances demonstrated to be of low acute toxicity by the oral route are also of low toxicity by the dermal route and, therefore, that dermal testing for acute systemic toxicity of such substances adds nothing to the hazard characterisation (e.g. Creton *et al.,* 2010; Seidle *et al.,* 2011, Moore *et al.,* 2013).

The revised legal text makes clear that testing by the dermal route can be waived if the substance is not classified via the oral route (LD50 > 2000 mg/kg bw (body weight)/d) and no systemic effects have been observed in *in vivo* studies with dermal exposure or, in the absence of such *in vivo* studies, no systemic effects after dermal exposure are predicted on the basis of non-testing approaches.

The revised legal text is expected to entry into force in autumn 2016 and, therefore, will have important implications for the REACH 2018 registration deadline.

5.3 Update of REACH Guidance on Information Requirements and Chemicals Safety Assessment

5.3.1 Skin Sensitisation

Advances in the area and more importantly the revision of the legal text in Annex VII to the REACH Regulation (see 5.2.1.2) have also prompted an update of the European Chemicals Agency's (ECHA) guidance to industry on Information Requirements and Chemical Safety Assessment (Chapter R 7.a, section R.7.3 Skin sensitisation; ECHA, 2016a). The revised ECHA draft guidance provides a description of the scope and limitations of the adopted alternative methods. Since the new *in chemico*/*in vitro* test methods should not be used as stand-alone methods due to test method specific limitations, a combination of these methods should be used. Additional information obtained from other approaches such as (Q)SARs or read-across may assist in making an adequate conclusion that is suitable for classification and risk assessment.

With a view to assist registrants fulfilling the information requirements under REACH the revised guidance proposes a testing and assessment strategy which comprises three

parts. Part 1 is about retrieving existing information, Part 2 consists of the Weight-of-Evidence (WoE) assessment and Part 3 is about the generation of new information by testing if needed. The strategy aims to help registrants to use information from *in chemico*/*in vitro* methods for skin sensitisation according to Annex VII, section 8.3.1, or in a WoE approach according to sections 1.2-1.5 of Annex XI on "General Rules for Adaptation of the Standard Testing Regime set out in Annexes VII to X".

Although the guidance recommends the testing and assessment strategy to be followed, it acknowledges that other approaches may be more appropriate depending on the specific case.

5.3.2 Acute Systemic Toxicity

ECHA is currently undergoing the process of updating section R.7.4 (acute toxicity) of the Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7a: Endpoint specific guidance[41].

EURL ECVAM has provided ECHA with strategy documents and data analyses to support the updating of the ECHA guidance document. In this regard, EURL ECVAM conducted in 2015 an online survey aimed at experts in the field of acute systemic toxicity testing to explore potential waiving opportunities for acute oral toxicity tests. The findings from this survey were supported by a retrospective data analysis of REACH-registered chemicals that explored whether it is possible to predict the outcome of an acute oral toxicity study (i.e. lethality) from the outcome of a 28-day repeated dose toxicity study. The JRC analysis and the outcome of the survey have been published (Graepel *et al.,* 2016).

Moreover, EURL ECVAM was invited by ECHA and nominated by JRC to participate in a Partner Expert Group (PEG) responsible for reviewing and commenting on ECHA's proposed updates to this section of the Guidance. The written consultation of the PEG was initiated in October 2015. ECHA received a total of 346 comments of which 59 were provided by EURL ECVAM. Forty nine of the 346 comments were prioritised by ECHA for discussion during the meeting. The great majority of EURL ECVAM's comments were accepted by the PEG members and ECHA. These were aimed mostly to making sure that 1) a proper reference is made in the updated guidance to the available scientific analysis of data that showed the predictive performance of several QSAR tools and the *in vitro* 3T3 NRU test method; 2) the Guidance text is aligned with the revised legal text of the REACH Annex VIII section 8.5.3. (i.e. waiving a dermal toxicity study if no acute oral toxicity is observed up to the limit dose of 2000 mg/kg b.w.) once it is published; 3) the update of the Guidance reflects the current status of development of the different new or revised EU Test Methods and/or OECD Test Guidelines for acute inhalation and dermal toxicity; 4) the dose range finding studies described under the WoE adaptation approach are revised to clarify whether all of them are needed and/or recommended and the real impact on animal testing; 5) the acute inhalation OECD TGs which use fewer animals are included in the figures of the acute testing strategy.

The PEG meeting took place at ECHA in December 2015 to discuss the comments received from the PEG members and to further consolidate the updated guidance. In May 2016 the consolidated version, which already considered the revised REACH Annex VIII voted by the REACH committee in December 2015, was sent to PEG members for final cross-check with the possibility to make additional comments. The new draft version was subsequently submitted to ECHA's Member State Committee and the Committee for Risk Assessment and no comments were received.

A final consultation with the Competent Authorities for REACH and CLP (CARACAL) was initiated in July 2016 (21st CARACAL meeting) and no comments were received by the

[41] https://echa.europa.eu/support/guidance/consultation-procedure/ongoing-reach

deadline of 16 September 2016. The target date for publication of this guidance update is foreseen in October 2016 on the ECHA website.

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 have been 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 vaccines was adopted in 2013 and is in force since 1st March 2014 (VICH, 2013). The comparable VICH GL55 for live veterinary vaccines and the revised VICH GL50 underwent public consultation in 2016 and are currently being finalised for adoption by the VICH Steering Committee.

A third guideline is under development aiming at the harmonisation of criteria to waive the general batch safety test in laboratory animals (e.g. abnormal toxicity test).

5.5 Activities in the OECD Task Force on Hazard Assessment

5.5.1 Update on Guidance Documents on Defined Approaches for Skin Sensitisation

EURL ECVAM led on behalf of the European Commission an OECD project aimed at the development of guidance documents on the reporting of Defined Approaches (DA). A defined approach to testing and assessment consists of a fixed data interpretation procedure (DIP) applied to data generated with a defined set of information sources to derive a result that can either be used on its own, or together with other information sources within an IATA, to satisfy a specific regulatory need. The concept of DIP, taken from OECD guidance document 34 (OECD, 2005), is defined as any algorithm for interpreting data from one or more information sources. The output of a DIP is typically a prediction (e.g. prediction of skin sensitisation potential from peptide binding data and/or chemical structure). Thus, a DA in contrast to the assessment process within IATA does not imply expert judgment in deriving the final prediction/assessment.

DA should be documented to the extent possible to facilitate their evaluation, or the evaluation of IATA in which they are used as one of the components, in regulatory decision-making.

In June 2016 the OECD Task Force on Hazard Assessment (TFHA) endorsed two GD on the reporting of DA. The first GD (OECD, 2016a) provides a set of principles for the reporting of DA. To facilitate its regulatory use a DA should be associated with the following set of information: 1) a defined endpoint, 2) a defined purpose, 3) a description of the underlying rationale, 4) a description of the individual information sources used within, 5) a description of how data from the individual information sources are processed and 6) a consideration of the known uncertainties associated with the application of the DA. The GD also provides reporting templates organised on the basis of these principles to enable the structured documentation of DA and the individual information sources. Beside other elements, emphasis is put in the templates on the proper reporting of the limitations in the application of the DA, of its predictive performance and of the sources of uncertainties that may impact on the final prediction. These templates should be used alongside the reporting formats for other IATA

components, such as QSARs (OECD, 2007a), grouping and read-across strategies (OECD, 2014a) and non-guideline test methods (OECD, 2014b).

The second GD (OECD, 2016b) exemplifies how the reporting templates have been used to document a number of DA developed in the area of skin sensitisation. These approaches are based on the use of information sources addressing key mechanisms/events of the skin sensitisation AOP and make use of a variety of specific methodologies, i.e. DIP, for converting the input data/parameters into a final prediction. The case studies provide a good overview of the different set of information sources and DIP that can be used within DA. The DIP can range from very simple rule-based sequential decision steps to mathematical and statistical approaches. It is not the intent of this document to seek for endorsement of any specific defined approach provided in the case studies, but rather provide a perspective of how individual information sources and defined approaches developed for skin sensitisation assessment should be reported.

Although there is not yet experience with the use of the developed templates for reporting DA in other areas of toxicology, it is envisaged that their application is not limited to the reporting of DA for skin sensitisation.

5.5.2 IATA Case Studies Project including the JRC-CosEU ab initio Case Study

The IATA Case Studies Project under the OECD TFHA was set up to support efforts of the OECD member countries to increase the use of alternative methods within Integrated Approaches for Testing and Assessment (IATA), in particular by developing respective guidance and tools. The project is investigating the practical applicability of IATA by discussing case studies, based on a draft template [42], and by summarising the considerations to create a common understanding of using the approaches. The first cycle in 2015 comprised four case studies focused on grouping and read-across for different hazard endpoints [43] (OECD, 2016d-g), including discussions on the need for specific guidance (OECD 2016h). In the 2016 cycle, five case studies [44] have been reviewed, including not only grouping approaches, but also two studies based on initial SEURAT-1 read-across work submitted by ICAPO, as well as the JRC/BIAC submission of the chemical safety assessment workflow based on exposure considerations and focusing on non-animal methods, developed from the SEURAT-1 cross-cluster *ab initio* case study (Richarz *et al.,* 2016). The workflow attempts to structure knowledge and data in a logical sequence for an integrated chemical safety assessment. It includes considerations of Threshold of Toxicological Concern (TTC), read-across approaches and application of physiologically-based kinetic (PBK) modelling to identify target organs and internal concentrations. *In silico* profilers and *in vitro* data generation contribute to building a weight of evidence, based on an AOP-anchored mode-of-action hypothesis, supported by *in vitro* to *in vivo* extrapolation (IVIVE) modelling and refinement. The case study highlights the challenge in integrating multiple data streams for safety assessment decisions.

[42] Developed based on OECD (2014c) and OECD (2014d)

[43] *In vitro* mutagenicity of 3,3' dimethoxybenzidine (DMOB) based direct dyes (Canada, United States), repeated-dose toxicity of substituted diphenylamines (SDPA) (Canada), hepatotoxicity of allyl esters category (Japan), bioaccumulation potential of degradation products of 4,4'-bis (chloromethyl)-1,1'-biphenyl (Japan)

[44] Structure related repeated-dose toxicity profiles assessment by using toxico-genomics data (Japan), pesticide cumulative risk assessment (United States), read-across of 90-day rat oral repeated-dose toxicity for selected n-alkanols and 2-alkyl-1-alkanols (ICAPO), chemical safety assessment workflow based on exposure considerations and non-animal methods (JRC/BIAC)

5.5.3 Combined Exposure to Chemical Mixtures

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 (a) the compositions are various and complex, (b) 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.

This gap in the EU regulatory assessment framework has recently gained more attention, following a 2012 Commission Communication on the Combined Effects of Chemicals (EC, 2012). The communication required further work to be accomplished in order to reach a consistent approach to the assessment of priority mixtures across the different relevant pieces of EU legislation.

After having reviewed the regulatory requirements, available guidance and approaches for the risk assessment of chemical mixtures (Kienzler *et al.,* 2016b; Kienzler *et al.,* 2014), EURL ECVAM investigated the applicability of novel, non-animal tools and scientific methodologies in the assessment of combined effects of chemicals on humans and the environment. These tools and methods allow meaningful information on individual mixture components or whole mixtures to be derived. Since it is practically impossible to test all possible chemical mixtures experimentally, the application of smart strategies using new, alternative tools that rely less on *in vivo* testing and incorporate instead alternative experimental and computational tools is of particular importance in the context of chemical mixtures. EURL ECVAM has conducted a review of recent literature and has surveyed the experience of experts on the different approaches, such as the adverse outcome pathway (AOP) concept, *in vitro* methods, omics techniques, *in silico* approaches such as quantitative structure activity relationships (QSARs) and read-across, toxicokinetic and dynamic energy budget (DEB) modelling, and on integrated approaches to testing and assessment (IATA) (Bopp *et al.,* 2015). These can help achieve a more effective regulatory assessment and at the same time reduce the reliance on animal testing. Their main strengths lie in their integrated use and putting into context different aspects of the hazard from combined exposure to multiple chemicals. But in order to benefit from these tools in the hazard assessment of mixtures, more guidance on their use is needed to facilitate a more widespread application.

In order to gain further insight into the issues linked to the risk assessment of chemical mixtures, EURL ECVAM further reviewed relevant case studies from the peer-reviewed literature (Bopp *et al.,* 2016). Of the 21 recent case studies identified, some show a potential concern for several groups of chemicals for highly exposed or particularly vulnerable population groups. 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 were 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. Future case studies on mixture risk assessment could fill the knowledge gaps identified. Such case studies could help by addressing differences between population groups, investigating different and emerging groups of substances, considering the relevance of interactions (i.e. synergisms), examining different approaches for the grouping of chemicals, and especially by investigating mixtures of potential concern that cross regulatory sectors.

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 Task Force on Hazard Assessment in collaboration with the Task Force on Exposure Assessment) and supports the development of consistent assessment approaches for combined exposure to chemical mixtures at international level.

5.6 Activities in the OECD Test Guideline Programme

At the 28th meeting of the Working Group of National Coordinators of the OECD Test Guideline Programme (TGP) held at the OECD headquarters in Paris on 19th to 22nd April 2016, 13 Test Guidelines were approved of which 4 were new TG and 9 were updated TG. Four TG out of the 13 were on *in vitro* methods, i.e. the *in vitro* skin sensitisation human Cell Line Activation Test (hCLAT); a Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity; update of TG 431 for skin corrosion *in vitro;* update of Performance-Based Test Guideline 455 for Stably Transfected Transactivation *In Vitro* Assays to Detect Estrogen Receptor Agonists and Antagonists to include the ER-CALUX test method. In addition TG 490 (approved in 2015) on *In Vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene for genetic toxicity testing was corrected.

Seven Guidance documents or supporting documents were approved as well.

The WNT agreed the new work plan that includes now twelve new projects, eight of which of relevance to the field of alternative methods/approaches.

More information on the OECD TG programme can be found on the OECD website of the Test Guideline Programme[45].

The following chapters mainly focus on TGs for which the EC (through JRC-EURL ECVAM) had the lead or co-lead or carried out validation studies. 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.

5.6.1 Development of an OECD Test Guideline on *in vitro* Fish Hepatic Metabolism

The OECD project on the development of a new OECD TG on *In vitro* Fish Hepatic Metabolism (under the lead of USA and the EC represented by JRC - EURL ECVAM) aims at standardising two *in vitro* methods using 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

[45] http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicalsandrelateddocuments.htm

the two *in vitro* methods (2014-2016). Based on the results of the ring trial two draft OECD TGs are in preparation and it is planned to start consultation with WNT experts in 2017.

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; (Nichols *et al.,* 2013; Laue *et al.,* 2014)). 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.

5.6.2 Update of OECD Guidance Document 23 on Aquatic Toxicity Testing of Difficult Substances and Mixtures

This project initiated by the International Council on Animal Protection in OECD Programmes (ICAPO) and co-led by the EC- EURL ECVAM) addresses the use of solvents in aquatic toxicity tests on fish. 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 aims at updating 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 GD23 (under the lead of USA) in view of the many comments received on all sections of the document during the 1st WNT commenting round.

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. The project was approved in 2015 and included in the OECD work plan.

5.6.3 Revision of OECD Guidance Document 126

The project aims at updating OECD GD126, the threshold approach for acute fish toxicity (OECD, 2010), and integrate the fish embryo acute toxicity test (OECD TG 236, 2013b) into the step-wise approach for determining acute fish toxicity data. The project started in 2015 under the lead of Austria and ICAPO.

5.6.4 Revision of OECD Test Guideline 203

OECD TG203 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.

5.6.5 Critical Assessment of Technical Requirements in OECD Test Guidelines using Vertebrate Non-mammalian Species

This new OECD project (led by UK) focuses on test guidelines using vertebrate (non-mammalian) species (e.g. fish, amphibian, birds) for ecotoxicity testing and follows up on a recommendation of the OECD Fish toxicity testing framework (OECD, 2012a). It will investigate whether specified deviations from the technical requirements laid down in the TGs may have an impact on the outcome of the individual studies. Reducing the uncertainty of the potential impact on study outcomes may avoid unnecessary repetition of studies and animal use, respectively.

5.6.6 OECD Validation Studies with Transgenic Fish and Amphibian Models to Identify Endocrine Disruptors

Within the OECD test guideline programme, France is leading two projects aiming at the validation of aquatic transgenic models, i.e. the EASZY and XETA assays. The EASZY assay is based on the use of transgenic cyp19a1b-GFP zebrafish embryos for detection of endocrine active substances acting through the estrogen pathway (Brion *et al.,* 2012).

The XETA (Xenopus Embryonic Thyroid Signalling Assay) allows the identification of endocrine active substances acting through the thyroid pathway. It uses transgenic ThβZIP-GFP Xenopus embryos where the GFP expression correlates with the level of expression of the ThβZIP gene, a direct thyroid hormone response gene and well characterised transcription factor involved in the control of amphibian metamorphosis (Fini *et al.,* 2007).

Both models have metabolic capacity and therefore have the potential of identifying substances requiring metabolic activation.

5.6.7 OECD Test Guideline on the Human Cell Line Activation Test (h-CLAT)

The h-CLAT Test Guideline was approved by the WNT at its April meeting in 2016. The h-CLAT method is proposed to address the third key event of the skin sensitisation AOP by quantifying changes in the expression of cell surface markers associated with the process of activation of monocytes and dendritic cells (i.e. cell surface markers CD86 and CD54), in the human monocytic leukaemia cell line THP-1, following exposure to sensitisers (Ashikaga *et al.,* 2006). The measured expression levels of CD86 and CD54 cell surface markers are then used for supporting the discrimination between skin sensitisers and non-sensitisers.

The h-CLAT method has been evaluated in an EURL ECVAM coordinated validation study and subsequent independent peer review by the EURL ECVAM Scientific Advisory Committee (ESAC). Considering all available evidence and input from regulators and stakeholders, the h-CLAT was recommended by EURL ECVAM (EURL ECVAM, 2015) 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.

5.6.8 Performance Based Test Guideline on CYP Induction assays

The human cytochrome P450 (CYP) activity n-fold induction *in vitro* test method which describes the methodology to assess the potential of test chemicals to induce three cytochrome P450 (CYP) enzyme activities (CYP1A2, CYP2B6, and CYP3A subfamily) in two human-derived metabolically competent hepatic *in vitro* test systems has been successfully validated and peer reviewed (see EURL ECVAM status reports 2014 and 2015, Zuang *et al.,* 2014 and 2015). The two test systems are: the cryopreserved human primary hepatocytes (cryohep) and the cryopreserved human HepaRG™ (cryoHepaRG™). An OECD Expert Group on Biotransformation Assays had been convened in May 2015 where the experts recognised the importance of developing *in vitro* metabolism OECD TGs based on CYP induction which could be used when establishing the hazard of both individual chemicals and mixtures. An OECD TG that would enable the induction to be determined *in vitro* using human hepatocytes was considered of benefit to all classes of chemicals.

Additional discussions revealed however, that, while for the development of drugs targeted to humans, the use of three different donors of cryohep was the gold standard within the pharmaceutical regulations, for chemicals screening on the other hand, a higher throughput assay showing more experiment to experiment consistency, would be more valuable. Based on the validation data, the cryoHepaRG™ is less variable.

The expert group indeed considered the cryoHepaRG™ model more consistent and possible to be tailored for chemical screening needs (rather than drug design and drug metabolism).

Considering the urgent need for TGs on *in vitro* metabolism, in terms of time frame for further evaluation of this methodology, the expert group considered that a PBTG was not appropriate at this point in time and suggested the development of a TG at short-term, ultimately a PBTG at medium-term, while keeping in mind AOP/IATA development at long-term. The expert group very much supported a TG on the cryoHepaRG™ model. Some experts offered to search for additional data on industrial chemicals generated with this test and also suggested to involve the WNT in this endeavour. At medium-stage a PBTG and an explanatory background review document (BRD) explaining the context of use of this type of PBTG could be prepared. Such a background document should address in particular the use purpose of a PBTG on CYP induction in a regulatory hazard characterisation and risk assessment context to generate wider understanding and appreciation of the value of information on the CYP inducing properties of a chemical.

5.6.9 Integrated Approaches to Testing and Assessment of Non-genotoxic Carcinogens

Non-genotoxic carcinogens contribute to an increased cancer risk by a variety of mechanisms that are not yet included in international regulatory approaches. To address this need, an integrated approach to testing and assessment (IATA) of non-genotoxic carcinogens is beginning to be developed internationally under the auspices of the OECD. An expert working group has in fact been set up and has met for the first time in March 2016 to examine the current international regulatory requirements and their limitations in respect to non-genotoxic carcinogenicity, and how an IATA could be developed to assist regulators in their assessment of non-genotoxic carcinogenicity. Moreover, the working group is tasked to review, describe and assess relevant *in vitro* assays with the aim of tentatively organising them into levels of testing, following the adverse outcome pathway format, such as that possible structure(s) of the future IATA(s) can be created. Some preliminary work to this activity has already been described in a publication of Jacobs and colleagues (Jacobs, 2016).

5.6.10 Integrated Approaches to Testing and Assessment on Eye Irritation/Serious Eye Damage

The assessment of the serious eye damage/eye irritation endpoint 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. Since 2002, the OECD TG 405 on *in vivo* "acute eye irritation/corrosion" contains a supplement describing a sequential testing and evaluation strategy. This strategy recommends that, prior to undertaking the described *in vivo* test, a weight-of-evidence analysis be performed on all existing relevant data. Where insufficient data are 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 the 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 (i.e., BCOP, ICE, FL, EpiOcular™ EIT and STE), they may in many cases satisfy all information requirements for serious eye damage/eye irritation. In addition, non-standards methods (i.e., 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 requires an update. The EC represented by JRC-EURL ECVAM and the US EPA are thus jointly leading the development of a Guidance Document on an IATA for serious eye damage/eye irritation at the OECD. The objective is 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 proposed by the EC and the U.S. EPA was approved by the WNT in April 2015 and a first draft of the Guidance Document was circulated to the WNT for commenting on the 18th of March 2016. An amended version of the document addressing the WNT comments will be discussed by the OECD Expert Group on eye irritation during a meeting at the OECD headquarters on 3-4 November 2016 and will be recirculate to the WNT before the end of the year for another commenting round. The final document is expected to be submitted for approval by the WNT in April 2017.

5.6.11 Draft 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 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. Such a GD aims at reducing the uncertainties in cell and tissue-based *in vitro* method derived predictions by applying all necessary good scientific, technical and quality practices from *in vitro* method development to *in vitro* method implementation for regulatory use. The draft GIVIMP guidance is coordinated by EURL ECVAM and it was accepted on the work plan of the OECD test guideline programme in April 2015 as a joint activity between the Working Group on Good Laboratory Practice and the Working Group of the National Coordinators of the Test Guideline Programme (WNT). During the first drafting stage, expert input was received from European regulatory agencies [i.e. 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, Three Rs 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 draft document was sent to the members of the EU-NETVAL (37 laboratories across Europe), to the experts of the Working Group on Good Laboratory Practice and the WNT in September and October 2016, respectively.

The Guidance aims to further facilitate the application of the OECD Mutual Acceptance of Data agreement for data generated by *in vitro* methods and as such contribute to avoidance of unnecessary additional testing. It describes the areas related to *in vitro* method development, standardisation, harmonisation and international acceptance that would benefit from more detailed scientific, technical and quality guidance.

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. The document has been discussed at the EU NETVAL meeting on 10th October 2016.

5.6.12 OECD Guidance Document on Considerations for Waiving or Bridging of Mammalian Acute Toxicity Tests

In November 2014 an OECD expert group on acute mammalian toxicity was established and EURL ECVAM was nominated as member of this group. The OECD project, that was included in the OECD Test Guidelines programme in 2014, was initiated by USA and Canada and it was based on guidance developed for pesticides (U.S. EPA 2012, Health Canada 2013).

The first draft of the guidance was circulated for review late in 2014 and based on the feedback received a revised draft was circulated to the expert group in May 2015 for another review/comment round. Two conference call meetings were then organised by the OECD Secretariat with the Expert group and lead countries to address and discuss the 55 comments received and the responses drafted by the lead countries. Most of EURL ECVAM's comments were aimed at clarifying 1) when a particular *in vivo* study could be waived based on skin corrosivity; 2) when the waiver of an acute oral toxicity test can be considered if the oral LD_{50} of the test material is predicted to be greater than 2000 mg/kg bw using alternative methods; 3) that the titles and text of the sections on dermal and eye irritation are aligned with the relevant ECHA guidance documents.

The guidance document introduces several considerations where a mammalian acute toxicity study may be waived. The criteria outlined in the document are applicable to mammalian acute toxicity via the oral, dermal and inhalation route, eye and skin irritation and skin sensitisation. Although the document is intended for the assessment of pesticides, the principles included can be extended to the assessment of other chemicals, formulations and biological materials. The disclaimer included in the guidance document encourage regulatory jurisdictions to the use of the approaches outlined herein as part of the weight of evidence in determining the need for a mammalian acute toxicity study and appropriate classification and/or labelling.

The Guidance document that was approved at the 28th meeting of the Working Group of the National Coordinators for the Test Guidelines Programme, has been recently published (OECD, 2016i) after being declassified by the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology in July 2016.

5.6.13 Proposal for the Deletion of Test Guideline on the Rodent Dominant Lethal Test (TG 478)

A revised TG 478 on Rodent Dominant Lethal Test was adopted in July 2015. Although it has been recognised that the revised TG had undergone significant improvements, several factors pointed to the potential merit of deleting this TG in the near future. These factors include: scientific limitations concerning the limited specificity and sensitivity of the test; the use of a huge number of animals; the fact that the test is labour-intensive and costly; the lack of laboratories that have experience in the conduct of this test; the availability of other tests to assess the same endpoint (i.e. germ cell genotoxicity). The test is rarely performed around the world and this is reflected by the fact that the references reported in TG 478 are from mid-70s to mid-90s, indicating the lack of recent reliable data. Literature search showed only a few more recent research publications of rather questionable quality. In addition, TG 478 already underlined that "due to its limitations and the use of a large number of animals, this assay is not intended for use as a primary method, but rather as a supplemental test method which can only be used when there is no alternative for regulatory requirements." Moreover, the summary record of the 27th WNT meeting stated "When timely for countries to re-open the discussion of the deletion of TG 478, a proposal via a Standard Project Submission Form (SPSF) could be submitted via a National Coordinator."

For the reasons listed above and after consultation with European Agencies (i.e. EMA, ECHA and EFSA), the EC considered that it was timely to submit an SPSF to propose TG

478 deletion. Although most of the EU Member States were in favour, no consensus was reached for its deletion at the 28th WNT meeting in 2016.

5.6.14 Workshop on Developmental Neurotoxicity

The initiative to organise a stakeholder workshop (18-19 October 2016) on an integrated testing strategy for developmental neurotoxicity (DNT) has been undertaken in collaboration with the EFSA secretariat, the OECD secretariat and the JRC/EURL ECVAM. The workshop's intended objective is to develop consensus on which testing battery of alternative DNT methods could be applied right now in a fit-for-purpose manner for chemical screening for prioritisation, or hazard identification for specific chemical risk assessment and which could lead to the development of IATA (Integrated Approaches to Testing and Assessment).

The programme of the workshop covers the perspectives of regulatory bodies, industry and academia on DNT testing strategies based on alternative approaches. During the workshop the JRC/EURL ECVAM will present its strategic aims, addressing the utility of non-animal approaches applied to the assessment of DNT for different regulatory purposes.

The intention of EFSA is to follow up the event with the initiation of an EFSA working group for the preparation of a scientific opinion on an integrated testing strategy for the identification and evaluation of hazards associated with DNT, for substances such as pesticide active substances and their metabolites. The publication of a report presenting the main outcomes of the workshop is envisaged.

5.7 Contributions to the OECD Adverse Outcome Pathway Development Programme

An Adverse Outcome Pathway (AOP) 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. AOPs are the central element of a toxicological knowledge framework, promoted by member countries through OECD, built to support chemical risk assessment based on mechanistic reasoning.

The EC (through JRC/EURL ECVAM) has shown commitment to the goals of the OECD programme on Adverse Outcome Pathways in many ways, including co-chairing with US EPA the Expert Advisory Group on Molecular Screening and Toxicogenomics (EAGMST) tasked with implementing the programme, contributing to the development of guidance on principles and best practices of AOP development, working in close collaboration with US EPA, US Army Corps of Engineers – Engineering, Research and Development Center (ERDC) and OECD on the development of the AOP Knowledge Base and its various components (see 6.4) developing and delivering training courses (see 6.5) and, not least, the actual development of a number of AOPs including entering them into the AOP Wiki. The first AOPs to go through the process of internal and external peer review within the OECD were finalised and published on the OECD website in August 2016[46]. Of these first five AOPs, three were developed by EURL ECVAM.

These were:

- Protein Alkylation leading to Liver Fibrosis (Landesmann B, 2016) [47]
- Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment[48]

[46] http://www.oecd-ilibrary.org/environment/oecd-series-on-adverse-outcome-pathways_2415170x;jsessionid=20oo8q54271qt.x-oecd-live-03
[47] https://aopwiki.org/wiki/index.php/Aop:38

- Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities[49]

In addition EURL ECVAM also introduced the OECD AOP for skin sensitisation, which had been drafted and agreed before the creation of the AOP Wiki, into the Wiki:

- Covalent Protein binding leading to Skin Sensitisation[50]

A number of other AOPs authored by EURL ECVAM are in different stages of development and review as follows:-

- Binding of inhibitor to the Complex I of mitochondrial ETC leads to motor deficit of Parkinson's disease (in collaboration with EFSA)
- PPARα activation leading to impaired fertility in adult males
- PPARγ activation leading to impaired fertility in adult females
- Sodium Iodide Symporter (NIS) inhibition and subsequent adverse neurodevelopmental outcomes in mammals
- Lysosomal damage (caused by nanoparticles) leading to liver inflammation

EURL ECVAM has undertaken the first steps in applying the AOP framework to neurodevelopmental toxicity evaluation (Bal-Price *et al.,* 2016) and identified potential AOPs relevant to both developmental and adult neurotoxic outcomes (Bal-Price *et al.,* 2015; Bal-Price *et al.,* 2016).

The AOPs elaborated to date are mainly qualitative pathway descriptions, and further research efforts are necessary to develop a more-precise characterisation of the dynamic relationships between key events in quantitative terms. In an effort to move forward on this aspect, EURL ECVAM hosted a workshop in September 2015 on *AOP-Informed Predictive Modelling Approaches for Regulatory Toxicology* to discuss how the systematic organisation of knowledge into AOP frameworks can inform and help direct the design and development of computational prediction models that can further enhance the utility of mechanistic and *in silico* data for chemical safety assessment. The workshop report (in press) lays out a vision for how AOPs might be used to facilitate development of computational prediction models needed to support the next generation of chemical safety assessment.

Even though the level of information currently available is not sufficient to perform a comprehensive risk assessment, a well-described AOP may still provide useful information for many purposes, such as priority setting for further testing, hazard identification, read-across for categorisation of chemicals or contributing to the development of integrated approaches to testing and assessment (IATA). It is anticipated that all the work on AOPs will help towards refinement, reduction and ultimately replacement of conventional *in vivo* animal testing.

[48] https://aopwiki.org/wiki/index.php/Aop:48
[49] https://aopwiki.org/wiki/index.php/Aop:13
[50] https://aopwiki.org/wiki/index.php/Aop:40

5.8 Promoting Regulatory Acceptance in the Frame of EMA: JEG 3Rs

The European Medicines Agency (EMA) decided in 2010 to establish an expert group, JEG 3Rs[51], which should provide advice and recommendations to the Committee for Medicinal Products for Veterinary Use (CVMP) and Committee for Medicinal Products for Human Use (CHMP) on all matters relating to the use of animals and the application of the Three Rs in the testing of medicines for regulatory purposes. Members of the JEG 3Rs are European experts of the CVMP and CHMP working parties for which animal testing is relevant, other named Three Rs experts, and representatives from EDQM and the European Commission (e.g. EURL ECVAM). JEG 3Rs recently published a draft *Reflection paper providing an overview of the current regulatory testing requirements for veterinary medicinal products and opportunities for implementation of the 3Rs*[52] and a comparable document is in preparation for human pharmaceuticals. Further ongoing work relates to compliance checking of EMA guidelines with Three Rs principles and proposals for revision, the development of guidance on the acceptance of Three Rs testing approaches[53] in pharmaceuticals testing; Three Rs issues related to batch release testing of vaccines as well as guidance on the use of transferring quality control methods validated in collaborative trials to a product/laboratory specific context[54].

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

The partnership runs a number of projects and organises or financially supports workshops and conferences which aim at promoting the regulatory acceptance of alternative methods and approaches.

5.9.1 Waiving of Two-year Carcinogenicity Studies

The University of Wageningen has collaborated with the Dutch Medicines Evaluation Board to compile and analyse 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-years carcinogenicity studies based on *in vitro* genotoxicity testing and the results of (sub-)chronic toxicity studies. Moreover, the combination of the histopathological approach as proposed by Sistare *et al.,* 2011 with the pharmacological approach as presented in a recent paper of van der Laan *et al.,* 2016 is investigated. A manuscript describing the outcome of this project has been submitted.

5.9.2 Acute toxicity

A data mining exercise was initiated in 2015 in close collaboration with the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs)

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[51] JEG 3Rs = The Joint Committee for Medicinal Products for Veterinary Use/Committee for Medicinal Products for Human Use Ad-hoc Expert Group on the Application of the 3Rs in Regulatory Testing of Medicinal Products http://www.ema.europa.eu/ema/index.jsp?curl=pages/contacts/CVMP/people_listing_000094.jsp&mid=WC0b01ac05803a9d6d

[52] http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/04/WC500205609.pdf

[53] http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2014/10/WC500174977.pdf

[54] http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/07/WC500211432.pdf

and the UK Chemicals Regulation Directorate (CRD) to enable the identification of clinical signs predictive of mortality (EPAA, 2015). The whole exercise was delayed due to the large number of studies that need to be screened, which was larger than originally expected. Nevertheless, the team is currently finalising the screening, data copying and data coding of a large enough number of studies to allow a robust statistical analysis of data to identify predictive clinical signs of mortality.

The ultimate aim is to build a decision framework document 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.

Discussions were held between members of the EPAA project and ECHA to ensure that the outcomes of this scientific data analysis were relevant in the context of the update of ECHA guidance on acute toxicity and the 2018 REACH registration deadline.

5.9.3 The Vaccines Consistency Approach

In order to facilitate the introduction of the consistency approach for the quality control of established human and veterinary vaccines, EPAA has initiated a project aimed at developing and validating non-animal methods with the support of stakeholders from academia, regulators, Official Medicines Control Laboratories (OMCLs), EDQM, European Commission and vaccine manufacturers. A recent publication summarises the work carried out within the four priority vaccines/vaccine groups (diphtheria/tetanus/acellular pertussis vaccines; human rabies vaccines; veterinary rabies vaccines; clostridial vaccines) defined at the start of the project in 2011 (De Mattia *et al.,* 2011).

EPAA decided in 2016 to close the overarching Vaccines Consistency Approach project and continue the two sub-projects on clostridial vaccines (see 5.9.4) and human rabies vaccines (see 5.9.5) as individual projects. The work on implementation of the consistency approach, in general, and in particular for diphtheria, tetanus, acellular pertussis vaccines and veterinary rabies vaccines will be continued in the VAC2VAC research project launched in March 2016 (see 2.6.1).

5.9.4 Clostridial vaccine project

The 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 satellite workshop on 15-16 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 (Sinitskaya *et al.,* 2016). However, in order to fully exploit the advantage of the Vero cell assays, further work is needed. A follow-up study started in 2016 aiming at the further optimisation of the Vero cell assays to increase their sensitivity and accuracy.

Adaptations of the Vero cell assays to other clostridial vaccines will be evaluated in the VAC2VAC project.

5.9.5 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 have been discussed at a workshop in May 2015 (a paper is in preparation). One out of the three ELISAs correctly quantified the antigen content of all vaccine samples (including degraded samples). After additional work carried out

during 2016, this ELISA was presented to and accepted by the EDQM BSP for full validation.

5.9.6 Harmonisation on Biologicals

The 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.

During 2013-2014, key requirements and differences in the various regions were mapped and possible areas for harmonisation defined. As a follow-up, EPAA convened an international workshop (15-16 September 2015) with representatives from regulatory bodies and manufacturers to discuss steps towards deletion of general safety tests and to identify means towards implementation of *in vitro* methods for potency testing of human and veterinary vaccines. The workshop report *Modern science for better quality control of medicinal products: Towards global harmonisation of 3Rs in biologicals*[55] is available on the EPAA website[56]. 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.

5.9.7 Optimised Evaluation of Skin Sensitisation

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. The ultimate purpose of this project is to explore whether methods using reconstituted human epidermis as a model system can overcome some of the limitations of the OECD adopted *in chemico* / *in vitro* tests. Such limitations are mainly related to difficulties in testing highly hydrophobic molecules including mixtures and to accurately predict potency, potential reasons being the fact that application to cells cultured in an aqueous-based medium differs from topical application to the skin as is the case in the clinical situation or animal test, and differences in metabolic profiles of cultured cells versus *in vivo* cells.

5.10 ILSI HESI activities: Framework for Intelligent Non-Animal Alternative Methods for Safety Assessment

The Health and Environmental Sciences Institute (ILSI HESI) has created a multi-sector forum to develop criteria to develop confidence in the use and acceptance of non-animal methods to support regulatory decisions. The objective of the group is to determine criteria to be used in assessing fitness-for-purpose methods and approaches for decision-making, provide general guidance for establishing sufficient confidence in non-animal methods and ensure peer engagement and transparency to assess the acceptability of the group's proposals. Three working groups have been established covering the following issues: 1) performance characterisation; 2) model predictive performance; and 3) utilisation. The final outcome of this activity will be the development of a white paper/publication that reflects the consensus of the group.

[55] https://circabc.europa.eu/sd/a/4a081e45-f19f-47f7-8d8d-65f4f10fccff/ihb%20sept%202015%20report.pdf
[56] http://ec.europa.eu/growth/sectors/chemicals/epaa/

5.11 Standard documentation of exposure models – MERLIN-Expo case study CEN Workshop Agreement

An important step in facilitating the acceptance and use of a computational model is the development of comprehensive and structured documentation. This is particularly challenging for environmental and/or human exposure models that aim to establish quantitative relationships between personal exposure levels and their determinants. Exposure models conceptually simulate the transport and fate of a contaminant from the source to the receptor and may then involve a large set of entities (e.g. all the environmental media the contaminants may pass through). Such complex models are difficult to describe in a comprehensive, unambiguous and accessible way. To address this difficulty, EURL ECVAM participated in a CEN (European Committee for Standardisation) initiative[57,58] that aimed to agree on minimum requirements for the amount and type of information to be provided on exposure models documentation along with guidelines for the structure and presentation of the information (Altenpohl *et al.,* in press; Ciffroy *et al.,* 2016).

5.12 Analysis of Carcinogenicity Testing for Regulatory Purposes in the European Union

The approaches for evaluating the carcinogenic potential of substances, including whether carcinogenicity studies should be conducted, differ substantially across sectors. Despite variations in testing schemes, the two-year bioassay study in rodents represents the standard element across all sectors. The validity of the two-year bioassay has however been questioned in the last decade. Uncertainty is associated with the extrapolation of data from rodents to humans. Furthermore, these studies are extremely time and resource-consuming and the high animal burden has raised ethical concerns. For all these reasons, there is a strong demand for alternative strategies and methods in this area. The development of new *in vitro* methods for carcinogenicity testing, however, has progressed slowly and those available are far from being accepted for regulatory decision making, especially when evaluating the carcinogenicity of non-genotoxic chemicals or specific classes of compounds such as biologicals and nanomaterials.

In this context, EURL ECVAM has carried out an analysis of carcinogenicity testing across sectors in the European Union (Madia *et al.,* 2016). This consisted of a systematic review of the different regulatory testing schemes; an analysis of the number of animals used per sector and an estimation of the number of carcinogenicity and genotoxicity studies conducted or waived in respect of the number of substances authorised per sector per year. Moreover, a review of the types of justification for waiving the two-year bioassay has been conducted. Results from this analysis will provide context for initiatives aimed at: 1) reducing the need for animal use where animal testing is still a requirement; 2) ensuring an adequate hazard identification and characterisation in sectors where animal use is banned or limited; and 3) identifying areas where existing methods are not suitable.

5.13 Strategic Aims for Improving Developmental Neurotoxicity (DNT) testing for Different Regulatory Purposes using non-animal methods

Currently there is a recognised need for neurotoxicity evaluation at the regulatory level (Bal-Price *et al.,* 2012). However, systematic testing for DNT is not a mandatory requirement in Europe for pesticides or chemical safety assessments and it is performed only as higher tiered tests triggered by, and based on, structure activity relationships or

[57] http://cordis.europa.eu/news/rcn/128194_en.html
[58] https://www.cen.eu/work/areas/chemical/Pages/WS-MerlinExpo.aspx

evidence of neurotoxicity in standard systemic adult, developmental or reproduction studies (Makris *et al.,* 2009; Bal-Price *et al.,* 2010 and 2012).

The OECD Test Guideline for DNT evaluation (OECD, 2007b; TG 426) is entirely based on the use of animal tests that are centered on neurobehavioral evaluation of cognitive, sensory and motor functions accompanied by morphometric and histopathological studies. This *in vivo* based guideline is very resource intensive in terms of animals, time and overall costs. Therefore, there is the pressing need for developing alternative methodologies that can more rapidly and cost-effectively screen large numbers of chemicals for their potential to cause DNT.

Based on an analysis of different regulatory scenarios, EURL ECVAM is considering a variety of possible approaches addressing the utility of non-animal approaches for DNT assessment, such as providing supplementary information on DNT to support screening, priority setting and hazard assessment, as well as supporting the development and evaluation of Integrated Approaches to Testing and Assessment (IATA), including considerations relevant for cumulative risk assessment (CRA).

6 Dissemination of Information on Alternatives

6.1 Second Meeting of European 3Rs Centres

Since 2015, EURL ECVAM has hosted two meetings with 3Rs Centres from across the EU, including EURL ECVAM. These centres focus on advancing the Reduction, Replacement and Refinement (Three Rs) of animal use for scientific purposes, through a variety of organisations and activities. Whilst the expertise within the centres may vary, shared priorities have been identified and explored as a means of achieving impact in the Three Rs. These priorities include:

- Efforts to reduce animal use in biomedical research
- Communication and dissemination
- Promoting the use of alternative methods/models as biotechnological resources
- Education and training
- Validation towards regulatory acceptance
- Research initiatives supported by 3Rs Centres

The second meeting was held from May 31st to June 1st 2016 to explore these common interests further. Each organisation provided updates on their activities since the last meeting in 2015, as well as specific updates on ventures to enhance knowledge sharing such as Norecopa's new website[59] and search engine, Johns Hopkins University Center for Alternatives to Animal Testing (CAAT) and CAAT-Europe's CAAT Academy[60] and the Education and Training Platform for Laboratory Animal Science (ETPLAS[61]).

A proposal put forward during the meeting was for the 3Rs Centres to become hubs of information to assist with a well-established knowledge chain. Each of the 3Rs Centres specialise in different elements and could formulate a network of 3Rs Centres of excellence to progress knowledge flow. Education and training was highlighted as an area which requires better coordination and 3Rs Centres could play a very important role here. All centres consider this to be a highly important aspect of their functions. As such, there is a large range of educational and training activities offered by the centres. These include producing teaching materials for schools, hosting large events and workshops and providing practical laboratory based training for professional scientists and technicians. The Three Rs focus of the training also varies depending on the specific competences of the centre. The opportunities for sharing expertise and materials are rich and need to be explored further.

The summary record of the meeting will be made public on EURL ECVAM's website.

6.2 EURL ECVAM Databases

6.2.1 *In vitro* methods: DB-ALM - EURL ECVAM's DataBase service on ALternative Methods to animal experimentation

The DB-ALM[62] provides *ready-to-use* and *evaluated* information about the application and development status of advanced and alternative methods in a standardised manner. Information at various level of detail is provided and defined according to pre-determined criteria for data content by experts in the field (Table 6.1). Current focus is given to *in vitro* methods and non-experimental approaches used for safety assessments

[59] https://norecopa.no/
[60] http://www.caat-academy.org/
[61] http://www.etplas.eu/index.php?id=4325
[62] https://ecvam-dbalm.jrc.ec.europa.eu

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 accepted format[63]

Table 6.1 The online information content originates from research projects, validation studies or individual submissions and covers as of September 2016:

Information Sector	Number of Documents
Topic Summaries	5
Method Summaries	178
Protocols	157
Method Evaluations, EU projects, Validation studies	90
Test Results (individual investigations)	9128
Contacts to People active in the field of alternative methods	93
Bibliographic References	7003

Growing interest in the DB-ALM was observed amounting to a total of nearly 5,000 registrations from 82 countries with 499 new in 2015 being the fourth highest since its existence. The usage increased by 29% compared to the year before with over 40,000 accesses to the website contents. The DB-ALM is referenced in formal OECD documents; scientific books and cited in scientific articles. The European Chemicals Agency (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.

Further information can be obtained from the DB-ALM Progress Report 2014-2016[64].

6.2.2 *In silico* methods: QSAR Model Database

The JRC QSAR Model Database[65] 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 information on QSARs by using a downloadable QMRF editor which is reviewed for adequacy and completeness by the JRC-EURL ECVAM before publishing it through the JRC database. Properly documented QSAR Models are provided as robust summaries including results of any validation studies. However, inclusion of the model does not imply acceptance or endorsement by the JRC or the EC, and responsibility for use of the models lies with the end-users.

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[63] OECD Guidance Document N◦211 for describing non-guideline *in vitro* test methods
[64] Access to the DB-ALM Report 2014-2016: http://publications.jrc.ec.europa.eu/repository/handle/JRC102254
[65] http://qsardb.jrc.ec.europa.eu

At the time of writing (July 2016), the online information content of JRC QSAR Model Database covers 109 QSAR Model Descriptions grouped according to OECD defined (regulatory) endpoints as indicated in Figure 6.1 out of which 39 QSAR Reports were published during the past 2 years. A progress report summarising the activities of the QSAR model database during 2014-2016 can be made available on request (JRC102362).

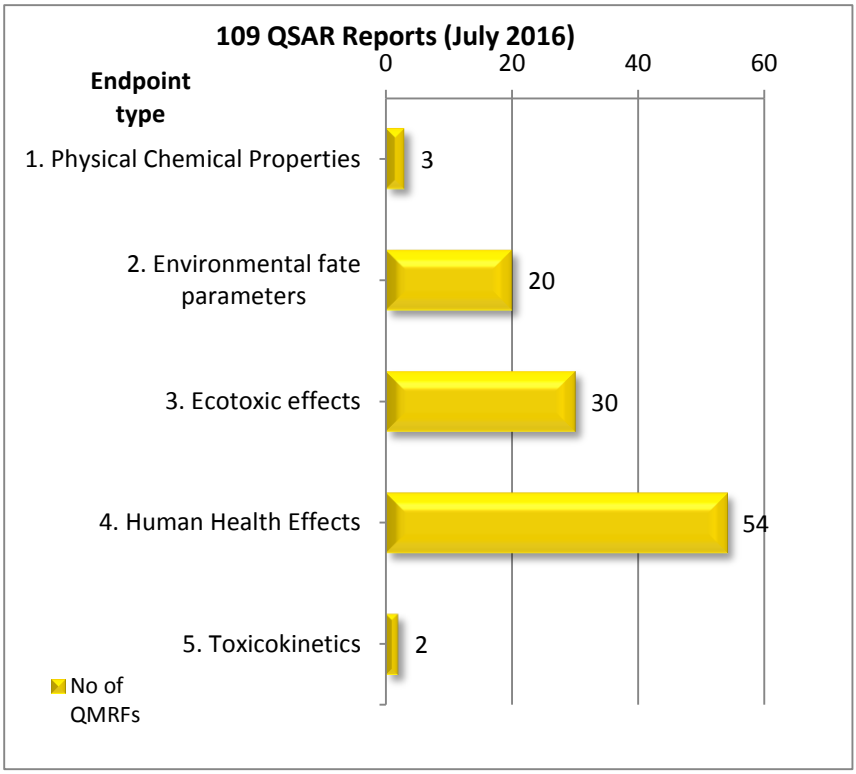

Figure 6.1 QSAR Model Database Online Information Content

As an indication of usage, in July 2016 (last update 28 July), the JRC QSAR Model database had a total of 435 visitors (354 unique), with an average of 16.0 per day (13.1 unique).

The public users worldwide originate from Poland, United Kingdom, Italy, Greece, Switzerland, Japan, India, United States of America, Colombia and Mexico.

Period	Total Visitors	Visitors per Day	Unique Visitors	Unique Ratio
Nov-14	99	3.3	62	63%
↓	↓	↓	↓	↓
Jul-16	435	16	354	81%

Figure 6.2 QSAR Model Database Visitors (extract)[66]

[66] http://stats.jrc.cec.eu.int/?config=qsardb.jrc.it&year=2016&month=7&view=allmonths.all&lang=en-gb

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

TSAR[67] serves to track progress of an alternative method, in a transparent manner, from proposal for validation through to its final adoption by its inclusion into the regulatory framework (EU, OECD and related standards). The currently developed revised TSAR version will also cover the needs of the individual partners of EURL ECVAM participating in the International Collaboration on Alternative Testing Methods (ICATM). In this way an overall view of the methods under evaluation by all international validation centres is provided from one access point. In order to be able to best cover all potential needs, more time has been invested in the project, creating a first beta version that has now technically been finalised. EURL ECVAM is currently in the process of compiling and reviewing the information content to be included in the first release. It is planned to present TSAR to its ICATM partners in October 2016 before TSAR will finally be released to the general public.

6.3 Information Retrieval Guidance: EURL ECVAM Search Guide

The EURL ECVAM Search Guide (first published in 2012 with a re-edition in 2013) 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 has now entered the Asian market where it was translated and re-published as a handbook and E-book in Korean. A Portuguese version is under development together with Brazilian Authorities within the framework of International Cooperation on Alternative Test Methods (ICATM).

The EURL ECVAM Search Guide[68] 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.

6.4 Update on the Adverse Outcome Pathway Knowledge Base (AOP-KB)

AOPs are the central element of a toxicological knowledge framework being built to support chemical risk assessment based on mechanistic reasoning (see 5.7). 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 Adverse Outcome Pathway Knowledge Base (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 will consist of several modules, each tailored to specific needs; a module titled e.AOP.Portal will be 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 is 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 simplify the entry of standardised information. In 2015 and early 2016, the introduction of ontologies to further harmonise the naming of

[67] TSAR table on test submissions: https://eurl-ecvam.jrc.ec.europa.eu/test-submission

[68] http://bookshop.europa.eu/en/the-eurl-ecvam-search-guide-pbLBN124391/

AOP objects was discussed and will be implemented in the AOP-KB Wiki in the second half of 2016.

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 Integrated Approaches to Testing and Assessment (IATA) will profit from the AOP concept as it informs scientists and regulators about the biology behind a chemical's mode of action.

6.5 AOP Training Activities

EURL ECVAM is strongly committed to the development and dissemination of AOPs and is part of the OECD AOP training group.

During the reporting period EURL ECVAM has given four AOP training courses for the European Food Safety Authority (EFSA), for both EFSA staff and external experts at EFSA premises in Parma, Italy (via a Service Level Agreement); these four courses were held in September and November 2015 and in March and June 2016. In total, there were 99 participants, i.e. an average of 25 per course.

The courses aimed 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.

Course facilitators were EURL ECVAM staff together with (varying) external experts from the US (EPA and University of St. Thomas in St. Paul, Minnesota) and Canada (University of Ottawa).

Besides lectures, one or two case studies were dealt with in four or five break out groups. In addition, an interactive exercise showed how AOPs can provide input for Integrated Approaches to Testing and Assessment (IATA) building. Besides plenary discussions, a final brainstorming on how AOP thinking could be incorporated into daily work was held.

Experience and feedback from each course was integrated in the preparation of the following course and also shared with the OECD training group.

6.6 Update on the 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 database compiling available genotoxicity and carcinogenicity data for 726 Ames positive chemicals originating from different sources (Kirkland *et al.,* 2014a).

It 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.,* 2014a). Its development, was one of the main objective of the published EURL ECVAM strategy to Avoid and Reduce 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 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. In addition, it is used as a platform for detailed structural characterisation of specific groups of compounds with or without carcinogenic or genotoxic activity. Inconsistencies (e.g. contradictory data derived from different sources) and poor data quality have been addressed through rigorous curation which included expert peer review.

Since its launch, the Genotoxicity & Carcinogenicity Database has become a reference database for the regulatory and scientific community as demonstrated by a number of activities carried out recently:

- The database and the subsequent analysis of Ames positive chemicals contributed to the 9th revision of SCCS's Notes of Guidance for testing cosmetic ingredients and their safety evaluation in the area of mutagenicity and genotoxicity (SCCS, 2015);
- The database has been considered the starting point for a huge project launched by the CEFIC Long-Range Research Initiative (LRI-B18);
- It is contributing to an on-going activity on *in vivo* follow up studies of the Genetic Toxicology Technical Committee of ILSI/HESI;
- It has been used to investigate the performance of an integrated approach to testing and assessment (IATA) designed to cover different genotoxic mechanisms causing cancer (Petkov *et al.,* 2016).

The database has been linked to two other JRC databases, ChEList (see 6.7) and ChemAgora (see 6.8) 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 the retrieval of additional information on the chemicals of interest using a single platform.

The database is meant as a living project with possibilities of update as new genotoxicity and carcinogenicity data are made available. In fact, an extension of the database is currently on-going to include chemicals with Ames negative results.

6.7 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.

6.8 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 ChemAgora continued to grow, with EURL ECVAM monitoring the chemical DB landscape to identify more databases to be searched via ChemAgora.

6.9 The Endocrine Active Substances Information System (EASIS)

The endocrine system is very complex, affecting many organs and regulating numerous biological processes (such as development, growth, reproduction, metabolism, immunity and behaviour). Hormones (synthesised by the endocrine system) act in very small amounts and at precise moments in time. Chemicals that interfere with the endocrine system can potentially have adverse effects on both humans and wildlife.

"*An endocrine disruptor 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*" (IPCS/WHO, 2002).

The term "endocrine active substance" (EAS) is used to describe any chemical that can interact directly or in-directly 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, i.e. not only with Endocrine Disruptors.

The starting point of EASIS was a database created by the European Commission's Environment Directorate-General (DG ENV) in 2006. A new application, EASIS, was developed and the data from the DG ENV database were migrated into EASIS. More data to cover the period after 2006 were added. There are currently data from around 9000 studies across 513 compounds in EASIS (see table 6.2).

Table 6.2 Compounds and Studies in EASIS

Study Type	Number of studies	Number of compounds
In vivo mammals	4690	375
In vivo humans	140	65
In vivo ecotoxicity	2398	368
In vitro	1926	365

Table 6.2 shows the four study types into which EASIS data are divided. The total number of compounds is higher than 513 due to multiple study types per compound. EASIS has recently been made available to the public.

6.10 Dissemination and training activities of EPAA

In the framework of its collaboration with the Institute for *In vitro* Sciences (IIVS), the European Partnership for Alternative Approaches to Animal Testing (EPAA) supported the production of a 13-minutes video that demonstrates how to perform the BCOP (Bovine Corneal Opacity and Permeability) assay according to OECD TG 437. The video focuses on steps that are critical to the success of the assay such as handling of the isolated cornea and removal of the test material from the cornea at the conclusion of the exposure time. The video is available in an English version as well as two subtitled versions (Chinese, Portuguese) free of charge on the EPAA website[69] and on YouTube[70].

In the framework of the EPAA/IIVS collaboration, a second educational training video on the 3T3 Neutral Red Uptake Phototoxicity test has been produced. It shows how to perform the assay as described in the OECD TG no. 432. The video is available online in an English version [71] as well as two subtitled versions, notably Portuguese [72] and Chinese[73].

Other dissemination activities of EPAA in the reporting period comprised the co-organisation of scientific workshops on clostridial vaccines[74] (see 5.9.4) and biologicals[75] (see 5.9.6).

6.11 European Citizens' Initiative Action 1: Accelerating progress in the Three Rs through knowledge sharing

In the context of the European Commission's Communication (Action 1)[76], published in response to the European Citizens' Initiative "Stop Vivisection" [77], EURL ECVAM conducted a public survey to solicit input from individuals (based on personal experience) and organisations, regarding i) the availability of knowledge sources for their activities with potential relevance to Replace, Reduce or Refine (the 'Three Rs') the use of animals for scientific purposes, ii) to understand how such knowledge is currently used, disseminated or shared, and iii) to highlight opportunities which could fill apparent knowledge gaps and enhance knowledge sharing. The survey was widely disseminated by stakeholders and scientific networks and 351 responses were received. These replies included individual responses as well as 36% who replied on behalf of their organisations (some of which employ over 1000 people).

The results of this survey were fed into a comprehensive, systematic study currently being undertaken by the Commission to map data, information and knowledge resources of different types which are readily available in the public domain in the different scientific fields and industry sectors. The study addresses those that could be exploited to replace animal studies with alternative approaches not involving animals, to adapt

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[69] https://ec.europa.eu/growth/sectors/chemicals/epaa/project-platform_en
[70] https://www.youtube.com/playlist?list=PLLt7eTk-UdPiH0jWO16YfIwnp-gmeb5Uo
[71] https://vimeo.com/156328249
[72] https://vimeo.com/175576942
[73] https://vimeo.com/175590221
[74] EPAA-EDQM workshop: Clostridial Vaccines workshop BSP 130, Egmond Aan Zee, the Netherlands, 15-16 September 2015
[75] EPAA Workshop: Modern science for better quality control of medicinal products 'Towards global harmonization of 3Rs in biologicals, Egmond Aan Zee, the Netherlands, 15-16 September 2015
[76] http://ec.europa.eu/environment/chemicals/lab_animals/pdf/vivisection/en.pdf
[77] http://ec.europa.eu/citizensinitiative/public/initiatives/successful/details/2012/000007

studies to reduce the number of animals needed, or to refine studies so as to minimise pain, suffering and distress of the animal, and to improve its welfare. Moreover, the study aims to explore how sharing of knowhow and access to resources could be enhanced to accelerate overall progress in the Three Rs in every domain where animals are used for a scientific purpose, whether it be for basic biological research, toxicological testing, or for training and education. The outcome of the study, together with the survey results, will be made public by the end of 2016 and presented in December 2016 at a European Commission scientific conference, Non-Animal Approaches - The Way Forward[78], to engage the scientific community and relevant stakeholders in a debate on how to accelerate progress in the Three Rs.

7 International Cooperation on Alternative Test Methods (ICATM)

ICATM Meeting 2015

The meeting of the International Cooperation on Alternative Test Methods (ICATM) partners was held on 10th – 11th November 2015 at the JRC in Ispra. Alongside EURL ECVAM, ICATM includes the US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the Japanese Center for the Validation of Alternative Methods (JaCVAM), the Korean Centre for the Validation of Alternative Methods (KoCVAM) and Health Canada (HC). China and Brazil participated as observers at this meeting, represented by the National Institutes for Food and Drug Control (NIFDC) and the Brazilian Centre for the Validation of Alternative Methods (BraCVAM), respectively.

A 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 will take place from 4th to 5th October 2016. Regulators from each of the ICATM partners' jurisdictions will also participate. The aim of this workshop is to facilitate a common understanding of the non-animal approaches which are available and their current proposed use, particularly within defined approaches and IATA. It will also seek to determine what still needs to be done in this area in order to establish general criteria for the evaluation and acceptance of IATA. Another goal will be to identify the obstacles that hamper the use of non-animal approaches in certain regulatory areas and regions by chemical sector and clearly define what steps should be taken to support their regulatory application.

8 EURL ECVAM strategies

8.1 Follow-up to the EURL ECVAM Strategy for Achieving 3Rs Impact in the Assessment of Toxicokinetics and Systemic Toxicity

The EURL ECVAM Toxicokinetics (TK) Strategy[79] was set up to promote a better use of TK data in chemical safety assessment while respecting the Three Rs. It is centred around four strategic aims:

[78] http://www.euconf.eu/non-animal-approaches-the-way-forward/en/registration/index.html

[79] https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-publishes-its-strategy-in-the-area-of-toxicokinetics

(1) Development of standards for human *in vitro* absorption, distribution, metabolism and excretion (ADME) methods. There is growing awareness that Toxicokinetic (TK) data are an indispensable information source for human chemical risk assessment and trigger the need to develop *in vitro* methods to obtain TK information. This includes, for example, the *in vitro* characterisation of metabolism to identify key metabolic routes or to estimate clearance as a surrogate for *in vivo* metabolism. Hepatic metabolic clearance is an important factor in toxicokinetics; therefore many *in vitro* clearance methods have been developed and are already in use. There is the need to standardise the way these methods are described and characterised to increase their reliability and relevance when used to inform risk assessment-decision making. With respect to this, EURL ECVAM is currently defining harmonised descriptive standards for characterising *in vitro* methods as guidance to measure human *in vitro* hepatic metabolic clearance and to facilitate method comparison. This work is following up on the collective knowledge-gathering exercise (involving literature survey, test submission e-survey and an expert workshop) carried out from 2013 to 2015 (see 3.10 of EURL ECVAM status report 2015, Zuang V. *et al.,* 2015).

(2) Good modelling practice: So far a number of biokinetic models (such as physiologically based kinetic (PBK) models) have enabled the investigation of potential TK interactions and facilitate the incorporation of TK data in risk assessment (i.e applying IVIVE). The foundation of good modelling practice is based on harmonised understanding (terminology/definition) and good knowledge as well as on the relevance and reliability of available input data. The needs and challenges now are in building and reporting of an animal-free PBK modelling and how such a model would then be validated. To achieve international consensus a workshop entitled: "Physiologically-based kinetic modelling in risk assessment–Reaching a whole new level in regulatory decision-making" will be held at JRC, EURL ECVAM in November 2016.

(3) Data collection: An ongoing collection of already existing databases reporting on chemical parameters, *in vitro* data and/or *in silico* predictions, is currently being performed. This information will be used for the development of kinetic modelling.

(4) Regulatory anchoring: Current efforts are on the development of a guidance on hepatic clearance and on good modelling practice of PBK models. This will give insight into the acceptance of these methods and on how to generate and use ADME/TK data in a regulatory setting for better risk assessment processes for both exposure and co-exposure of single chemicals or mixture.

Implementation of the strategy is the way forward for increasing the development, harmonisation and acceptance of human TK data in several areas of toxicology.

8.2 Implementing the EURL ECVAM Strategy to Avoid and Reduce Animal use in Genotoxicity Testing

A strategic plan to avoid and reduce animal use in genotoxicity testing had previously been described by EURL ECVAM, based on the regulatory requirements across different EU legislations, state of the science, and latest and ongoing efforts undertaken by various organisations, including EURL ECVAM (ECVAM, 2013). It was proposed that in the short- and medium-term efforts should be directed towards the overall improvement of the current testing strategy for better hazard assessment with the use of fewer or no animals to satisfy the information requirements of various EU legislations. In recent years several activities have taken place to address one of the main strategic aims, which focused at enhancing the performance of the *in vitro* testing battery to reduce the need for *in vivo* follow-up tests. These activities and the related achievements have been summarised by Corvi and Madia (2016). 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. Furthermore useful tools, such as

databases of reference chemical lists have been developed to support advance in the field.

9 Conclusions

Research and development activities continued to be funded during 2016 for the complex endpoints such as repeated dose and reproductive toxicology, where the toxicological processes and the mechanistic understanding have not been sufficiently elucidated yet and for which Three Rs solutions are more difficult to find. These projects focus on the integration of *in vitro* non-animal methods and *in silico* computational technologies to translate molecular mechanistic understanding of toxicity into safety testing strategies. Several R&D projects are also ongoing for fish toxicity and bioaccumulation testing ranging from the use of a fish cell line-based cytotoxicity assay for acute fish toxicity testing over the development of AOPs for chronic fish toxicity testing and of a tiered testing strategy based on i*n vitro* approaches for fish bioaccumulation testing, to the application of threshold of toxicological concern in aquatic toxicity assessment.

For the quality control of vaccines, research projects aim to develop, optimise and evaluate non-animal methods for routine batch quality, safety and efficacy testing of vaccines.

Good progress in the validation and regulatory acceptance is made in areas where non-animal alternative methods have been developed and validated and where the focus lies in an intelligent combination/ integration of the various non-animal approaches. This has been achieved in the areas of topical toxicity and skin sensitisation. In the latter area, several OECD test guidelines have recently been adopted and Guidance Documents have been prepared. These GDs aim to provide a set of principles for the proper reporting of defined approaches to testing and assessment and the related reporting templates in order to facilitate their regulatory use. The GDs also show how the reporting templates have been used to document a number of defined approaches developed in that area. In the area of genotoxicity, efforts have been made on the overall improvement of the current testing strategy for better hazard assessment with the use of fewer or no animals to satisfy the information requirements of various EU legislation. Since toxicokinetic (TK) data are crucial for human chemical risk assessment, efforts are currently focused on the development of standards for human *in vitro* absorption, distribution, metabolism and excretion (ADME) methods and on good modelling practice.

EURL ECVAM intensified its discussions with regulators and stakeholders on the latest developments in the field such as e.g. the validation and regulatory use of IATAs and continues 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 also in international fora.

Information on alternatives has been disseminated through a variety of dedicated meetings, training efforts and specialised database services.

Annex I – Summary status of the adoption of Test Guidelines based on alternative methods in the OECD TG programme (2012-2016)

Table 1 summarises the status of adoption of OECD test guidelines on alternative methods from 2012 to 2016. It should be noted that beside TGs, also Guidance Documents and new projects on alternative methods were respectively adopted and included on the OECD Work programme during that period. For additional information, please consult the OECD website of the Test Guideline Programme: http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicalsandrelateddocuments.htm

Table 1. Status of adoption of OECD Test Guidelines based on alternative methods 2012-2016

Nr.	Toxicity area	Test method description	Acceptance status
1	Skin corrosion	Reconstructed human Epidermis (RhE) test methods as included in OECD TG 431/EU TM B.40 bis	Adopted 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 prediction models of EpiDerm™, SkinEthic™ RHE and epiCS®) for a more accurate prediction of Sub-Categories 1A and 1B-and-1C combined
2	Skin corrosion	Transcutaneous Electrical Resistance (TER) test method as included in OECD TG 430/EU TM B.40	Adopted in 2004; updated version (inclusion of performance standards) adopted in 2013

Nr.	Toxicity area	Test method description	Acceptance status
			Updated in 2015 for the deletion of the performance standards (published separately on 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)
3	Skin corrosion	*In vitro* Membrane Barrier Test Method for Skin Corrosion as included in OECD TG 435/EU TM B.40	Adopted 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)
4	Skin irritation	Reconstructed human Epidermis (RhE) test methods as included in OECD TG 439/EU B.46	Adopted 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)
5	Serious eye damage/eye irritation	Fluorescein Leakage (FL) test method as included in OECD TG 460	Adopted in 2012
6	Serious eye damage/eye	Bovine Corneal Opacity and Permeability (BCOP) test method as included in OECD TG 437/EU TM	Adopted in 2009; updated version (revision of positive controls, use to identify non-classified chemicals and

Nr.	Toxicity area	Test method description	Acceptance status
	irritation	B.47	several other revisions) adopted in 2013
7	Serious eye damage/eye irritation	Isolated Chicken Eye (ICE) test method as included in OECD TG 438/EU TM B.48	Adopted in 2009, updated version (use to identify non-classified chemicals and several other revisions) adopted in 2013
8	Serious eye damage/eye irritation	Cytosensor Microphysiometer (CM) test method	New draft TG first discussed at WNT in 2013 but not adopted, pending further clarification on its use to identify non-classified chemicals. The additional data requested by the WNT that should have been submitted by US to support the project were not received and thus the project has been discontinued because of lower priority for the EC
9	Serious eye damage/eye irritation	Short Time Exposure (STE) test method for the detection of chemicals causing serious eye damage and chemicals not requiring classification for serious eye damage or eye irritation, as included in OECD TG 491	Adopted as a new TG in 2015
10	Serious eye damage/eye irritation	Reconstructed human Cornea-like Epithelium (RhCE) for the detection of chemicals not requiring classification and labelling for eye irritation or serious eye damage as included in OECD TG 492	Adopted as a new TG in 2015
11	Skin sensitisation	*In chemico* skin sensitisation: Direct Peptide Reactivity Assay (DPRA), as included in OECD TG 442C	Adopted as a new TG in 2015
12	Skin sensitisation	*In vitro* skin sensitisation: ARE-Nrf2 Luciferase test method (KeratinoSens™), as included in OECD TG 442D	Adopted as a new TG in 2015

Nr.	Toxicity area	Test method description	Acceptance status
13	Skin sensitisation	*In vitro* Skin Sensitisation Test: Human Cell Line Activation Test (h-CLAT), as included in OECD TG 442E	Adopted as a new TG in 2016.
14	Carcinogenicity	*In vitro* Syrian Hamster Embryo (SHE) Cell Transformation Assay (CTA) as included in OECD GD no 214*	Adopted as a new GD in 2015
15	Carcinogenicity	*In vitro* Bhas 42 Cell Transformation Assay (CTA) as included in OECD GD no 231*	Adoption as a new GD in 2016
16	Genotoxicity	*In vitro* Mammalian Chromosome Aberration Assay as included in OECD TG 473	Updated OECD TG 473 (originally adopted in 1983) adopted in 2014. Updated in 2016 to reference the Guidance Document on genetic toxicology Test Guidelines
17	Genotoxicity	*In vitro* Mammalian Cell Micronucleus Assay as included in OECD TG 487	Updated OECD TG 487 (originally adopted in 2010) adopted in 2014. Updated in 2016 to reference the Guidance Document on genetic toxicology Test Guidelines
18	Genotoxicity	*In vitro* Mammalian Cell Gene Mutation Test using *Hprt* and *xprt* genes as included in OECD TG 476	OECD TG 476 (originally adopted in 1984) "*In vitro* Mammalian Cell Gene Mutation Test" has been split up into two TGs: 1. The updated TG 476 now using the *Hprt* and *xprt* genes was adopted in 2015; 2. OECD TG 490 using *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

Nr.	Toxicity area	Test method description	Acceptance status
			corrected (see below)
19	Genotoxicity	*In vitro* Mammalian Cell Gene Mutation Tests Using the *Thymidine Kinase* Gene as included in OECD TG 490	Adopted as TG 490 in 2015 (see above). Updated in 2016 to reference the Guidance Document on genetic toxicology Test Guidelines and to correct a paragraph related to the maximum concentration that is based on cytotoxicity
20	Endocrine disruption	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)
21	Endocrine disruption	Performance-Based Test Guideline for Stably Transfected Transactivation *In Vitro* Assays to Detect Estrogen Receptor Agonists and Antagonists	OECD 455 adopted in 2009 (STTA assay using the hERα-HeLa-9903 cell line); updated version (PBTG, inclusion of BG1Luc ER TA assay using the BG1Luc-4E2 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 was approved in 2016
22	Endocrine disruption	Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) *In Vitro* Assays to Detect Chemicals with ER Binding Affinity as included in OECD TG 493	Adopted as new TG in 2015. It includes two reference test methods: • *In Vitro* Estrogen Receptor (ER) Binding Assay Using a Full Length Human Recombinant ERα; • *In Vitro* Estrogen Receptor Binding Assay Using a Human Recombinant Ligand Binding Domain Protein

Nr.	Toxicity area	Test method description	Acceptance status
23	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
24	Acute fish toxicity	Fish Embryo Acute Toxicity (FET) Test as included in OECD TG 236	Adopted in 2013

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

Annex II – ICATM Alternative Test Methods Validation and Status of Regulatory Acceptance

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

Method	Current Status	Lead Action Organisation	International Acceptance
Dermal Corrosivity Test Methods			
CORROSITEX Skin Corrosivity Test	**Completed**		**OECD TG 435 (2006)** Updated version (including the Corrositex® prediction model, the deletion of the performance standards (to be published separately in the Series on Testing and Assessment), including paragraphs referring to the IATA for Skin Corrosion and Irritation in OECD GD No. 203 and the updating of the list of proficiency substances) adopted in 2015
EpiSkin™, EpiDerm™, SkinEthic™, epiCS® Skin Corrosivity Tests	**Completed**		**OECD TG 431 (2004),** updated version (sub-categorisation, inclusion of performance standards, inclusion of SkinEthic™ RHE and epiCS™) adopted in 2013. Revised version including the sub-categorization with the epiCS™ test method adopted in 2014 Updated version [deleting the performance standards (published separately in the Series on Testing and Assessment No. 219), including paragraphs referring to

Method	Current Status	Lead Action Organisation	International Acceptance
			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 prediction models of EpiDerm™, SkinEthic™ RHE and epiCS®) for a more accurate prediction of Sub-Categories 1A and 1B-and-1C combined.
Rat TER Skin Corrosivity Test	**Completed**		**OECD TG 430 (2004**), updated version (inclusion of performance standards) adopted in 2013 Updated version (deleting the performance standards (published separately in the Series on Testing and Assessment No. 218) and including paragraphs referring to the IATA for Skin Corrosion and Irritation in OECD GD No. 203) adopted in 2015
Dermal Irritation Test Methods			
***In vitro* reconstructed human epidermis (RhE) test methods:**	**Completed**		**OECD TG 439 (2010),** updated version (including the LabCyte™

Method	Current Status	Lead Action Organisation	International Acceptance
EpiDerm™, EpiSkin™, SkinEthic™ RHE and LabCyte EPI-MODEL24 *SIT*			EPI-model) adopted in 2013 Updated version [deleting the performance standards (published separately in the Series on Testing and Assessment No. 220, including paragraphs referring to the IATA for Skin Corrosion and Irritation in OECD GD No. 203 and including the use of HPLC/UPLC-spectrophotometry as an alternative procedure to measure tissue viability (increasing the applicability domain of the test methods to coloured substances interfering with the measurement of MTT-formazan)] adopted in 2015
In vitro reconstructed human epidermis (RhE) test methods: Korean epidermis model	KoCVAM sponsored validation study is ongoing	KoCVAM	
Phototoxicity Test Methods			
3T3 NRU Phototoxicity Test	**Completed**		**OECD TG 432 (2004)** **ICH S10 (2014)**
Test method battery to predict phototoxicity (yeast growth inhibition phototoxicity assay and red blood cell photohemolysis assay)	Japanese Regulatory Acceptance Board recommended additional work be performed	JaCVAM	

Method	Current Status	Lead Action Organisation	International Acceptance
***In vitro* test method based on reactive oxygen species (ROS) and photostability**	**Completed**		**ICH S10 (2014)** SPSF to develop an OECD TG was approved in 2016
Ocular Toxicity Test Methods			
Bovine Corneal Opacity and Permeability (BCOP) Test Method	**Completed**		**OECD TG 437 (2009),** updated version (positive control, use in a bottom-up approach to identify non-classified chemicals and several other revisions) adopted in 2013
Isolated Chicken Eye (ICE) Test Method	**Completed**		**OECD TG 438 (2009),** updated version (use in a bottom-up approach to identify non-classified chemicals and several other revisions) adopted at WNT in 2013
Use of Histopathology as an additional endpoint in Ocular Safety Testing	**Completed**		**OECD GD 160 (2011)**
Cytotoxicity test: SIRC CVS	Peer review coordinated by JaCVAM is ongoing	JaCVAM; EURL ECVAM, ICCVAM, KoCVAM and Health Canada VMT	
Cytotoxicity test: three-dimensional dermal model (MATREX)	JaCVAM-sponsored validation study in the planning stage	JaCVAM	
Cytotoxicity test: Short Time Exposure (STE) test	**Completed**		**OECD TG 491 (2015)**

Method	Current Status	Lead Action Organisation	International Acceptance
Use of anaesthetics, analgesics, and humane endpoints for routine use in TG 405	**Completed**		**OECD updated TG 405 (2012b)**
In vitro approach for categorisation of anti-microbial cleaning products: recommendations for further studies	Completed. EPA/OPP[80] 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 http://www.epa.gov/pesticides/regulating/eye-policy.pdf for the details of the scope of the policy).	ICCVAM	
Cytosensor Microphysiometer® (CM) Test method	The draft TG was submitted to OECD for comments including a set of Performance Standards	EURL ECVAM; ICCVAM	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.

[80] Environmental Protection Agency/Office of Pesticide Program

Method	Current Status	Lead Action Organisation	International Acceptance
Fluorescein Leakage (FL) test method	**Completed**		**OECD TG 460 (2012)**
Human reconstructed tissue models for eye irritation **EpiOcular™ EIT**	**Completed**		**OECD TG 492 (2015)**
Vitrigel-EIT	Peer review coordinated by JaCVAM is ongoing	JaCVAM; EURL ECVAM, NICEATM, ICCVAM, Health Canada and KoCVAM VMT liaisons	
Human reconstructed tissue models for eye irritation LabCyte Cornea-model	Peer review coordinated by JaCVAM is ongoing	JaCVAM; and Korean expert VMT liaisons	
OptiSafe	Validation Study coordinated by NICEATM is currently in Phase 1	NICEATM; ICCVAM and EURL ECVAM VMT liaison	
In vitro reconstructed human Cornea-epithelium model (RhCE) test method: Korean Cornea-model	KoCVAM-sponsored validation study started in 2016	KoCVAM	
Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) Test Method	Validation study sponsored by Brazilian Ministry of Science, Technology Innovation and Communication (MCTIC). Preliminary phase of validation study ongoing		

Method	Current Status	Lead Action Organisation	International Acceptance
Immunotoxicity (Allergic Contact Dermatitis) Test Methods			
Murine local lymph node assay (LLNA) for skin sensitization	**Completed**		**OECD TG 429 (2002)** **ISO (2002)**
Updated Murine local lymph node assay (LLNA) for skin sensitization (20% reduction)	**Completed**		**Update to TG 429 OECD (2010)** **ISO (2010)**
Reduced LLNA (rLLNA)	**Completed**		**Update to TG 429 OECD (2010)**
Nonradioactive LLNA protocol (LLNA: BrdU-ELISA)	**Completed**		**OECD TG 442B OECD (2010)**
Nonradioactive LLNA protocol, LLNA:DA	**Completed**		**OECD TG 442A OECD (2010)**
Harmonized performance standards for the LLNA	**Completed**		**Update to TG 429 OECD (2010)**
Nonradioactive LLNA protocol (LLNA: BrdU-Flow Cytometry)	KoCVAM validation study is on-going	KoCVAM	SPSF to develop a TG approved in 2016
***In vitro* skin sensitisation assay (DPRA)**	**Completed**		**OECD TG 442C (2015)**
***In vitro* skin sensitisation assay**	**Completed**	EURL ECVAM; JaCVAM and ICCVAM VMT	**OECD TG 442E (2016)**

Method	Current Status	Lead Action Organisation	International Acceptance
(h-CLAT)		liaison members	
***In vitro* skin sensitisation assay KeratinoSens™**	**Completed**		**OECD TG 442D (2015)**
In vitro skin sensitisation assay IL-8 Luc assay	Peer review coordinated by JaCVAM was completed	JaCVAM; EURL ECVAM, NICEATM,, KoCVAM and Health Canada VMT liaisons	SPSF to develop a TG approved in 2015 New draft TG discussed at OECD WNT meeting in 2016
In vitro skin sensitisation assay Vitrigel-SST	MAFF[81]-sponsored validation study is pending	JaCVAM; EURL ECVAM, NICEATM, KoCVAM and Health Canada VMT liaisons	
In vitro skin sensitisation assay Amino acid derivative reactivity assay (ARDA)	JCIA[82] and JSAAE[83] validation study is ongoing	JaCVAM; NICEATM, EU experts and KoCVAM VMT liaisons	
IL-2 Luc assay for the evaluation of the immunotoxic potential of chemicals	JaCVAM validation study is ongoing	JaCVAM; NICEATM and EU experts VMT liaisons	
Electrophilic allergen screening assay (EASA)	Validation study coordinated by NICEATM is currently being planned. Testing currently anticipated to begin Spring 2017.	NICEATM; VMT currently being established from ICCVAM working group	

[81] Ministry of Agriculture, Forestry and Fisheries
[82] Japan Cosmetic Industry Association
[83] Japanese Society for Alternatives to Animal Experiments

Method	Current Status	Lead Action Organisation	International Acceptance
		members and liaisons	
Acute Toxicity Test Methods			
Up and Down Procedure (UDP)	**Completed**		**OECD TG 425 (2008)**
***In vitro* cytotoxicity test methods for estimating starting doses for acute oral systemic toxicity tests**	**Completed**		**OECD GD 129 (2010)**
In vitro cytotoxicity test (3T3 Neutral Red Uptake) for identifying substances with acute oral LD50 > 2000 mg/kg b.w.	EURL ECVAM ESAC peer review completed, and EURL ECVAM Recommendation published in 2013	EURL ECVAM and ICATM organisations	
Zebrafish Embryo Toxicity test (ZFET)	**Completed**		**OECD TG 236 (2013)**
Toxicokinetic Test Methods			
In vitro hepatic biotransformation – CYP induction: Hepa RG and cryopreserved human hepatocytes		EURL ECVAM; NICEATM, and JaCVAM VMT liaisons	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.
In vitro Fish Hepatic Metabolism - Two *in vitro* systems for deriving information on biotransformation and improving reliability of bioconcentration and	Ring trial conducted under the auspices of the OECD close to completion; ring trial report, draft test guidelines and draft guidance document in preparation; 1st WNT commenting round planned for	United States and European Commission (through JRC-EURL ECVAM)	SPSF for a TG on *in vitro* Fish Hepatic Metabolism approved in April 2014

Method	Current Status	Lead Action Organisation	International Acceptance
bioaccumulation factors (BCF & BAF) and avoiding use of fish bio-concentration tests	early 2017.		
Endocrine Disruptor Test Methods			
Stably transfected human estrogen receptor-α transcriptional activation assay for detection of estrogenic agonist-activity of chemicals (STTA and BG1-Luc assays)	**Completed**		**OECD TG 455 (2009), updated 2012 and 2015,** inclusion of the antagonist protocols in addition to the agonist protocols, deletion of OECD TG 457 in parallel as it is no longer needed
BG1Luc® human estrogen receptor transcriptional activation assay: agonist and antagonist protocols	**Completed**		**OECD TG 457 (2012)** TG 457 has been deleted in parallel to TG 455 updates (see previous table entry)
CertiChem MCF-7 cell proliferation assay for the detection of human estrogen receptor agonists and antagonists	International validation study completed. Protocol must be revised for adequate transferability.	NICEATM; EURL ECVAM, JaCVAM and KoCVAM VMT liaisons	
CertiChem MDA-Kb2 assay for the detection of human androgen receptor agonists and antagonists	NICEATM coordinated single lab validation study ongoing	NICEATM; ICCVAM and EURL ECVAM VMT liaison	
Stably transfected CHO Androgen receptor-α transcriptional activation assay for detection of androgenic agonist and antagonist activity of chemicals	**Completed**	JaCVAM and VMG NA liaisons	**OECD TG 458 (2016)**

Method	Current Status	Lead Action Organisation	International Acceptance
(AR-STTA)			
MELN® human estrogen receptor transcriptional activation assay: agonist and antagonist protocols	Validation stopped		
Stably Transfected Transactivation *in vitro* Assay to detect Androgen Receptor Agonists and Antagonists	Validation study ongoing	EURL ECVAM, NICEATM VMT, JaCVAM and KoCVAM VMT liaison	SPSF to develop a PBTG on ARTA approved in April 2013
Transactivation assay for the detection of compounds with (anti)androgenic potential using 22Rv1/MMTV cells	Validation study ongoing	Ministry of Food and Drug Safety (MFDS) South Korea, EURL ECVAM and JACVAM VMT liaisons	
Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) *In Vitro* Assays to Detect Chemicals with ER Binding Affinity	**Completed**		**OECD TG 493**
Genetic Toxicity Test Methods			
***In vitro mammalian cell* micronucleus test**	**Completed**		**OECD TG 487 (2010), updated TG adopted in 2014**

Method	Current Status	Lead Action Organisation	International Acceptance
***In vitro* mammalian cell chromosome aberration assay**[84]	**Completed**		**OECD TG 473 (1997), updated TG adopted in 2014**
***In vivo* comet assay**	**Completed**		**OECD TG 489 (2014)**
In vitro comet assay	Validation study for the *in vitro* comet assay stopped	JaCVAM; EURL ECVAM, NICEATM and ICCVAM VMT liaisons	
Genotoxicity assays (micronucleus and comet) in 3D skin models	Validation study ongoing	Cosmetics Europe (lead); EURL ECVAM support	
Carcinogenicity Test Methods			
***In vitro* Bhas 42 cell transformation assay (CTA)**	**Completed**	JaCVAM; EURL ECVAM, NICEATM-ICCVAM, and Health Canada VMT liaisons	**OECD GD 231 (2016)**
***In vitro* Syrian hamster embryonic cells (SHE) cell transformation assays (CTAs)**	**Completed**		**OECD GD 214 (2015)**
Reproductive Test Methods			
Hand-1 Luc assay	METI[85]-sponsored validation is ongoing	JaCVAM; EURL ECVAM, NICEATM-ICCVAM, and	

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

Method	Current Status	Lead Action Organisation	International Acceptance
		Health Canada, and KoCVAM VMT liaisons	
Quality Control testing of Biologicals			
Monocyte Activation Test (MAT)	Collaborative Studies – 1. Applicability of MAT to biological (hyperimmune sera and vaccines) 2. MAT response to non-endotoxin pyrogens	BraCVAM/INCQS and Renama	Brazilian Pharmacopoeia
Toxin Binding Inhibition (ToBI) test	Collaborative Study - Applicability of ToBI for lot release - Tetanus Antitoxin for Human Use	BraCVAM/INCQS and Renama	Brazilian Pharmacopoeia

[85] Ministry of Economy, Trade and Industry

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List of figures

List of tables

LA-NA-28156-EN-N

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doi: 10.2787/400140
ISBN 978-92-79-63031-6